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HOMENAJE A OSVALDO CORI

In Honour of Osvaldo Cori

Sociedad de Biología de Chile

Los
ARCHIVOS DE BIOLOGIA Y MEDICINA EXPERIMENTALES

órgano oficial de la Sociedad de Biología de Chile, están destinados a publicar trabajos originales sobre problemas de las distintas ramas de la Biología y de la Medicina Experimental. Además publicarán trabajos de revisión y el texto de conferencias acerca del estado actual de problemas de interés general en las disciplinas mencionadas. Asimismo, darán cabida a los resúmenes de comunicaciones presentadas en reuniones anuales o especiales de las Secciones de la Sociedad y de las Sociedades afiliadas. Ocasionalmente, *Archivos* publicará, especialmente, los trabajos *in extenso* presentados en simposios realizados en el país.

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Envíe el original y un duplicado del manuscrito a

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El texto deberá ser escrito a máquina a doble espacio en toda su extensión, lo que incluye referencias, tablas, notas al pie de página y leyendas, en hojas de 21 por 27 cm (tamaño carta) con un margen de 2,5 cm por lado. El texto debe ser tan corto como sea posible. Comience en hoja separada cada uno de los siguientes items: a) Título del trabajo, nombre de los autores, Institución en que se efectuó el trabajo (Laboratorio, Departamento, Instituto, etc.) seguido de su dirección postal y el subtítulo (no más de 60 caracteres incluyendo los espacios); b) resumen; c) texto; d) referencias; e) notas al pie de página; f) leyendas de figuras (pueden agruparse en una o más páginas); g) cada una de las tablas. Numere sucesivamente todas las páginas del manuscrito, empezando por aquella que contiene el título. Indique la localización apropiada en el texto de tablas y figuras mediante notas marginales.

Los manuscritos deben escribirse en correcto español,evitando el uso de neologismos técnicos o abreviaturas que no sean las internacionalmente aceptadas.

La adopción del idioma inglés, por la mayoría de las publicaciones científicas en el momento actual, hace aconsejable la recepción y consideración editorial de manuscritos en ese idioma. Estas contribuciones deben ceñirse estrictamente a las normas usuales y aceptadas de la gramática inglesa.

ORGANIZACION DEL MANUSCRITO

Se sugiere que cada trabajo conste de las siguientes secciones: a) Abstract, en inglés; b) Introducción; c) Métodos; d) Resultados; e) Discusión; f) Referencias. En algunos casos puede ser aconsejable la combinación de Resultados y Discusión en una sola sección, especialmente si se trata de comunicaciones breves.

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5. La descripción de los Métodos debe ser breve, pero adecuada, para permitir la repetición del trabajo por otros investigadores.

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Aebi, H., Koblet, H. Wartburg, J.E., *Helvet. Physiol. Pharmacol.* Acta *15:* 384-392, 1957.

Jellinek, E.M. *The Disease Concept of Alcoholism.* New Haven, Hillhouse Press, 1960.

Richter, C.P. En *Neuropharmacology. Transactions of the Third Conference* (Abramson, H.A., Ed.). New York, Josiah Macy Jr. Foundation, 1957, pp. 39-63.

2) Se indicarán en el texto con la mención de los autores y el año de la publicación entre paréntesis, *e.g.* "... para la medición de proteínas (Lowry et al., 1951)." o "de acuerdo con las indicaciones de Miller (1959).". Al final del texto se agruparán por orden alfabético y, si hay más de una cita del mismo autor, según la antigüedad de la cita. Se incluirá el título completo del artículo citado. Incluya las páginas INICIAL Y FINAL.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. y Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem. 193,* 265-275.

Miller, G.L. (1959) Protein determination for large number of samples. *Anal. Chem. 31,* 964.

Cite en las REFERENCIAS artículos que hayan sido aceptados para publicación; el nombre de la revista deberá ir seguido por las palabras "en prensa" y por el año probable de publicación. Incluya en el texto entre paréntesis, o en una nota al pie de página, no en REFERENCIAS, cualquier mención a "manuscrito en preparación", "experimentos no publicados", "comunicación personal". En este último caso, verifique la redacción de la comunicación personal con la persona que proporcionó la información y obtenga su aprobación para usar su nombre en relación a la cita. Es de responsabilidad de los autores obtener autorización específica de toda "comunicación personal" como también de los "Agradecimientos". Se da por entendido que los autores han enviado copia del manuscrito a las personas mencionadas y que se ha obtenido su aprobación específica; los editores pueden requerir copia de tal autorización.

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9. Deberán incluirse los nombres latinos binomiales subrayados de los organismos de experimentación, excepto los de los animales comunes de laboratorio. No use abreviaturas si son mencionados en el título y en el resumen. Es conveniente indicar el lugar de obtención de los animales. Para los efectos de nomenclatura deberán seguirse las reglas establecidas en el "International Code of Zoological Nomenclature". Deben incluirse también los nombres latinos binomiales de todas las especies de plantas y microorganismos que se mencionen. Las

variedades y fuentes de obtención deben especificarse.

Al referirse a experimentos en genética bacteriana deberán seguirse las recomendaciones propuestas por Demerec, M., Adelberg, E.A., Clark, A.J. y Hartman, P.E. (*Genetics 54:* 61-76, 1966).

La Sociedad de Biología de Chile y el Comité Editorial de *Archivos* hacen suyos los principios expresados en la Declaración de Helsinki (1964). Los autores que describan experimentos en humanos deberán asegurar que su trabajo concuerda con tal declaración e incluir en el texto una nota que específicamente indique que los sujetos han expresado su consentimiento informado a su participación en el trabajo.

10. La denominación de las drogas que se mencionen se ajustará al criterio establecido en *Pharmacological Reviews.*

11. Las enzimas deberán identificarse en su primera mención de acuerdo con la Enzyme Commision de la International Union of Biochemistry.

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1. Las tablas se enumerarán en forma correlativa con números romanos. Cada tabla debe tener un título explicativo y suficiente detalle experimental como para ser comprensible sin referencia al texto. El encabezamiento de cada columna debe expresar claramente su contenido y la unidad. No se incluirán datos que puedan ser deducidos aritméticamente de otros contenidos en ella. Si una condición experimental, por ejemplo, número de animales, dosis, concentración de un compuesto, etc., es la misma para todos los experimentos presentados, proporcione esa información en una nota al pie de la tabla y no en una columna de números iguales en la tabla. Para conservar espacio, no tabule repetidas observaciones individuales y prefiera el empleo de valores promedio y alguna medida aceptable de dispersión (desviación estándar, rango), indicando el número de las observaciones individuales que contribuyen a la estadística. Cualquier comentario con respecto a la significación de las medidas debe ir acompañado de los valores de probabilidad deducidos de algún análisis estadístico.

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La Sociedad de Bioquímica de Chile a través del presente número de *Archivos de Biología y Medicina Experimentales* rinde homenaje a la memoria del profesor Dr. Osvaldo Cori Moully, maestro y formador de la mayoría de los bioquímicos del país y destacado miembro fundador de nuestra Sociedad.

Esta es una colección de trabajos de investigación en el área de la bioquímica que han sido realizados por muchos de sus discípulos y constituyen un modesto homenaje pero, sin duda, el más apropiado para quien dedicara toda una vida a la investigación científica, a la formación de jóvenes investigadores y a dar impulso a proyectos y políticas para el fortalecimiento de la estructura científica nacional. Sin lugar a dudas, personalidades como la del profesor Cori son características de los grandes hombres de ciencia.

SOCIEDAD DE BIOQUIMICA DE CHILE

La edición de este volumen especial fue posible gracias al apoyo económico de las siguientes instituciones:

Departamento Técnico de Investigación, Universidad de Chile

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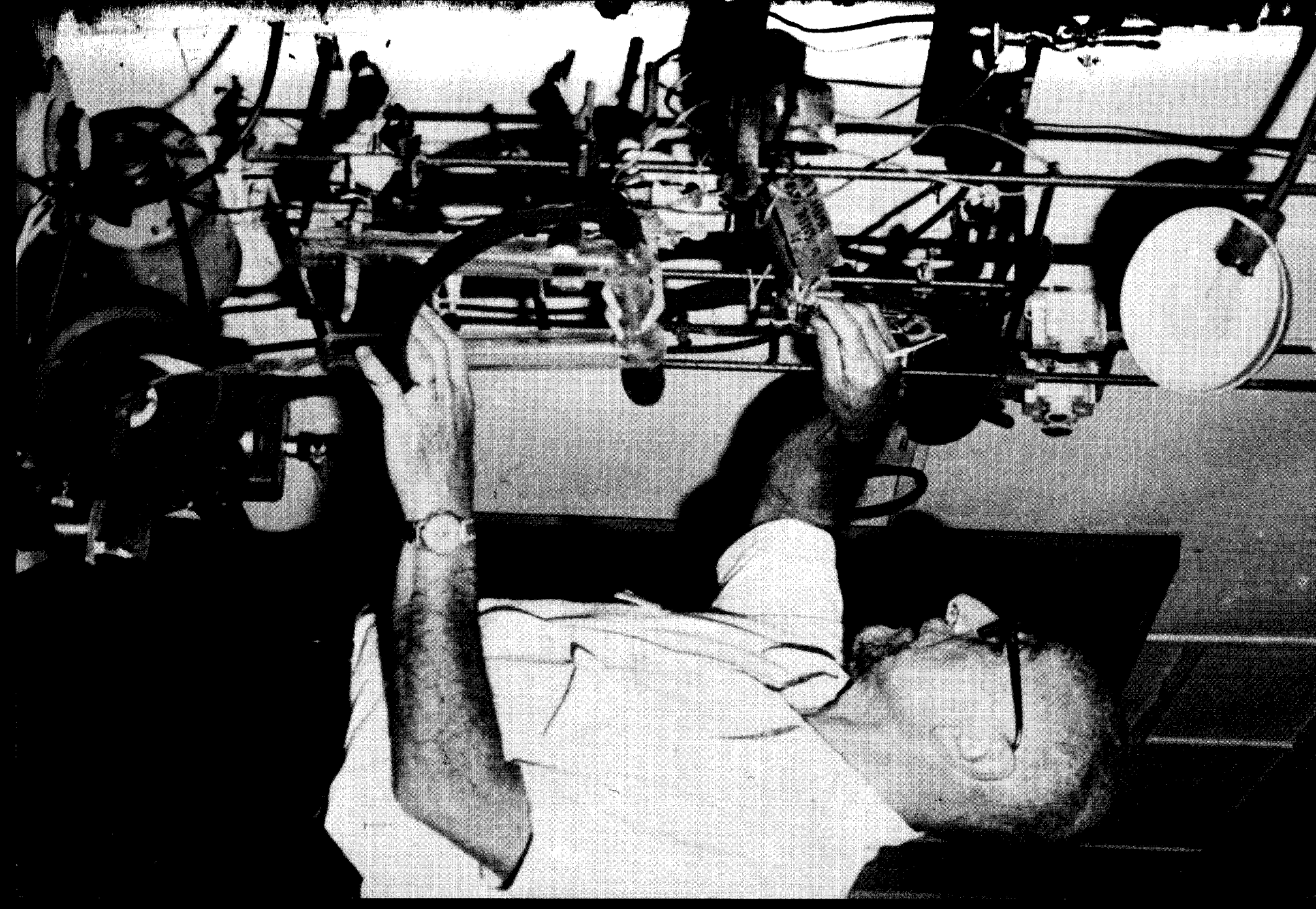

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Osvaldo Cori Moully

Antofagasta, 11 de agosto de 1921 - Santiago, 2 de abril de 1987

Padres: Alejandro Cori Alaloff - Abigail Moully Levy
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EDUCACION Y PERFECCIONAMIENTO

Humanidades en Deutsche Schule y Liceo Eduardo de la Barra, Valparaíso. Bachiller en Humanidades con mención en Biología y Química (1940).

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1958-1959: Becario Fundación Rockefeller y Comisión Fulbright: Public Health Research Institute City of New York (Prof. Ephraim Racker).
1959: Becario British Council y Burroughs Foundation: Visita a varios centros de investigación en Inglaterra.
1964: Subvención de viaje de la Rockefeller Foundation: Visita a varios centros de investigación en Estados Unidos.
1965-1966: Becario Comisión Fulbright: Profesor visitante Universidad de California Los Angeles.
1971: Becario Welcome Trust: Visita a varios centros de investigación en Inglaterra.
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1975: Decano, Facultad de Ciencias, Universidad de Chile.
1975: Vicerrector de la Sede Oriente de la Universidad de Chile.
1976-1978: Director del Servicio de Desarrollo Científico y Creación Artística de la Universidad de Chile.
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1965: Miembro de Número de la Academia de Ciencias del Instituto de Chile.
1983: Miembro de la Academia de Ciencias de América Latina.
1986: Miembro de la Academia de Ciencias Exactas de Buenos Aires.
1986: Doctor Honoris Scientiae, Pontificia Universidad Católica de Chile.

TESISTAS

QF = Químico-Farmacéutico; BQ = Bioquímico; DC = Doctor en Ciencias; MagBQ = Magister en Bioquímica; MC = Médico Cirujano.

Aedo, Ana Rosa (1969 QF).
Behrens, Willi (1963 BQ).
Beytía, Enrique (1965 BQ).
Canelo, Eliana (1960 QF).
Cardemil, Emilio (1970 BQ).
Chaimovich, Hernán (1962 BQ).
Chayet, Liliana (1972 BQ).
De la Fuente, Milton (1976 DC).

Devés, Rosa (1974 BQ).
Durandin, Rose Marie (1974 BQ).
Faini, Francesca (1970 QF).
Fernández, Luis (1978 BQ).
Foster, Richard (1974 BQ).
Frenkel, René (1956 QF).
George-Nascimento, Carlos (1969 BQ).
Hashagen, Uta (1977 BQ).
Holuige, Loreto (1979 BQ).
Israel, Silvia (1958 QF).
Israel, Yedi (1962 BQ).
Jabalquinto, Ana María (1973 BQ).
Jacob, Germaine (1970 BQ).
Jedlicki, Eugenia (1972 BQ).
Loyola, Gustavo (1979 BQ).
Lozada, Ramón (1981 MagBQ).
Marcus, Ditta (1963 BQ).
Marcus, Frank (1958 QF).
Montecinos, Víctor (1950 MC).
Muñoz, Marietta (1959 QF).
Pérez, Luz M. (1975 BQ, 1985 DC).
Pino, Ana María (1969 BQ).
Rabí, Jaime (1966 BQ).
Rojas, María Cecilia (1976 BQ).
Rosemblatt, Mario (1966 BQ).
Ruiz, Francisca (1969 BQ).
Sánchez, Gina (1979 BQ).
Segura, Marina (1949 QF).
Tarragó, Laura (1966 BQ).
Taucher, Gisela (1977 BQ).
Téllez, Rowena (1972 BQ, QF).
Traverso, Aída (1949 QF).
Valenzuela, Pablo (1964 BQ).
Vial, María Victoria (1979 BQ).
Yudelevich, Arturo (1965 BQ).

PUBLICACIONES

Artículos científicos:

1. HOFFMANN, F., CORI, O. & TRAVERSO-CORI, A. (1951) Effect of thyroxin on the oxygen consumption and nitrogen excretion in adrenalectomized rats. *Acta Physiol. Lat. 2,* 84-90.
2. CORI, O. & LIPMANN, F. (1952) The primary oxidation product of enzymatic glucose-6-phosphate oxidation. *J. Biol. Chem. 194,* 417-425.
3. KAUFMAN, S., GILVARG, C., CORI, A. & OCHOA, S. (1953) Enzymatic oxidation of -ketoglutarate and coupled phosphorylation. *J. Biol. Chem. 202,* 869-888.
4. ROTHSCHILD, H.A., CORI, O. & GUZMÁN BARRON, E.S. (1954) The components of choline oxidase and aerobic phosphorylation coupled with choline oxidation. *J. Biol. Chem. 208,* 41-53.
5. CORI, O., ABARCA, F., FRENKEL, R. & TRAVERSO-CORI, A. (1956) Synthesis of phosphocreatine by enzymes from heart and skeletal muscle. *Nature, 178,* 1231-1233.
6. CORI, O. (1957) Propiedades generales de los sistemas enzimáticos. Su importancia en el diagnóstico y en la interpretación fisiopatológica. *Rev. Med. Valparaíso, 10,* 160-169.
7. CORI, O., TRAVERSO-CORI, A., LAGARRIGUE, M. & MARCUS, F. (1958) Enzymic phosphorylation of creatinine by 1:3 diphosphoglyceric acid. *Biochem. J. 70,* 633-641.
8. CORI, O., TRAVERSO-CORI, A., MARCUS, F., TETAS, M., MUÑOZ, M. & LAGARRIGUE, M. (1960) The role of DPN in the enzymatic phosphorylation of creatinine. *Biochim. Biophys. Acta, 42,* 560-561.
9. CORI, O., MARCUS, F. & TRAVERSO-CORI, A. (1962) Participation of adenine nucleotides in the proposed direct pathway of biosynthesis of N-phosphorylcreatinine. *Nature 194,* 476.
10. TRAVERSO-CORI, A. & CORI, O. (1962) Splitting of the terminal phosphate group of ATP by potato apyrase. *Biochim. Biophys. Acta, 57,* 158-160.
11. TRAVERSO-CORI, A., CHAIMOVICH, H. & CORI, O. (1965) Kinetic studies and properties of potato apyrase. *Arch. Biochem. Biophys. 109,* 173-184.
12. CORI, O. (1965) Uso de inhibidores en el estudio de la interacción enzima-sustrato. *Arch. Biol. Med. Exp. 2,* 109-116.
13. CORI, O., TRAVERSO-CORI, A., TETAS, M. & CHAIMOVICH, H. (1965) Substrate specificity and inhibition studies of potato apyrase. *Biochem. Z. 342,* 345-358.
14. VALENZUELA, P., BEYTÍA, E., CORI, O. & YUDELEVICH, A. (1966) Phosphorylated intermediates of terpene biosynthesis in *Pinus radiata. Arch. Biochem. Biophys. 113,* 536-539.

15. VALENZUELA, P., CORI, O. & YUDELEVICH, A. (1966) Occurrence of monoterpenes in *Pinus radiata* and utilization of labelled CO_2 and mevalonic acid. *Phytochemistry 5,* 1005-1011.
16. VALENZUELA, P. & CORI, O. (1967) Acid catalized hydrolysis of neryl pyrophosphate and geranyl pyrophosphate. *Tetrahedron Lett. 32,* 3089-3094.
17. CORI, O. (1968) Bioquímica de las resinas naturales. *Bol. Acad. Ciencias Chile 1,* 57-67.
18. BEYTÍA, E., VALENZUELA, P. & CORI, O. (1969) Terpene biosynthesis: formation of nerol, geraniol and other prenols by an enzyme system from *Pinus radiata. Arch. Biochem. Biophys. 129,* 346-356.
19. GEORGE-NASCIMENTO, C., BEYTÍA, E., AEDO, A.R. & CORI, O. (1969) Transformation of mevalonic acid into isoprenoid compound by enzymes from *Pinus radiata.* Activation of the overall reaction by phospholipids. *Arch. Biochem. Biophys. 132,* 470-476.
20. CORI, O. (1969) Terpene biosynthesis: utilization of neryl pyrophosphate by an enzyme system from *P. radiata* seedlings. *Arch. Biochem. Biophys. 135,* 416-418.
21. GEORGE-NASCIMENTO, C., PONT-LEZICA, R. & CORI, O. (1971) Non enzymic formation of nerolydol from farnesyl pyrophosphate in the presence of bivalent cations. *Biochem. Biophys. Res. Comm. 45,* 119-124.
22. GEORGE-NASCIMENTO, C. & CORI, O. (1971) Terpene biosynthesis from geranyl and neryl pyrophosphate by enzymes from orange flavedo. *Phytochemistry 10,* 1803-1810.
23. CORI, O. (1972) Complements to define R or S configuration viewing molecules from any side. *J. Chem. Ed. 49,* 461.
24. JACOB, G., CARDEMIL, E., CHAYET, L., TÉLLEZ, R., PONT-LEZICA, R. & CORI, O. (1972) Synthesis of isomeric farnesols by soluble enzymes from *Pinus radiata* seedlings. *Phytochemistry 11,* 1683-1688.
25. JEDLICKI, E. JACOB, G., FAINI, F., CORI, O. & BUNTON, C.A. (1972) Stereospecificity of isopentenylpyrophosphate isomerase and prenyltransferase from *Pinus* and *Citrus. Arch. Biochem. Biophys. 152,* 590-596.
26. TRAVERSO-CORI, A. & CORI, O. (1972) Algunos aspectos de la especificidad enzimática. *Rev. Méd. Chile 100,* 1467-1480.
27. CARDEMIL, E. & CORI, O. (1973) Synthesis of 1-^{3}H-3,3 dimethylallylpyrophosphate. *J. Lab. Comp. 9,* 15-21.
28. CHAYET, L., PONT-LEZICA, R., GEORGE-NASCIMENTO, C. & CORI, O. (1973) Biosynthesis of sesquiterpene alcohols and aldehydes by cell-free extracts from orange flavedo. *Phytochemistry 12,* 95-101.
29. CARDEMIL, E., VICUÑA, J.R., JABALQUINTO, A.M. & CORI, O. (1974) Separation of isoprenoid alcohols and aldehydes by thin layer chromatography. *Anal. Biochem. 59,* 636-638.
30. CORI, O., CARDEMIL, E., CHAYET, L., JABALQUINTO, A.M., VICUÑA, J.R. & DEVÉS, R. (1974) Biosíntesis de isoprenoides en plantas. *Ciencia Invest. 30,* 95-99.
31. CORI, O. (1976) Biosíntesis de los núcleos de las hormonas de estructura isoprénica. En *Bases Moleculares de la Acción Hormonal* (Perretta, M., Minguell, J. & Swaneck, J., eds.). Ediciones Sede Santiago Sur, pp. 11-29.
32. CHAYET, L., ROJAS, C., CARDEMIL, E., JABALQUINTO, A.M., VICUÑA, J.R. & CORI, O. (1977) Biosynthesis of monoterpene hydrocarbons from 1-^{3}H neryl-pyrophosphate and 1-^{3}H geranylpyrophosphate by soluble enzymes from *Citrus limonum. Arch. Biochem. Biophys. 180,* 318-327.
33. CORI, O. (1977) Mecanismos enzimáticos en la biosíntesis de isoprenoides. *Rev. Chilena Educ. Quím. 2,* 123-137.
34. DEL CAMPO, G., PUENTE, J., VALENZUELA, M.A., TRAVERSO-CORI, A. & CORI, O. (1977) Hydrolysis of synthetic pyrophosphoric esters by an isoenzyme of apyrase from *Solanum tuberosum. Biochem. J. 167,* 525-529.
35. PÉREZ, L.M., CHAYET, L., DE LA FUENTE, M., ROJAS, M.C., PORTILLA, G., HASHAGEN, U., FERNÁNDEZ, L.A. & CORI, O. (1978) Biosynthesis of mono- and sesquiterpenoids by soluble enzymes from *Citrus* flavedo. *Lloydia 41,* 650.

36. BUNTON, C.A. & CORI, O. (1979) Mechanistic aspects of terpenoid biochemistry. *Interciencia 3,* 291-297.
37. BUNTON, C.A., CORI, O., HACHEY, D. & LERESCHE, P. (1979) Cyclization and allylic rearrangements in solvolyses of monoterpenoids. *J. Org. Chem. 44,* 3238-3244.
38. PÉREZ, L.M., TAUCHER, G. & CORI, O. (1980) Hydrolysis of allylic phosphate by enzymes from the flavedo of *Citrus sinensis. Phytochemistry 19,* 183-187.
39. CORI, O., CHAYET, L., DE LA FUENTE, M., FERNÁNDEZ, L A., HASHAGEN, U., PÉREZ, L.M., ROJAS, M.C., PORTILLA, G., SÁNCHEZ, G. & VIAL, M.V. (1980) Stereochemical aspects of chain lenghthening and cyclation processes in terpenoid biosynthesis. *Mol. Biol. Biochem. Biophys. 32,* 97-110.
40. DE LA FUENTE, M., PÉREZ, L.M., HASHAGEN, U., CHAYET, L., ROJAS, C., PORTILLA, G. & CORI, O. (1981) Prenyltransferases from the flavedo of *Citrus sinensis. Phytochemistry 20,* 1551-1557.
41. VIAL, M.V., ROJAS, M.C., PORTILLA, G., CHAYET, L., PÉREZ, L.M., CORI, O. & BUNTON, C.A. (1981) Enhancement of geranyl pyrophosphate hydrolysis by bivalent metal ions: a model for the enzymic biosynthesis of cyclic monoterpenes. *Tetrahedron 37,* 2351-2357.
42. CORI, O., CHAYET, L., PÉREZ, L.M., ROJAS, M.C., PORTILLA, G., HOLUIGUE, L. & FERNÁNDEZ, L.A. (1981) Isoprenoid biosynthesis: sulfhydryl groups in prenylsynthetase and carbocyclase from *Citrus* flavedo. *Arch. Biol. Med. Exp. 14,* 129-137.
43. PORTILLA, G., ROJAS, M.C., CHAYET L. & CORI, O. (1982) Synthesis of monoterpene hydrocarbons from (1-^{3}H) linalylpyrophosphate by carbocyclase from *Citrus limonum. Arch. Biochem. Biophys. 218,* 614-618.
44. CORI, O., PORTILLA, G. & CHAYET, L. (1982) Secondary plant metabolism and enzyme mechanisms. *Arch. Biol. Med. Exp. 15,* 357-364.
45. PÉREZ, L.M., LOZADA, R. & CORI O. (1983) Biosynthesis of allylic isoprenoid pyrophosphates by an enzyme preparation from the flavedo of *Citrus paradisii. Phytochemistry 22,* 431-433.
46. ROJAS, M.C., CHAYET, L., PORTILLA, G. & CORI, O. (1983) Substrate and metal specificity in the enzymic synthesis of cyclic monoterpenes from geranyl and neryl pyrophosphates. *Arch. Biochem. Biophys. 222,* 389-396.
47. CORI, O. (1983) Enzymic aspects of the biosynthesis of monoterpenes in plants. *Phytochemistry 22,* 331-341.
48. CHAYET, L., ROJAS, M.C., CORI, O., BUNTON, C.A. & MCKENZIE, D.C. (1984) Complexes of bivalent cations with neryl and geranyl phyrophosphate. Their role in terpene biosynthesis. *Bioorg. Chem. 12,* 329-338.
49. CORI, O. (1984) Inhibidores e inactivadores de enzimas. *Rev. Col. Quím. Farm. 40,* 19-23.
50. CORI, O. & ROJAS, M.C. (1985) Carbocyclases from *Citrus limonum. Methods Enzymol. 110,* 406-417.
51. BANTHORPE, D.V., BUNTON, C.A., CORI O. & FRANCIS, M.J.O. (1985) Relation between loss of *pro* chiral hydrogen and E-Z geometry in isoprenoid biosynthesis. *Phytochemistry 24,* 251-252.
52. PORTILLA, G., ROJAS, M.C., CHIONG, E. & CORI, O. (1987) Hydrolysis of bismetallic complexes of linalyldiphosphate and their participation in the biosynthesis of cyclic monoterpenes. *J. Chem. Soc. Perkin Trans.* I, pp. 1479-1484.
53. PORTILLA, G. & CORI, O. (1988) Biosynthesis of cyclic monoterpenes: participation of bivalent cations in the C-O splitting process. *Rev. Lat. Quím.* en prensa.

Publicaciones de carácter general

1. CORI, O. (1970) Profesión y formación del Bioquímico en Chile. *Rev. Med. Chile 98,* 168-174.
2. CORI, O. (1975) Basic science as a premise for the transfer of technology. En Orrego, F. (ed.). Proceedings of the Transpacific Seminar on Science and Technology Transfer and Acquisition for National Development, pp. 52-57.

3. CORI, O. (1976) Investigación básica y enseñanza universitaria. En Cori, O. (ed.). *Las Ciencias Naturales en Chile. Visión Crítica y Perspectiva.* Ediciones C.P.U. Nº 36, pp. 77-109. Santiago.

4. CORI, O. (1978) Ciencia básica y transferencia de tecnología. *Interciencia 3,* 38-42.

5. CORI, O. (1978) La investigación científica como valor cultural. *Rev. Med. Chile. 106,* 132-137.

6. CORI, O. (1979) La Química Orgánica: Ausente. *Rev. Chilena Educ. Quím. 4,* 99-101.

7. CORI, O. (1980) Investigación científica y humanismo. *Realidades 2,* 41-45.

8. VICUÑA, J.R. & CORI, O. (1981) Biochemistry in Chile. *Trends Biochem. Sci. 9,* III-IV.

9. CORI, O. (1982) Francisco Hoffmann, Profesor de Fisiología (1902-1981). *Rev. Med. Chile 110,* 181-188.

10. CORI, O. (1982) Homenaje a Francisco Hoffmann. En *Figuras Señeras de la Ciencia Chilena* (Ed. Instituto de Chile, Academia de Ciencias). Nº 1, pp. 25-61.

11. CORI, O. (1984) Citation of prior discovery. *Accounts Chem. Res. 17,* 346.

12. CORI, O. (1983) La Química, una disciplina formativa. *Rev. Col. Quím. Farm. 39,* 9-13. Reimpresa en *Rev. Chilena Educ. Quím.* (1983) *8,* 16-20. Reimpreso en *Academia* 5/6, 175-181.

13. CORI, O. (1983) ¿Abolir la investigación en la Universidad? *Interciencia 8,* 238-239.

14. CORI, O. (1983) Ciencia y Humanismo. *An. Inst. Chile,* pp. 45-62. Reimpreso en *Occidente 40,* 27-36.

15. CORI, O. (1983) Ciencia y Humanismo integrados en la Educación. *Academia 8,* 201-205.

16. CORI, O. (1983) Los cimientos de la Bioquímica. *Bol. Pedagog. Univ., P. Univ. Cat. Chile, 17,* 73-79.

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Introducción

Aún tenemos Bioquímica, ciudadanos..., así **versa** la dedicatoria que Osvaldo me escri**bió** en septiembre de 1981, en un artículo **pub**licado en *Trends in Biochemical Sciences* junto a Rafael Vicuña. Esta frase es, a **mi** juicio, una brevísima muestra de la **titán**ica labor de Osvaldo Cori por la inves**tig**ación y la enseñanza de la Bioquímica **en** Chile. Esta "cruzada" que Osvaldo man**tuv**o toda su vida, la de mantener, educar **y** predicar la responsabilidad de los científicos para con la ciencia y la población de Chile, hicieron de él un ser querido por algunos e incomprendido por otros.

No pretendo enumerar los logros científicos y educativos de Osvaldo, para eso existen sus trabajos publicados. Sólo unas palabras para quienes no lo conocieron. Recibe el grado de médico cirujano, pero desde muy temprano en esos estudios su inclinación era la investigación científica. Ingresa a la Cátedra de Fisiología donde inicia los primeros pasos de lo que más tarde será su carrera como bioquímico. Más de alguna vez él dijo: "La última vez que vi un enfermo fue para mi examen de grado"... La medicina en Chile, en esos años, era el camino natural para llegar a la investigación científica. El hecho de que la medicina fuese el único sendero disponible para los estudiantes con inclinaciones por la investigación lleva a Osvaldo a impulsar lo que fue uno de sus mayores logros como universitario: la creación de la carrera de Bioquímica. No es exagerado decir que las nuevas generaciones de bioquímicos en Chile le deben su *status* a la visionaria tarea del Dr. Cori. Los primeros adiestramientos de bioquímicos se inician en la Facultad de Química y Farmacia, Universidad de Chile, en 1957. Los primeros graduados de la carrera de Bioquímica en 1962 y las posteriores generaciones son la semilla, junto a la "vieja guardia de bioquímicos", para la creación, al principio de la década del setenta, de los primeros programas de doctorado en Chile. En la continua búsqueda de expandir los horizontes del bioquímico, Osvaldo participa activamente en la educación del legislador y gobernante...; él decía: "cada vez que hay cambio de gobierno, tenemos que ir a conversar con el Ministro de Educación y plantearle la importancia del desarrollo científico en Chile", y así lo hizo. Esta aparente indefinición política trajo más de algún sinsabor a la pipa que fumaba. En esta constante lucha por la educación del significado de la ciencia a la comunidad no científica lleva al Dr. Cori a relacionarse con todas las universidades chilenas, las cuales, finalmente, cooperan a la creación de la Comisión Chilena de Investigación Científica y Tecnología en 1967. Osvaldo participa activamente y en esa época llega a ser vicepresidente de esta organización. Años más tarde es nombrado su presidente, cargo que tuvo hasta el momento de su fallecimiento.

Volvamos un poco al pasado, a esos años de Osvaldo en que sale de la Facultad de Medicina con una enseñanza de quien fue su tutor, y a quien jamás olvidará, el profesor Francisco Hoffmann.

Un paso decisivo en su vida profesional fue el hacerse cargo de la Cátedra de Bioquímica de la Facultad de Química y Farmacia. Es ahí donde termina de abandonar la Fisiología para imponer un tinte químico-bioquímico a su investigación. En su capacidad de profesor de esa Facultad y activo investigador con un laboratorio pletórico de actividad, me asoció a su grupo en enero de 1968. Menciono esta fecha para poner en perspectiva los futuros comentarios que haré. Me voy a referir a Osvaldo Cori como un amigo-educador, como un verdadero maestro, el cual me enseñó mucho más allá del metabolismo intermediario y las reacciones químicas celulares. He decidido contarles algunas anécdotas de su laboratorio para ilustrar el grado de amistad, comprensión y entereza que Osvaldo, como hombre, tenía para quienes éramos sus discípulos. Al llegar al laborato-

rio de Osvaldo, en el segundo piso, existía la sala de seminario-comedor-preparación de clases-biblioteca-sala de café-sala de juegos y la funcionalidad más temida de todos, sala de exámenes finales. Osvaldo se sentaba a la cabecera, a su mano derecha la o el ayudante más antiguo. Siempre próximo a él estaban los más "viejos". A continuación, los alumnos tesistas manteniendo la distancia adecuada. En el extremo opuesto, el último estudiante que tan sólo esperaba temporalmente haciendo una pasantía en el laboratorio. Esta jerarquización de los puestos permitía a los más cercanos a Osvaldo una fructífera discusión científica, política, artística. Por otro lado, el sueño del estudiante que entraba al laboratorio era el "avanzar" hasta llegar a sentarse al lado del Dr. Cori; esto implicaba que uno ya podría ser escuchado... Los almuerzos así eran siempre "presididos" por Osvaldo. Su almuerzo consistía en forma diaria de las así famosas "galletas de Osvaldo". Tienen que haber sido de un alto valor nutritivo. Fue curioso observar los momentos en que ocurría la transición, del trato formal de "Dr. Cori" a Osvaldo. Tal situación tenía lugar más o menos al llegar al tercio próximo a la cabecera de la mesa. Era un paso difícil, pero era el signo inicial que el Dr. Cori le daba al estudiante un futuro amistoso o tormentoso en sus relaciones.

Me hubiese gustado reproducir, finalmente, algunas cartas que él me escribió. La verdad es que habría que censurar ciertos pasajes de ellas, pues Osvaldo manejaba el idioma castellano en toda su extensión y propiedad, usando ocasionalmente palabras fuera de salón.

Quisiera terminar estas palabras recogiendo el sentir de todos quienes conocimos a Osvaldo Cori más allá del profesor. Para muchos de nosotros sigue siendo lo que él firmaba en sus cartas en vez de su nombre: "el chufinga". Es por eso que finalizo este escrito diciéndole al "chufinga" que su labor y su comprensión como investigador se proyectará más allá de la generación que directamente tuvo la suerte de cooperar con él. Para ti, Osvaldo, un adiós con la simpleza que tú comprendes de un verdadero amigo.

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Reactions in aqueous colloidal assemblies*

Reacciones en agregados coloidales en agua

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Micelles, formed by self assembly of aqueous surfactants, can bind reactants and control rates and products of reaction. Binding to ionic micelles is governed by coulombic, dispersive and hydrophobic interactions, and typically cationic micelles speed, and anionic micelles inhibit, bimolecular reactions of anionic nucleophiles. The rate enhancements are due largely to concentration of two reactants at the micellar surface, but micelles can also control rates of spontaneous reactions. These rate effects can be analyzed by estimating reactant concentrations at the micellar surface. Other colloidal assemblies, such as microemulsion droplets and synthetic vesicles, behave similarly to micelles in their effects upon chemical reactivity. These colloidal species can also influence product-regio-and stereoselectivity in chemical reactions.

Biological reactions occur at interfaces, and the high rates and specificities of these reactions has prompted a search for model systems which mimic, at least to some extent, the biological catalysts. The term "Biomimetic Chemistry" has been coined to describe this general subject, and "Membrane Mimetic Chemistry" is that aspect of the subject which is related to reactions at interfaces between colloidal particles or membranes and solvents (Fendler, 1982).

Many enzymic reactions take place in hydrophobic pockets of the protein, and one approach to the development of model systems is to examine reactions in submicroscopic particles which provide a hydrophobic environment distinct from the bulk solvent. Some of these materials are of biological origin, for example cyclodextrins are cyclic glucose oligomers which have a hydrophobic cylindrical cavity whose dimensions increase with increasing numbers of glucose units from six to eight (Komiyama and Bender, 1984). Appropriately sized substrates can enter; and react in, this cavity and cyclodextrins can catalyze reactions, often with a degree of specificity (Breslow *et al.*, 1983; Fornasier *et al.*, 1987; Menger and Ladika, 1987).

Synthetic cavitands are cyclic ethers or amines which provide cavities which take up solutes with a high degree of specificity (Cram *et al.*, 1984; Schneider *et al.*, 1987). Other general classes of biomimetic agents are assemblies of synthetic or naturally occurring amphiphiles. Anphiphiles have both polar or ionic residues and apolar, hydrophobic, groups (Lindman and Wennerström, 1980). Synthetic surfactants are typical amphiphiles, and some single chain surfactants are shown below.

C_{16} H_{33} N Me_3 Cl, cetyl (hexadecyl) trimethylammonium chloride, CTACl
C_{14} H_{29} N Me_3 Br, myristyl (tetradecyl) trimethylammonium bromide, MyTABr
C_{12} H_{25} OSO_3 Na, sodium lauryl (dodecvl) sulfate, SDS, NaLS.

The long chain alkyl groups are generally $C_{10}H_{21}$ or longer, and surfactants are monomeric at low concentrations in water, but above the so-called critical micelle concentration, cmc, they self assemble in water to form micelles.

The cmc in water decreases with increasing length of the alkyl group and is ca. 10^{-3} M for C_{16} surfactants (Mukerjee and Mysels, 1970). Added salts decrease the cmc of ionic surfactants by decreasing the coulombic repulsions between the head groups.

Surfactants which have two hydrophobic alkyl groups, C_{14} or longer, are generally

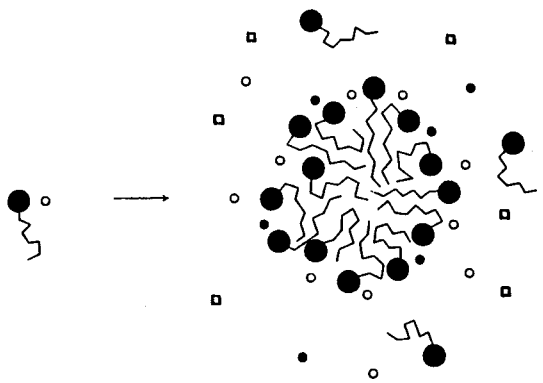
* This article is dedicated to the memory of Dr. Osvaldo Cori, who was for many years my valued coworker and very close friend.

insoluble in water, but they form bilayered vesicles on sonication or by solvent evaporation (Fendler, 1982; Cuccovia *et al.*, 1982). Vesicles are especially interesting because they have exterior and interior surfaces, and can, in principle, compartmentalize reagents (Moss and Hui, 1983; Moss and Schreck, 1983; Moss *et al.*, 1987B).

Micelles are in rapid equilibrium with monomer, which enters the micelle at a rate close to that of diffusion control, and they have highly mobile structures which depend upon the surfactant concentration and the presence of ionic and nonionic solutes (Lindman and Wennerström, 1980; Lang and Zana, 1987). Added solutes also enter the micelle at a rate close to that of diffusion control.

Formation of an ionic micelle is illustrated in Scheme 1. In the presence of added electrolyte, counterions of the electrolyte, denoted by small closed circles, will exchange with the counterions of the surfactant, open circles, but the added coins, denoted by open squares, will be repelled by the micelle.

SCHEME 1



Monomeric surfactant Micelle + free and bound ions

It is relatively easy to control the rates of reactions by the use of aqueous micelles and considerable progress has been made in delineating the factors responsible for the rate effects (Romsted, 1984; Bunton, 1984; Bunton and Savelli, 1986).

The following discussion will consider only relatively slow thermal reactions in aqueous micelles, so that reactants in water will be in equilibrium with those bound to the micelle (Romsted, 1977, 1984). Most quantitative treatments assume that the micelle acts as a pseudophase and provides a reaction medium distinct from water.

The Pseudophase Model

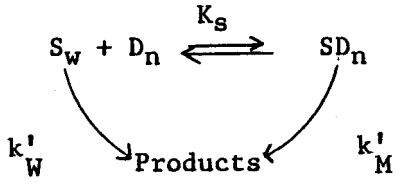
The treatment of spontaneous reactions is very simple if the substrate, S, is so dilute that it does not perturb the structure of the micelle, and its binding to the micelle can be estimated (Sepúlveda *et al.*, 1986).

The concentration of micellized, surfactant (detergent, D_n) is given by:

$$[D_n] = [D] - cm \qquad (1)$$

Where [D] is total concentration and that of monomeric surfactant is the critical micelle concentration, cmc. Reaction follows Scheme 2:

SCHEME 2



Where S_w is substrate in the water, K_s is a binding constant and k'_w and k'_M are first order rate constants in aqueous and micellar pseudophases. The observed first order rate constant is given by:

$$k\psi = \frac{k'_w + k'_M K_s [D_n]}{1 + K_s [D_n]} \qquad (2)$$

(Bunton, 1984; Romsted, 1984.)

It is easy to understand these micellar rate effects, at least qualitatively. Polar solutes, bind at, or close to, the micellar surface, which is less polar than water (Ramachandran *et al.*, 1982). Therefore aqueous micelles typically inhibit spontaneous deacylations and S_N reactions that are speeded by polar, solvents, and assist

decarboxylations and reactions that are fastest in apolar solvents (Romsted, 1984; Bunton and Savelli, 1986).

Eq. 2 has to be modified for bimolecular, nonsolvolytic, reactions whose rate depends upon the concentration of a reagent, e.g., a nucleophile, N, at the micellar surface. This concentration is conveniently expressed as a mole ratio of N to surfactant, so that:

$$k'_W = k_W [N_W] \text{ and} \qquad (3a, b)$$

$$k'_M = k_M m_N^s$$

where $m_N^s = [N_M] / [D_n]$ and W and M denote aqueous and micellar pseudophases, with N in excess over S:

$$k_\psi = \frac{k_W [N_W] + k_M K_s [N_M]}{1 + K_s [D_n]} \qquad (4)$$

with constant [N] values of k_ψ go through maxima with increasing $[D_n]$, as predicted by eq. 4. In favorable cases $[N_M]$ can be measured experimentally, and eq. 4 fits the data very well (Romsted, 1984; Bunton, 1984; Bunton and Savelli, 1986).

Eq. 4 has a simple form, but values of k_M cannot be compared directly with those of k_W, because concentrations are expressed in different units (eq. 3a and 3b). But rate constants at the micellar surface, k_2^m, $M^{-1} s^{-1}$ are given by eq. 5:

$$k_2^m = k_M V_M \qquad (5)$$

where V_M is the molar volume of the region in which reaction takes place. This volume could be that of the micelle, or of a reaction region at the micellar surface, and it probably depends upon the surfactant and the nature of the reaction. Estimates of V_M range from approximately 0·14-0·38 L (Martinek *et al.*, 1977; Quina and Chaimovich, 1979; Romsted, 1984; Bunton and Savelli, 1986) and I use the lower value.

Regardless of the chosen value of V_M values of k_2 and k_W for reactions of organic nucleophiles generally are within one order of magnitude, which suggests that concentration of reactants at the micellar surface is the main source of the rate enhancements (Table I). We can draw an analogy between rate enhancements due to the binding of two reactants to a micelle, and the high rates of intra- as compared to inter-molecular reactions (Kirby, 1980), which arise from bringing reagents into close proximity (Page and Jencks, 1971; Menger, 1985).

Another important conclusion is that hydrophobicity of reactants affects reactivity in micelles by changing the relative concentrations in the aqueous and micellar pseudophases, rather than by affecting the second order rate constants at the micellar surface.

The next question is that of binding of hydrophilic counterions. In favorable cases the concentrations at the micellar surface can be estimated electrochemically, or spectrophotometrically (Bunton *et al.*, 1977; Sepúlveda and Cortés, 1985; Abuin and Lissi, 1986; Olea and Lissi, 1986). But for some chemically interesting ions, e.g., OH^-, direct measurements are not feasible. In addition the concept of ion

TABLE I

Reactions of organic nucleophiles

Reaction			Surfactant	k / k_W
$C_6H_{13}CO_2C_6H_4NO_2$(4)	+	areneimidazolide ion	CTABr	ca. 10^a
$C_6H_{13}CO_2C_6H_4NO_2$(4)	+	areneimidazole	CTABr	ca. 10^{-2a}
$MeCO_2C_6H_4NO_2$(4)	+	PhS^-	CTABr	0·42[b]
$C_6H_3F(NO_2)_2$(2, 4)	+	$PhNH_2$	CTABr	0·17[c]
$C_6H_3F(NO_2)_2$(2, 4)	+	$PhNH_2$	SDS	0·12[c]

[a]Martinek *et al.*, 1977; [b]Cuccovia *et al.*, 1978; [c]Bunton and Savelli, 1986.

binding may have no strict physical meaning, because for counterions that bind largely coulombically concentration will decrease gradually with increasing distance from the micellar surface (Gunnarsson *et al.*, 1980).

It is not surprising that different experimental methods give different estimates of the extent of ion binding. For example ion-selective electrodes sense ion activities in the aqueous media, whereas quenching probes sense ions at or close to the micellar surface. As a result electrochemical and light scattering estimates of ion-binding may differ considerably (Sepúlveda and Cortés, 1985; Biresaw *et al.*, 1985).

The first general treatment of micellar effects was based on the assumption that ions competed for the micellar surface according to e.g. 6 (Romsted, 1984):

$$K_X^N = [N_W]\,[X_M] / ([N_M]\,[X_W]) \qquad (6)$$

where N^- and X^- are respectively reactive and inert counterions. The second key assumption was that, β, the fractional coverage of the micellar surface by counterions, was independent of their nature or concentrations. (The fractional micellar ionization, $\alpha = 1 - \beta$). It was then possible to calculate the concentration of N at the micellar surface in terms of β and K_X^N. This treatment fitted a great deal of experimental data for both reaction rates and equilibria and in a slightly different form has been applied to mixtures containing more than two competing counterions (Quina *et al.*, 1980).

Although this model fits a great of kinetic data it has serious problems. First, it is not readily applicable to mixtures of counterions of different valence, although it describes competition between chemically similar dianions (Lissi *et al.*, 1986) and modifications of the simple model have been discussed (Abuin *et al.*, 1983; Lissi *et al.*, 1984). Second, it generally fails for reactions of relatively concentrated counterions, and, reaction of OH^- in the water with micellar-bound substrate has been postulated (Nome *et al.*, 1982; Stadler *et al.*, 1984). Third, β depends upon the counterion and is much higher for Br^- ($\beta \approx 0{\cdot}8$) than for OH^- ($\beta \approx 0{\cdot}5$) (Athanassakis *et al.*, 1985). The simple model is predicted to fail if β for the predominant counterion is very low, as it is for OH^- (Hall, 1987). This conclusion is consistent with evidence on reactions in mixtures of hydrophilic anions (Broxton and Sango, 1983).

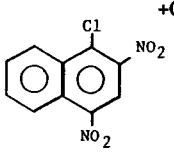
A variant of the ion-exchange model is based on the assumption that β is not constant and that counterions bind to micelles according to a Langmuir isotherm (Bunton *et al.*, 1981; 1983; Rodenas and Vera, 1985) and seems to be better than the original model in fitting rate data for reactions in relatively concentrated OH^- (Cipiciani A., Germani R., Moffatt, J.R. and Savelli G., unpublished results).

Coulombic contributions to ion-binding can be calculated by solving the Poisson-Boltzmann equation (PBE). This equation can be solved analytically for plane surfaces, but for micelles and similar colloids the solution involves numerical integration and equations based on the cell model of solutions. (Mille and Vanderkooi, 1977; Gunnarsson *et al.*, 1980). The treatment involves several assumptions, e.g., ions are treated as point charges and the micellar surface is assumed to be smooth and uniform, but it can be applied successfully to micellar effects upon ionic reactions (Bunton and Moffatt, 1985; Rodenas and Ortega, 1987), if a term for specific binding is added (Bunton and Moffart, 1986, 1987).

These models share the assumption that micelles act as a pseudophase, but they differ in some key respects. Ion-exchange equations are linear in concentration, whereas the PBE allows coulombic and specific interactions to have different dependence upon ion concentration. The ion-exchange models assume a sharp cut-off between the reaction regions provided by the micelle and water, and the PBE model does not involve this explicit assumption. Despite these differences the various models lead to similar general conclusions about the source of micellar rate effects. Some examples are given in Table II.

TABLE II

Reaction of hydrophilic anions[a]

Reaction		Surfactant			
			I	II	III
$(PhO)_2PO{\cdot}OC_6H_4NO_2$ (4)	OH^{-b}	CTABr	0·15	0·2	0·12
	$+OH^{-b}$	CTABr	1·9	3	1·6
		CTACl	2·3	2·8	1·6
$Ph\ SO_3\ Me + Cl^{-c}$		CTACl		0·8	0·9
$Ph\ SO_3\ Me + Br^{-c}$		CTABr		1·7	1·9

[a]Models; I, ion exchange, II, Langmuir isotherm, III, by solution of PBE; [b]Bunton and Moffatt (1986); [c]Al-Lohedan *et al.* (1983); Moffatt (1987); Bunton and Moffatt (1987).

Reactions in functional micelles

Reactive groups can be incorporated into the surfactant and its micellization generates functional micelles which typically have the reactive groups at their surfaces, and often give large rate enhancements that in favorable cases are comparable in magnitude to some enzymic rate enhancements (Kunitake and Shinkai, 1980). Typical reactive groups include amino, hydroxyl, thiolate, imidazole, oximate and hydroxamate, and they can react nucleophilically with a variety of organic substrates.

These rate enhancements are readily understandable. The functional group introduces a new reaction path, relative to that in water, and it generally reacts in its deprotonated form and cationic micelles should increase deprotonation. Most importantly the concentration of the functional group is very high at the micellar surface, and this effect can be predicted on the assumption that, as in nonfunctional micelles, $k_2^m \approx k_W$. In a functional micelle every surfactant head group carries a reactive group which is typically a nucleophile, N, and for fully micellar-bound substrate the first order rate constant is given by:

$$k\psi = k_M = k_2^m / V_M \qquad (7)$$

because $m_N^s = 1$, eq. 3b, and if $k_2^m \approx k_W$

$$k\psi \approx k_W / V_M \qquad (8)$$

where k_W is the second order rate constant for the bimolecular reaction of the nonmicellized functional reagent in water and on the assumption that $V_M \approx 0{\cdot}14\ L\ M^{-1}$:

$$k\psi \approx 7kW \qquad (9)$$

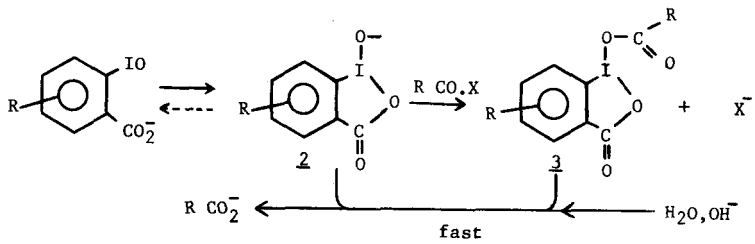
Deacylation, dephosphorylation and nucleophilic attack upon nitrohaloarenes and performed carbocations have been examined, and the rate enhancements fit the model reasonably well (Fornasier and Tonellato, 1980; Bunton, 1984). These results show how high reactant concentrations at a surface give rate enhancements, and they should also be important in enzymic reactions.

It is difficult to develop a catalytic system, because generally the functional groups are stoichiometric reagents, and turnover is slow. Two turnover catalysts have recently been developed. Iodosobenzoate ions have the cyclic structure, *2*, and acylation or phosphorylation is rapid (Scheme 4) (Moss *et al.*, 1983, 1986).

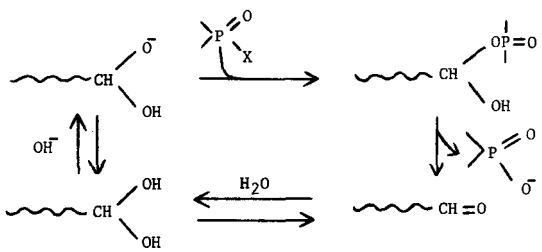
The first formed covalent intermediate (*3*) is then rapidly decomposed and the catalyst is regenerated.

The second system involves nucleophilic attack by diolate ion which is in equilibrium with the *gem*-diol and the aldehyde (Menger and Whitesell, 1985) (Scheme 5).

SCHEME 4



SCHEME 5



Menger and coworkers (1987) have also shown that cupric ion complexes of hydrophobic diamines are very effective dephosphorylating agents.

Rate effects depend upon the difference in the free energies of initial and transition states. The pseudophase model factors out the free energy contribution of increased reactant concentration and the rate constant at the surface. An alternative approach combines transition state theory with solution thermodynamics to predict the dependence of the rate constant of the overall reaction with concentration of the reactive ion, and makes predictions about micellar effects upon reactions of hydrophilic counterions (Hall, 1987). These two models are analogous to discussions of enzymic catalysis in terms of substrate binding to the active site in the proximity of various functional groups which interact with the substrate, or the alternative which simply considers binding of substrate, transition state and products (Knowles, 1987).

Effects on reaction products

Micelles, can give large rate enhancements for many nucleophilic reactions but they are poor enzymic models in that they are generally very unspecific, although they can discriminate between reactions of different molecularity. For example, cationic micelles speed bimolecular E2 eliminations by OH^- from alkyl halides, and inhibit spontaneous S_N1 reactions, and they can change the course of reaction from substitution to elimination (Bunton *et al.*, 1974).

Efforts to control product or regiospecificity in a variety of reactions of the same molecularity include halogenations (Jaeger and Robertson, 1977; Onyivruka *et al.*, 1983; Bianchi *et al.*, 1984), alkene mercuration (Sutter and Sukenik, 1984), borohydride reduction (Nikles and Sukenik, 1982) and oxidation (Jaeger and Frey, 1982). Micelles can control the direction of nucleophilic attack upon carbocations formed by ionization of chiral alkyl arene sulfonates or by deamination of chiral alkylamines (Moss *et al.*, 1973; Okamoto *et al.*, 1975; Sukenik and Bergman, 1976).

Functional micelles derived from amino acids give limited stereoselectivity in deacylations of chiral esters (Bunton and Savelli, 1986). Moss and coworkers (1987A) have investigated this problem in great detail, and have observed a high degree of diastereoselectivity in deacylations, and they have used molecular dynamics to model the interactions of the initial and transition states with the micelle, and thereby rationalize the observed stereochemical control. Micelles will also control the stereochemistry of reduction of propellane carbonyl derivatives by borohydride ion (Natrajan *et al.*, 1987).

These recent developments in micellar control of stereochemistry illustrate the potential of micelles and similar colloids in the development of synthetic methods,

and the way in which the surface of a submicroscopic colloid can control transition state geometry.

ABBREVIATIONS

α: degree of fractional micellar ionization; β: fractional micellar coverage by counterions; β: $1-\alpha$; cmc: critical micelle concentration; [D]: stoichiometric concentration of surfactant (detergent); [Dn]: concentration of micellized surfactant; K_X^N: ion exchange constant for ions of the same charge; K_S: binding constant of solute based on concentration of micellized surfactant; $k\psi$: observed first-order rate constant; k_W': first-order rate constant (s^{-1}) in aqueous pseudophase; k_W: second-order rate constant ($M^{-1}s^{-1}$) in aqueous pseudophase; k_M': first-order rate constant (s^{-1}) in micellar pseudophase; k_M: second-rate constant (s^{-1}) in micellar pseudophase with concentration expressed as a mole ratio; k_2^m: second-order rate constant ($M^{-1}s^{-1}$) in micellar pseudophase, $k_2^m = k_M V_M$; V_M- molar volume element of reaction the micelle; m_N^S: mole ratio of N in the micelle; [ ]: molarity in terms of total solution volume.

ACKNOWLEDGMENTS

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REFERENCES

ABUIN, E.; LISSI, E. (1986) *J. Colloid Interface Sci., 112:* 178.

ABUIN, E.; LISSI, E.; BIANCHI, N.; MIOLA, L.; QUINA, F. (1983) *J. Phys. Chem., 87:* 5166.

AL-LOHEDAN; BUNTON, C.A.; MOFFATT, J.R. (1983) *J. Phys. Chem., 87:* 332.

ATHANASSAKIS, V.; MOFFATT, J.R.; BUNTON, C. A.; DORSHOW, R.B.; SAVELLI, G.; NICOLI, D.F. (1985) *Chem. Phys. Lett. 115:* 467.

BIRESAW, G.; McKENZIE, D.C.; BUNTON, C.A.; NICOLI, D.F. (1985) *J. Phys. Chem., 89:* 5144.

BRESLOW, R.; TRAINOR, G.; VENO, A. (1983) *J. Am. Chem. Soc., 105:* 2739.

BROXTON, T.J.; SANGO, D.B. (1983) *Aust. J. Chem., 36:* 711.

BUNTON, C.A. (1984) In The Chemistry of Enzyme Action, Chap. 13 (M.I. Page, editor). Elsevier, Amsterdam.

BUNTON, C.A.; MOFFATT, J.R. (1986) *J. Phys. Chem., 90:* 538.

BUNTON, C.A.; SAVELLI, G. (1986) *Adv. Phys. Org. Chem., 22:* 213.

BUNTON, C.A.; MOFFATT, J.R. (1987) *Ann. Chim. (Rome) 77:* 117.

BUNTON, C.A.; KAMEGO, A.A.; NG, P. (1974) *J. Org. Chem. 39:* 3469.

BUNTON, C.A.; OHMENZETTER, K.; SEPULVEDA, L. (1977) *J. Phys. Chem., 81:* 2000.

BUNTON, C.A.; GAN, L. - H.; MOFFATT, J.R.; ROMSTED, L.S.; SAVELLI, G. (1981) *J. Phys. Chem., 85:* 4118.

BUNTON, C.A.; GAN, L. - H.; HAMED, F.H.; MOFFATT, J.R. (1983) *J. Phys. Chem., 87:* 336.

CRAM, D.J.; DICKER, I.B.; LAUER, M.; KNOBLER, C.B. (1984) *J. Am. Chem. Soc., 106:* 7150.

CUCCOVIA, I.M.; SCHROTER, E.M.; MONTIERO, P.M.; CHAIMOVICH, H. (1978) *J. Orga. Chem., 43:* 2248.

CUCCOVIA, I.M.; QUINA, F.H.; CHAIMOVICH, H. (1982) *Tetrahedron, 38:* 917.

FENDLER, J.H. (1982) Membrane Mimetic Chemistry, Wiley-Interscience, New York.

FORNASIER, R.; TONELLATO, U. (1980) *J. Chem. Soc. Faraday Trans. 1, 76:* 1301.

FORNASIER, R.; RENIERO, F.; SCRIMIN, P.; TONELLATO, U. (1987) *J. Chem. Soc. Perkin Trans. 2:* 193.

GUNNARSSON, G.; JOHNSON, B.; WENNERSTROM, H. (1980) *J. Phys. Chem. 84:* 3114.

HALL, D.G. (1987) *J. Phys. Chem.*, in press.

JAEGER, D.A.; ROBERTSON, R.E. (1977) *J. Org. Chem., 42:* 3298.

JAEGER, D.A.; FREY, M.R. (1982) *J. Org. Chem., 47:* 311.

KIRBY, A.J. (1980) *Adv. Phys. Org. Chem., 17:* 183.

KOMIYAMA, M.; BENDER, M.L. (1984) In The Chemistry of Enzyme Action, Chap. 14 (m.I. Page, editor) Elsevier, Amsterdam.

KNOWLES, J.R. (1987) *Science, 236:* 1252.

KUNITAKE, T.; SHINKAI, S. (1980) *Adv. Phys. Org. Chem., 17:* 435.

LANG, J.; ZANA, R. (1987) In Surfactant Solutions. New Methods of Investigation Chap. 8 (R. Zana, editor) Marcel Dekker, New York.

LINDMAN, B.; WENNERSTROM, H. (1980) *Top. Curr. Chem., 87:* 1.

LISSI, E.A.; ABUIN, E.B.; SEPULVEDA, L.; QUINA, F. (1984) *J. Phys. Chem., 88:* 81.

LISSI, E.; ABUIN, E.; CUCCOVIA, I.M.; CHAIMOVICH, H. (1986) *J. Colloid Interface Sci., 112:* 513.

MARTINEK, K.; YATSIMIRSKI, A.K.; LEVASHOV, A.V.; BREZEIN, I.V. (1977) In Micellization, Solubilization and Microemulsion, Vol. 2, p. 489 (K.L. Mittal, editor). Plenum Press, New York.

MENGER, F.M. (1985) *Acc. Chem Res., 18:* 128.

MENGER, F.M.; WHITESELL, L.G. (1985) *J. Am. Chem. Soc., 107:* 707.

MENGER, F.M.; LADIKA, M. (1987) *J. Am. Chem. Soc., 109:* 3145.

MENGER, F.M.; GAN, L.H.; JOHNSON, E.; DURST, D.H. (1987) *J. Am. Chem. Soc., 109:* 2800.

MILLE, M.; VANDERKOOI, G. (1977) *J. Colloid Interface Sci., 59:* 211.

MOFFATT, J.R. (1987) Thesis, University of California, Santa Bárbara.

MOSS, R.A.; TALKOWSKI, C.J.; REGER, D.W.; POWELL, C.E. (1973) *J. Am. Chem. Soc., 95:* 5215.

MOSS, R.A.; HUI, Y. (1983) *Tetrahedron Lett., 24:* 1961.

MOSS, R.A.; SCHRECK, R.P. (1983) *J. Am. Chem. Soc., 105:* 6767.

MOSS, R.A.; ALWIS, K.W.; BIZZIGOTTI, G.O. (1983) *J. Am. Chem. Soc., 105:* 681.

MOSS, R.A.; KIM, K.Y.; SWARUP, S. (1986) *J. Am. Chem. Soc., 108:* 788.

MOSS, R.A.; HENDRICKSON, T.F.; UEOKA, R.; KIM, K.Y.; WEINER, P.K. (1987A) *J. Am. Chem. Soc., 109:* 4363.

MOSS, R.A.; BHATTAGHARYA, S.; SCRIMIN, P.; SWARUP, S. (1987B) *J. Am. Chem. Soc.*, in press.

MUKERJEE, P.; MYSELS, K.J. (1970) Critical Micelle Concentrations of Aqueous Surfactant Systems. National Bureau of Standards, Washington, D.C.

NATRAJAN, A.; FERRARA, J.D.; YOUNGS, W.J.; SUKENIK, C.N. (1987) *J. Am. Chem. Soc.*, in press.

NOME, F.; RUBIRA, A.F.; FRANCO, C.; IONESCU, L.G. (1982) *J. Phys. Chem., 86:* 1881.

NIKLES, J.A.; SUKENIK, C.N. (1982) *Tetrahedron Lett., 23:* 4211.

OKAMOTO, K.; KINOSHITA, T.; YONEDA, H. (1975) *J. Chem. Soc. Chem. Commun. 922.*

OLEAS, A.; LISSI, E.A. (1986) *Langmuir, 2:* 216.

ONYIVRUKA, S.O.; SUCKLING, C.J.; WILSON, A.A. (1983) *J. Chem. Soc., 68:* 1678.

PAGE, M.I.; JENCKS, W.P. (1971) Proc. Natl. Acad. Sci. USA. *Perkin Trans. 2:* 1103.

QUINA, F.H.; CHAIMOVICH, H. (1979) *J. Phys. Chem., 83:* 1844.

QUINA, F.H.; POLITI, M.J.; CUCCOVIA, I.M.; BAUMGARTEN, E.; MARTINS-FRANCHETTI, S.M.; CHAIMOVICH, H. (1980) *J. Phys. Chem., 84:* 361.

RAMACHANDRAN, C.; PYTER, R.A.; MUKERJEE, P. (1982) *J. Phys. Chem., 86:* 3198.

RODENAS, E.; VERA, S. (1985) *J. Phys. Chem., 89:* 513.

RODENAS, E.; ORTEGA, F. (1987) *J. Phys. Chem., 91:* 837.

ROMSTED, L.S. (1984) In Surfactants in Solution, Vol. 2, p. 1015 (K.L. Mittal and B. Lindman, editors) Plenum Press, New York.

SCHNEIDER, H. - J.; BUSCH, R.; KRAMER, R.; SCHNEIDER, U.; THEIS, I. (1987) In Nucleophilicity, Chapter 31 (j. M. Harris and S.P. McManus) Advances in Chemistry Series, American Chemical Society, Washington, D.C.

SEPULVEDA, L.; CORTES, J. (1985) *J. Phys. Chem., 89:* 5322.

SEPULVEDA, L.; LISSI, E.; QUINA, F. (1986) *Advances Colloid Interface Sci., 25:* 1.

STADLER, E.; ZANETTE, D.; REZENDE, M.C.; NOME, F. (1984) *J. Phys. Chem., 88:* 1892.

SUKENIK, C.N.; BERGMAN, R.G. (1976) *J. Am. Chem. Soc., 98:* 6613.

SUTTER, J.K.; SUKENIK, C.N. (1984) *J. Org. Chem., 49:* 1295.

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Solvolysis of metal complexes of prenyl diphosphates. Its role in monoterpene biosynthesis.

Solvólisis de complejos metálicos de prenil difosfatos. Su rol en la biosíntesis de monoterpenos.

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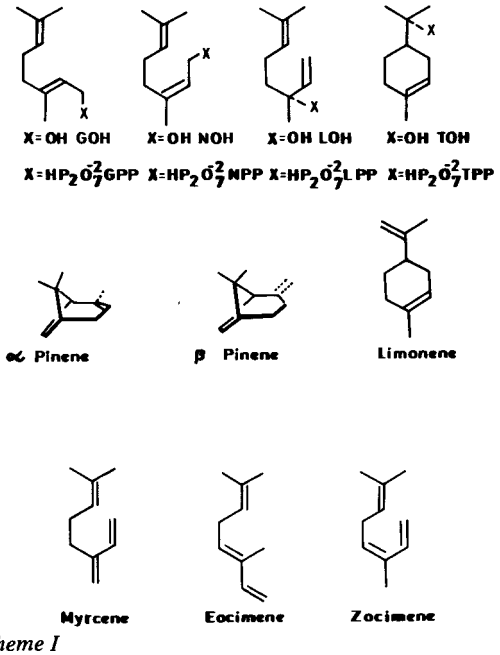
Solvolysis of [1−^{3}H] GPP and [1−^{3}H] NPP in sodium dodecyl sulfate is enhanced in the presence of Mn^{2+}. Elimination products, hydrocarbons, are favoured as compared with those obtained in water.

In some reactions of the metabolism of terpenes, solvolysis is a competitive process. Evidence is given that alcohols and hydrocarbons formed through a solvolytic reaction could interfere both in the qualitative and quantitative determination of the biosynthetic products.

INTRODUCTION

A wide variety of terpenic compounds constituents of essential oils, are found in the plant kingdom. Among them the major and simpler, with the exception of isoprene C_5H_8, are monoterpenes (C_{10}). It is possible to find several functional groups such as hydrocarbons $C_{10}H_{16}$, alcohols $C_{10}H_{15}OH$, aldehydes, ketones, esters, ethers either cyclic or open chain.

The pattern of biosynthesis of these compounds was hypothetically rationalized on the basis of the known chemical structures and reactions well before any biochemical studies were available (1). They would arise through cyclization of nerol, or alternatively of linalool, being the proposed pathways, with some exceptions (2), verified by latter work. Although it must be considered that the amount of monoterpene in a plant in a determined phase of biological cycle is genetically determined (3, 4) and that an enzyme is involved, this enzyme is only catalyzing a chemically feasible reaction. Thus, α- and β-pinene (Scheme I) occur in almost all *Pinus* species while limonene is the major compound in *Citrus limonum* and *Citrus sinensis* (5). On the other hand citronellal is predominant in



Scheme I

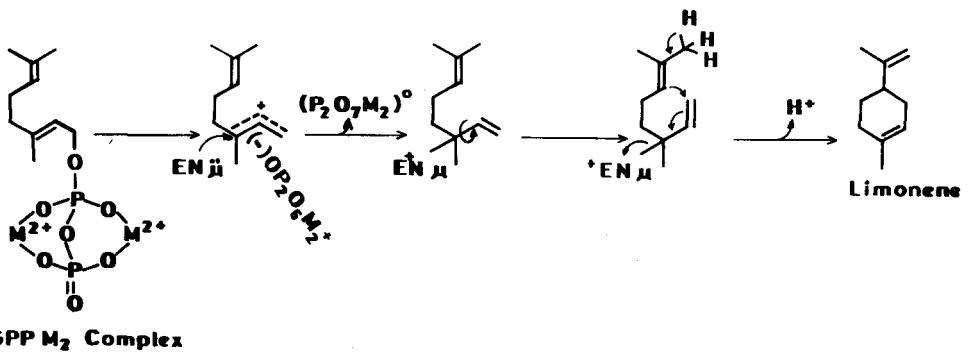
Eucaliptus citriodora and 1,8 cineole in *Eucaliptus globulus labilis* (6).

On the basis of the first biosynthetic experiments, neryl diphosphate, NPP, was accepted as the more likely direct precursor for the biosynthesis of cyclic monoterpenoids (7-11). Chemical experiments were in line with those results (12, 13).

Expressing adequately my admiration and affection for and my indebtedness to Osvaldo Cori I find beyond my abilities. He alone can know its extent. Thanks Osvaldo.

During the last fifteen years, however, it has been shown that geranyl diphosphate, GPP, is as good, or even a better substrate than NPP (14-16). Isomerization between them has been excluded (8, 17-19) and no free intermediates have been isolated (20, 21). Conversion of isopentenyl diphosphate, IPP, into GPP, NPP, or higher isoprenoids, is evidenced by the presence of prenyl transferases (22, 23). Cell free extracts from *Citrus sinensis* or *Pinus radiata* seem to have stereospecific prenyl transferases which could generate these two isomers and the corresponding C_{15} isomers (24, 25).

The E conformation of GPP around the C_2-C_3 double bond does not have the appropriate structure for cyclization. The formation of the cyclohexanoid ring from an acyclic precursor requires proximity between C_1-C_6 to form the new bond. To rationalize this steric problem, a reaction sequence in which the E isomer must be transformed to a suitable intermediate for cyclization was implied (8, 26). A new version is in Scheme II. It must first react with the enzyme with concomitant C-O fission to afford an ionic species or a bound linalyl intermediate. An enzyme bound linalyl structure would permit rotation around the C_2-C_3 bond which enables the GPP precursor to attain the appropriate conformation for cyclization. Carbocyclases from *Citrus limonum* have been described that require bivalent metals which would contribute to this process. Nucleophiles such as cysteine and methionine would stabilize either the ion pair or the linalyl structure (27). LPP is efficiently converted to cyclic compounds either in *Citrus, Mentha* or *Salvia* species (21, 28-30).



Scheme II
Biosynthesis of a cyclic hydrocarbon from GPP through an ion pair or a linalyl structure.

Evidence for this stage of terpene metabolism comes from a study of the chemical reactivities of GPP, NPP and of the tertiary derivative LPP. (30-32). Some additional properties of these compounds are here reported.

Experimental procedures

Tritium-labeled [1–^{3}H] GPP, [1–^{3}H] NPP and [1–^{3}H] LPP were prepared by phosphorylation of the corresponding labelled prenols (33). Sodium dodecyl sulfate, SDS, was recrystallized three times from ethanol. The hydrolysis of the substrates in the presence of Mn^{2+} was performed either in water or in SDS in glass stoppered tubes in 3×10^{-3} M TES buffer pH 7 or 6.7 at 30°, containing the metal ion at the corresponding pH. The reactions were stopped by adding NaCl, thus disrupting the micelle and vigorous stirring with 2 ml hexane extracted the organic products. The product concentration was estimated as has been described (14, 31).

Carbocyclase was partially purified from the flavedo of *Citrus limonum* and assayed to obtain initial reaction rates for 3.5 min at 30° in 50×10^{-3} M TES buffer pH 7 in a final volume of 1 ml (sp. act, 2-7 nmol/min/mg). Hydrocarbons were separated from prenols by absorbing the latter on silicic

acid, and were identified by G.L.C. coupled to a gas phase Geiger counter on Apiezon L and ββ' - oxydipropionitrile columns (33).

RESULTS AND DISCUSSION

Chemical properties of allylic diphosphate:

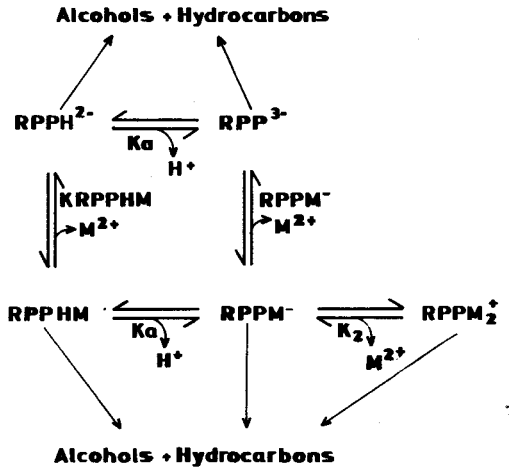
Some of the significant chemical properties of GPP, NPP and LPP are centered on their diphosphate groups and on their allylic character. Allylic esters hydrolyze with C-O bond cleavage by an apparent acid-catalyzed S_{N1} mechanism (12, 13). The pKa value of $GPPH^{2-}$ in 0.1 M KCl is 6.55 and pKa values of NPP and LPP are in the same range (30). Solutions of these esters are markedly unstable at pH<7, the rate of hydrolysis being pH dependent (30, 32). The dianion is the major component at this pH.

The tertiary allylic diphosphate, LPP, is much more labile at neutral pH than the primary isomers. Its rate constant, ko = $1{,}2\times10^{-4}$ sec^{-1} at 30° and pH 7, which means that LPP is 67% hydrolyzed in 90 min.

As has been described for farnesyl diphosphate and chrysanthemyl diphosphate (34, 35) bivalent cations such as Mg^{2+} and Mn^{2+} enhance the hydrolysis of these allylic esters at pH 7 with C-O fission. Although monometallic and bismetallic species are formed, the actual reacting species is the bismetallic complex $RPPM_2$ (30-32).

From the pKa values of $RPPH^{2-}$ it may be estimated that at pH 7 ca. 36% of the esters are in the form of the dianion $RPPH^{2-}$, which binds only weakly to the metal ion. The trianion RPP^{3-} makes an important contribution to the total binding to form the monometallic species. Scheme III includes the reactive species formed on adding bivalent metal ions.

The apparent dissociation constant for GPP-Mn_2, Kdiss = $1{,}5\times10^{-3}$ M, is lower by two orders of magnitude than the respective constants for NPP and LPP. Thus, while GPP predominantly exists as a bismetallic complex, NPP and LPP exist as mono and bismetallic species over a wide range of metal concentration, probably due



Scheme III
Binding of bivalent cations to differently protonated ligand species.

to steric factors which hinder the binding of a second metal ion (36). Considering that an anionic surfactant effectively does not bind negative substrates, such as RPP^{3-}, $RPPH^{2-}$ and $RPPM^{-}$ but could bind the neutral RPPHM or the bismetallic $RPPM_2$ the solvolysis of these species in SDS was studied.

The dependence of the rate constant of solvolysis of NPP on $[Mn^{2+}]$ in water and in the presence of 30×10^{-3} M SDS is shown in Fig. 1. In the absence of metal ion the rate constant is the same either in the presence of SDS or in water, but as $[Mn^{2+}]$ increases, the rate constant increases

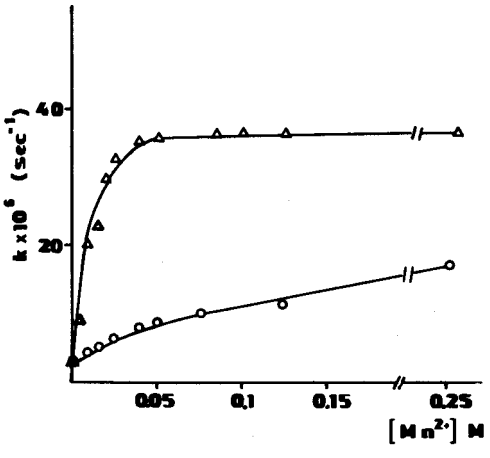


Fig. 1: Mn^{2+} – catalyzed solvolysis of NPP in water O-O and in SDS 30×10^{-3} M △–△; [NPP] = 2.6×10^{-6} M.

but only reaches a limiting value in the presence of SDS.

The metallic cation binds very strongly to micellar SDS which will favour formation of neutral or cationic complexes with a prenyl diphosphate, the latter being responsible for the plateau observed. Although GPP behaves similarly to NPP in the absence of metal, with increasing Mn^{2+} concentration saturation is reached in both water and SDS (Fig. 2). If the micelle is only concentrating the components one would expect to reach the same limiting rate constant at high $[Mn^{2+}]$. Therefore it is necessary to explain this kinetic difference.

Table I shows the product distribution obtained in these experiments. For GPP the products are qualitatively and quantitatively essentially the same in the presence and absence of SDS but the percentage of cyclic alcohols and hydrocarbons is greater than from acid catalyzed solvolysis (12-13).

Nevertheless, in SDS for NPP there is a remarkable enhancement of the cyclic hydrocarbons limonene and terpinolene, as compared with reaction in water. From reported data (30) it is possible to calculate that at pH 6.7 Kdiss NPPMn = 2.7×10^{-5} M *and Kdiss* $NPPMn_2$ = *0.9 M. So, in water* and at $[Mn^{2+}]$ = 0.25 M, only 28% of the substrate is as bismetallic complex, and the

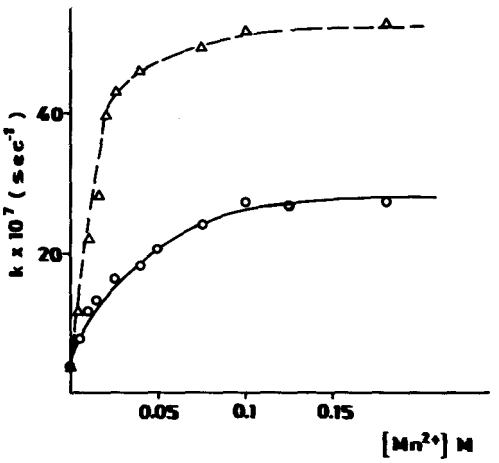


Fig. 2: Mn^{2+} – catalyzed solvolysis of GPP in water O-O and in SDS 30×10^{-3} M △–△; $[GPP] = 6 \times 10^{-6}$ M.

rest is as monometallic complex. In water the products are derived largely from the monometallic species and do not differ considerably from those obtained at acidic or neutral pH. The products obtained in the presence of SDS probably come largely from the bismetallic complex which would favour hydrocarbon formation. In the bismetallic complex the two metal ions are, certainly, disminishing the negative charge of the leaving diphosphate group but, it is possible that at the same time this group may be protecting the cation from water attack and assisting the elimination.

TABLE I

Solvolysis Products of Geranyl and Neryl Diphosphate*

	GPP – Mn^{2+}		NPP – Mn^{2+}	
	water	SDS	water	SDS
Alcohols	96	95.2	94.6	75
Hydrocarbons	4	4.5	6.3	23.3
Linalool	72.5	70	16.5	14.3
Terpineol	3.4	3.2	65.8	62
Nerol	–	–	10.3	–
Geraniol	20	22	2	–
Myrcene	1.8	2.1	–	–
Ocymenes	1.1	0.7	–	–
Limonene	0.8	1.0	4.2	17.7
Terpinolene	0.2	0.7	2.1	5.6

* At 25°, Tes 3×10^{-3} M pH 6.7
$[GPP] = 11 \times 10^{-6}$ M and $[Mn^{2+}] = 40 \times 10^{-3}$ M.
$[NPP] = 14 \times 10^{-6}$ M and $[Mn^{2+}] = 25 \times 10^{-2}$ M.
$[SDS] = 30 \times 10^{-3}$ M.

Solvolysis of LPP and its role in the biosynthetic process

The possible intermediacy of linalyl diphosphate in the biosynthesis of cyclic monoterpenes was suggested on the basis of seasonal fluctuation of linalool in *Citrus* species (37). There are several reports of linalool formation from mevalonic acid, or from IPP or GPP (8, 28, 38, 39), but there is also evidence against its participation. A linalyl derivative is formed from [4 - ^{14}C] IPP by enzymes from *Citrus sinensis* (8) and from [1 - ^{14}C, 1 - ^{3}H] GPP by enzymes from *Mentha spicata* (39). As shown above and elsewhere (13, 36) the major product of solvolysis of GPP in the presence of bivalent metal ions is linalool. Thus the observed linalool in the biosynthetic process could have been a solvolysis product.

Together with the study of the biosynthesis of cyclic hydrocarbons from LPP in a partially purified enzyme from *Citrus limonum*, we studied the chemical reactivity of LPP in the presence of Mn^{2+} (30). LPP increases its rate of solvolysis 6 fold at 30° in the bismetallic complex (k_{LPPMn_2} = 7.2×10^{-4} sec^{-1}) which is the true substrate of carbocyclase.

A serious difficulty is presented if this reactivity of LPP is not properly considered. Fig. 3 shows the relationship between the concentration of LPP and the rate of hydrocarbon formation in the reaction catalyzed by carbocyclase, and in its absence. As the substrate concentration increases, the enzymatic reaction rate reaches a typical saturation value, and the non enzymatic reaction rate continues to increases.

The formation of hydrocarbons at two different points of the plot is shown in Fig. 4. At [LPP] = 0.5×10^{-6} M (Fig. 4(A)), α and β-pinene, limonene and γ-terpinene are obtained whereas, at [LPP] = 31.8×10^{-6} M (Fig. 4(B)) there are, in addition, myrcene and ocimenes, the latter coming from solvolysis of LPP (Fig. 4C). Similar artefacts could be obtained if the reaction is prolonged beyond 10 min or if the enzymatic activity is too low. It is possible that these "unusual effects" were not perceived in the experiments performed with enzymes from *Salvia officinalis* (29).

Although the preferential incorporation sequence LPP>GPP>NPP into some cyclic monoterpenes in cell-free extracts is an

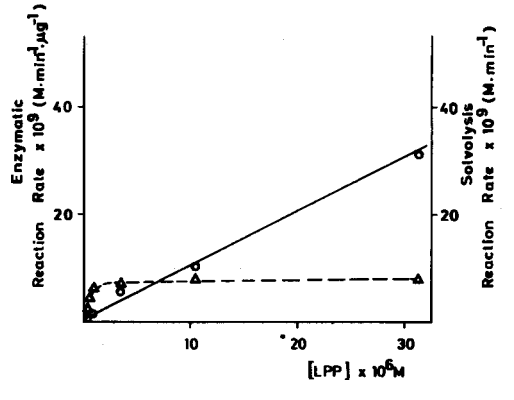


Fig. 3: Effect of the concentration of LPP over the rate of LPP carbocyclase reaction △–△ and the rate of solvolysis of LPP O-O. $[Mn^{2+}] = 9\times10^{-4}$ M.

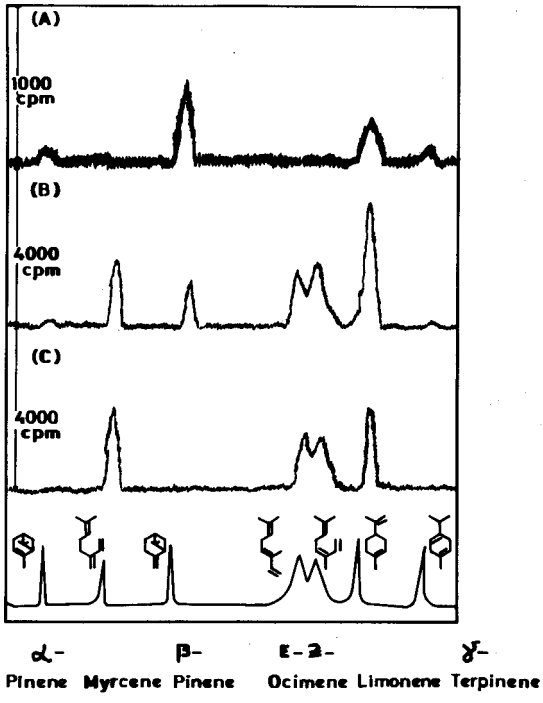


Fig. 4: Hydrocarbon formation from [1 – ^{3}H] GPP by carbocyclase from *Citrus limonum* and by non enzymatic solvolysis at 3,5 min and 30°. The upper tracings indicate response of the radioactivity monitored by means of an attached gas-phase Geiger counter, whereas the smooth lower tracings indicate response to coinjected standards detected by GLC.

(A) Carbocyclase reaction at [LPP]=0.5×10^{-6} M

(B) Carbocyclase reaction at [LPP]=30×10^{-6} M

(C) Non enzymatic solvolysis at [LPP]=30×10^{-6} M

argument for the role of LPP as a precursor, the evidence is not conclusive. On the one hand LPP may be a good alternative substrate, and on the other, LOH that in some occasions has been detected in vitro, may emerge from solvolysis of GPP and not necessarily from LPP.

The nature of the direct precursor of the LOH biosynthesized by some higher plants is not known. Should GPP be the precursor then, besides an isomerization to LPP with subsequent hydrolysis by phosphatases (cleavage of O-P), there may be other explanations of its formation: attack by water at the allylic center of GPP with departure of the diphosphate group as in an S_N2' reaction or through a cationic or ion pair mechanism.

Abbreviations used: (See Scheme I)

GPP : Geranyl diphosphate C_{10} Δ^2 E, Δ^6
NPP : Neryl diphosphate C_{10} Δ^2 Z, Δ^6
LPP : Linalyl diphosphate C_{10} Δ^1 , Δ^6
RPP : Free allylic diphosphate
RPPM : Monometallic complex of allylic diphosphates
$RPPM_2$: Bismetallic complex of allylic diphosphates
GOH : Geraniol
NOH : Nerol
LOH : Linalool
TOH : α-Terpineol
SDS : Sodium dodecyl sulfate
TES : N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid.

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REFERENCES

1. RUZCICKA, L.; ESCHENMOSER, A. and HEUSSER, H. (1953) *Experientia 9:* 357.
2. BANTHORPE, D.V. and CHARLWOOD, B.V. (1980) In Encyclopedia of Plant Physiology, New Series (Bell, E.A. and Charlwood, B.V., eds.) Vol. 8 pp. 185-220. Springer.
3. HEFENDEHL, F.W.; MURRAY, M.J. (1976) *LLoydia 39:* 39.
4. LAWRENCE, B.M. (1978) Doctoral Thesis Rijsuniversiteit te Groningen. Print Three, Hamilton, Ontario, Canada.
5. KEFFORD, J.F. and CHANDLER, B.V. (1970) In the Chemical Constituents of Citrus Fruits, Advances in Food Research. Supplement 2. (Chichester C.O., Mrak E.M. and Stewart G.F., eds) pp. 93. Academic Press, London.
6. JAJAM, J. (1974-1976) Anales Fac. Cienc. Quim. U. Chile. *26-28:* 115.
7. LOOMIS, W.D. (1967) In Terpenoids in Plants (Pridham, J.B., ed.) pp. 59-82. Academic Press, New York.
8. GEORGE-MASCIMENTO, C. and Cori, O. (1971) *Phytochemistry 10:* 1803.
9. BANTHORPE, D.V., EKUNDAYO, O. and ROWAN, M.G. (1978) *Phytochemistry 17:* 1111.
10. BANTHORPE, D.V., LE PATOUREL, G.N.J. and FRANCIS, M.J.O. (1972) *Biochem. J. 130:* 1045.
11. CROTEAU, R. and KARP, F. (1977) *Arch. Biochem. Biophys. 179:* 257.
12. VALENZUELA, P. and CORI, O. (1967) Tetrahedron Letters 2431.
13. CRAMER, F. and RITTERSDORF, W. (1967) *Tetrahedron 23:* 3015.
14. CHAYET, L., ROJAS, C., CARDEMIL, E., JABALQUINTO, A.M. and CORI, O. (1977) *Arch. Biochem. Biophys. 180:* 318.
15. CROTEAU, R. and KARP, F. (1979) *Arch. Biochem. Biophys. 198:* 512.
16. CROTEAU, R.; FELTON, M. and RONALD, R.C. (1980) *Arch. Biochem. Biophys. 200:* 534.
17. POULOSE, A.J. and CROTEAU, R. (1978) *Arch. Biochem. Biophys. 191:* 400.
18. BANTHORPE, D.V., BUCKNALL, G.A.; DOONAN, H.J.; DOONAN, S. and ROWAN, M.G. (1976) *Phytochemistry 15:* 91.
19. CORI, O.; CHAYET, L.; PEREZ, L.M.; DE LA FUENTE, M.; ROJAS, M.C.; PORTILLA, G. and Vial, M.V. (1980) *Mol. Biol. Biochem. Biophys. 32:* 97.
20. CROTEAU, R. (1981) In "Biosynthesis of Isoprenoid Compounds" (Porter J.W., and Spurgeon, S.L. eds). Vol. 1 p. 225 Wiley, N.Y.
21. PORTILLA, G.; ROJAS, C.; CHAYET, L. and CORI, O. (1982) *Arch. Biochem. Biophys. 218:* 614.
22. BLOCK, M.A., JOYARD, J. and DOUCE, R. (1980) *Biochim. Biophys. Acta. 631:* 210.
23. CORI, O. (1983) *Phytochem. 22:* 331.
24. DE LA FUENTE, M.; PEREZ, L.M.; HASHAGEN, U.; CHAYET, L.; ROJAS, M.C.; PORTILLA, G. and CORI, O. (1981) *Phytochem. 20:* 1551.
25. PEREZ, L.M.; LOZADA, R. and CORI, O. (1983) *Phytochemistry, 22:* 431.
26. BUNTON, C.A. and CORI, O. (1978) *Interciencia 3:* (5), 291.
27. CORI, O.; CHAYET, L.; PEREZ, L.M.; ROJAS, M.C.; PORTILLA, G.; HOLUIGUE, L. and FERNANDEZ, L.A. (1981) *Arch. Med. Biol. Exp. 14:* 129.
28. SUGA, T.; SHISHIBORI, T. and MORINAKA, H. (1980) *J. Chem. Soc. Chem. Commun.* 167.
29. GAMBIEL, H. and CROTEAU, R. (1982) *J. Biol. Chem. 257:* 2335.
30. PORTILLA, G.; ROJAS, M.C.; CHIONG, E. and CORI, O. (1987) J. Chem. Soc. Perkin Trans. I. 1479.
31. VIAL, M.V.; ROJAS, M.C.; PORTILLA, G.; CHAYET, L.; PEREZ L.M.; CORI, O. and BUNTON, C.A. (1981) *Tetrahedron 37:* 2351.

32. CHAYET, L.; ROJAS, M.C. and CORI, O. (1984) *Biorg. Chem. 12:* 329.
33. CORI, O. and ROJAS, C. (1984) *Methods Enzymol. 110A:* 406.
34. GEORGE-NASCIMENTO, C.; PONT-LEZICA, R. and CORI. O. (1971) *Biochem. Biophys. Res. Comm. 45:* 119.
35. BREMS, D.N. and RILLING, H.J. (1977) *J. Am. Chem. Soc. 99:* 8351.
36. PORTILLA, G. and CORI, O. (1988) *Rev. Lat. Quim.* In Press.
37. ATTAWAY, J.A. and BUSLING, B.S. (1969) *Phytochemistry. 8:* 1671.
38. POTTY, V.H. and BRUEMMER, J.H. (1970) *Phytochemistry. 9:* 1001.
39. SUGA, T.; HIRATA, T.; AOKI, T. and SHISHIBORI, T. (1986) *Phytochemistry. 25:* 2769.

Arch. Biol. Med. Exp. *21*: 25-31 (1988)
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Substitution and elimination reactions on phosphorylated monoterpene precursors*

Reacciones de sustitución y eliminación en precursores fosforilados de monoterpenos

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Some chemical features of monoterpene diphosphates in neutral aqueous media have been studied. Substitution by azide ion on the acyclic ester geranyl diphosphate (GPP) resulted in the formation of a product that cochromatographs with standard geranyl azide, besides alcohols normally detected under these conditions. Total rate was zero order on NaN_3 concentration, consistent with an ionic mechanism in which the nucleophile attacks after the rate-limiting step. Different ratios betwen the second order rate constant of azide and water attack on the intermediate (k_N/k_H) were obtained for GPP and GP, suggesting the participation of ion pairs.

The tertiary cyclic ester terpinyl diphosphate (TPP) was transformed into α-terpineol and a great amount of hydrocarbons. The reaction was also zero order on NaN_3. Addition of $MnSO_4$ enhanced ten times the rate of TPP transformation, and showed saturating kinetics which was interpreted as the formation of a TPP-Mn complex (Kd= 38 mM). This complex seems to be $TPPMn_2$ since it is transformed into limonene by carbocyclase, an enzyme that is known to utilyze only the bismetallic complexes of monoterpene diphosphates.

INTRODUCTION

Compounds biosynthesized from mevalonic acid (terpenes or isoprenoids) play a wide variety of functions in plants and animals such as hormonal control, light uptake in photosynthesis or interaction of a plant with its surroundings (1, 2).

Chemically speaking these molecules present some features that have encouraged the study of their reactions, and explain that the mechanims involved in terpene biosynthesis are unique within metabolism. Heterolytic reactions with C-0 fission and pyrophosphate elimination are common in this pathway, due to the fact that many terpene precursors are allylic diphosphates that may originate stable carbocations. These are not usual intermediates in biochemistry; the participation of a cationic intermediate has been clearly established only for the reaction catalyzed by some glucosidases as lysozyme (3, 4).

On the other hand, allylic derivatives are reactive species with nucleophiles such as the double bonds characteristic of isoprenic structure (Fig. 1). Olefinic intermolecular

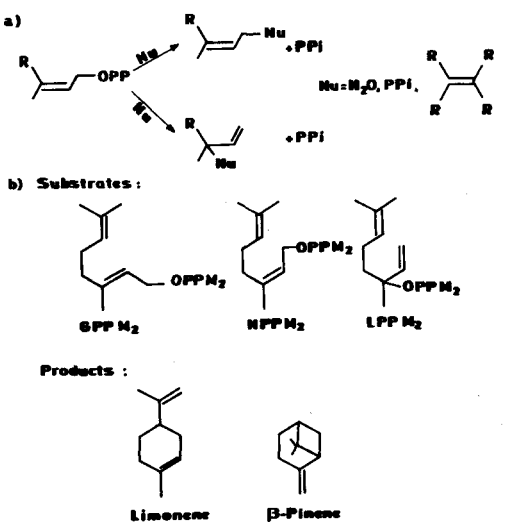

Fig. 1: a) Nucleophilic substitution on allylic diphosphates. b) Carbocyclase substrates and products.

* Dedicamos este trabajo a la memoria del Dr. Osvaldo Cori, como expresión de gratitud por las innumerables valiosas experiencias que nos dio la oportunidad de vivir.

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attack on C_1 of an allylic diphosphate results in isoprenic chain lenghthening while analogous reactions with intramolecular double bonds form the different sketetons of cyclic terpenes. The proposal of ionic mechanisms for substitution reactions in terpene biosynthesis seems reasonable considering the chemical properties of allylic derivatives (5, 6), and it has been supported by the demonstration of a mechanism of this type for the reaction catalyzed by prenyltransferase (7). Moreover, some enzymes related to triterpene and monoterpene metabolism, bind strongly ammonium and sulfonium derivatives, designed as analogs of presumptive cationic intermediates (8, 9). Nevertheless we must consider that the possibility of concerted mechanisms for some reactions of terpene metabolism cannot be excluded.

Mechanistic features common to the biosynthesis of different terpenes, such as the participation of ionic intermediates, may be studied in a simple enzymic reaction like cyclic monoterpene hydrocarbon synthesis from neryl and geranyl diphosphate (NPP and GPP, Fig. 1b). The reaction is an intramolecular nucleophilic substitution and is catalyzed by enzymes (carbocyclases) present in plant tissues that synthesize essential oils (11, 14). On the other hand, investigation of the chemical properties of carbocyclase substrates, provide additional information that help to understand biochemical problems related to terpenes. This point was clearly illustrated by the demonstration of a bismetallic $GPPMn_2$ complex as the reactive species in the non enzymic hydrolysis of GPP in the presence of Mn^{+2} (10). This information cleared the way for the understanding of bivalent metal requirement by carbocyclase and established that $GPPMn_2$ is the true substrate of the enzyme (14). The participation of bismetallic complexes could be limited to terpene biosynthesis and is probably related to the ease of pyrophosphate elimination in C-O fission reactions from allylic derivatives.

This report describes some evidence about the involvement of ionic intermediates in non enzymic substitution reactions over monoterpene diphosphates (GPP, TPP) as an approach to the understanding of carbocyclase mechanism.

MATERIALS AND METHODS

Radioactive substrates

Tritium labeled [1-^{3}H] - GPP and GP were synthesized as described (10, 11) and had a specific radioactivity of 25,7 Ci·mol^{-1}. Tritiated terpinil dipohosphate, [3-^{3}H] TPP (26,2 Ci·mol^{-1}), was prepared by phosphorylation of [3-^{3}H] - TOH obtained by acid hydrolysis of tritiated neryl phosphate esters (12).

Kinetic measurements (model reaction)

Transformation of [1 - ^{3}H] - GPP, [1-^{3}H] - GP or [3 - ^{3}H] - TPP to alcohols, hydrocarbons or azide derivatives were followed by measuring the liberation of hexane soluble radioactivity from the water soluble substrates by conventional β-scintillation spectrometry (10, 13). The reactions were carried out in 0.1 M TES-NaOH buffer at pH 7.0 and 40°. Sodium azide (Merck) was added from a stock solution at pH 7.0. Extraction controls showed that 97% of the tritiated azide derivatives were transferred to the hexane phase under the conditions employed.

Product analysis were carried out by radio-GLC (11) using a 3 mtx 0,25 inch column with 3% SE-30 as liquid phase.

Enzymic reactions

Partially purified carbocyclase (specific activity 3-5 nmol·min^{-1}·mg^{-1}) was prepared from the flavedo of *Citrus limonum* (11). Incubations were made in 0.1 M TES-NaOH buffer pH 7.0 at 30° in the presence of [1 - ^{3}H] GPP or [3 - ^{3}H] TPP and $MnSO_4$. Hydrocarbons were separated from prenols in the hexane phase by adsorbing the latter on silicic acid. Products were analyzed by radio-GLC (14).

Standard geranyl azide

Geranyl azide was synthesized and kindly supplied by Dr. Hernán Pavez from Facultad de Ciencias Químicas y Farmacéuticas Universidad de Chile. IR spectrum (between NaCl plates) showed absorptions at 3.36; 3.41 and 3.5μ (C-H); 4.75μ (N_3), 6.02 μ (C=C) and 11.4μ ($^{H}\!\!>\!C=C<$). NMR spectrum showed a contaminant that may be attributed to linalyl azide or to alkenes (myrcene, ocimene).

RESULTS

I. *Water and azide ion substitution on geranyl phosphate esters*

Tritiated geranyl diphosphate hydrolyzes in TES buffer at pH 7.0 and 40° (Fig. 2a). Figure 2b shows that when sodium azide is added to the reaction mixture, a radioactive product that cochromatographs with standard geranyl azide in GLC is formed. The rate of appearence of this derivative has a hyperbolic dependence on the concentration of NaN_3.

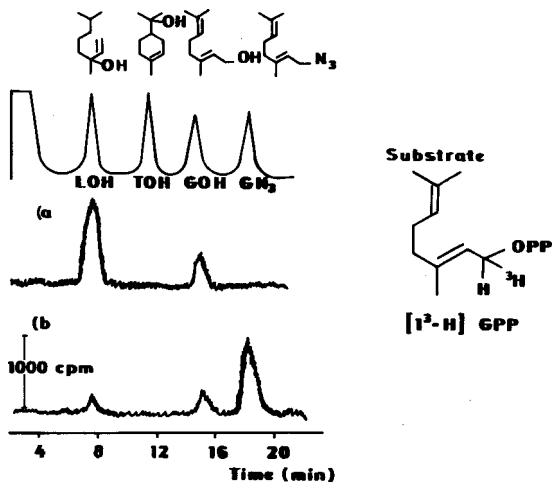

Fig. 2: Radio-GLC of products formed from [1–^{3}H] GPP. a) in 0.1M TES pH 7.0. b) equal conditions with added 1M NaN_3. GLC conditions as described, oven temperature 140°C. Upper tracing: detector response to carrier monoterpenes. Lower tracing: radioactivity detection by a gas phase geiger coupled to the chromatograph.

Total rate [rate of alcohols (ROH) plus azide derivatives (RN_3) formation] does not vary with N_3^- concentrations up to 2M (Fig. 3), even when 75% of the products are azide derivatives under the latter conditions.

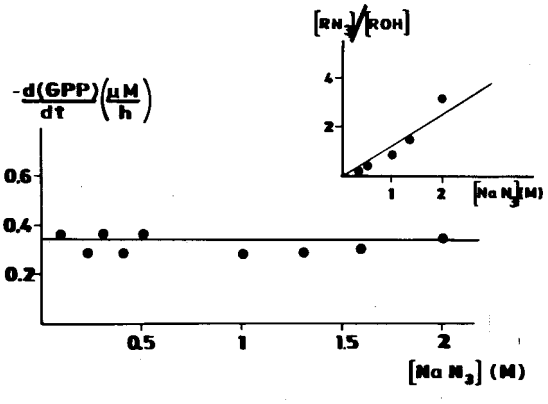

Fig. 3: Effect of NaN_3 on GPP hydrolisis. Insert: The slope of the straight line corresponds to $k_N/k_H \cdot [H_2O]$

Zero order kinetics on the nucleophile, evidences a stepwise mechanism in which the nucleophile attacks after the rate limiting step, that corresponds to the ionization of the substrate.

$$GP \underset{k-1}{\overset{k_1}{\rightleftharpoons}} I \begin{cases} \xrightarrow{k_H[H_2O]} ROH & k_N, k_H \gg k_1 \\ \xrightarrow{k_N[N_3^-]} RN_3 & \end{cases} \qquad I = \text{intermediate}$$

The ratio between the second order rate constants of N_3^- and water attack on the intermediate (k_N/k_H) can be determined from ROH and RN_3 concentrations:

$$\frac{k_N}{k_H} = \frac{[RN_3]\,[H_2O]}{[ROH]\,[N_3^-]}$$

A value of k_N/k_H of 69 was obtained for GPP (insert Fig. 3) showing that the intermediate makes little discrimination between water and azide ion, compounds that differ markedly in nucleophilicity (15).

The intermediate evidenced by the unimolecular kinetics may be a free carbocation or an ion pair in which the leaving group still interacts with the organic moiety after C-O fission. Determination of the k_N/k_H ratio for a geranyl derivative with another leaving group should throw some light at this point. The geranyl phosphomonoester (GP) reacts with NaN_3 to give the same azide derivative than GPP. Likewise the reaction is zero order on the nucleophile whereas it has a k_N/k_H ratio of 9, clearly different than the value of 69 obtained for GPP. This indicates that different intermediates are formed from both substrates (ion pairs) since a single k_N/k_H value would be expected for the free geranyl cation arising from GPP or GP.

II. *Terpinyl diphosphate (TPP) as monoterpene hydrocarbons precursor in enzymic and model reactions*

a) *Enzymic reaction*

Partially purified carbocyclase from *Citrus limonum* transforms the bis metallic com-

plexes of GPP or NPP (13, 14), into limonene and β-pinene (Fig. 1). Neryl and geranyl diphosphates have been isolated and identified as normal intermediates of terpene biosynthesis in plants (16), and the complex $NPPMg_2$ has been proposed as the direct precursor of monoterpenes *in vivo* (13).

The same enzyme preparation catalyzes the transformation of the cyclic diphosphate TPP into limonene, with a specific activity of 4 $\mu M\ min^{-1}\ mg^{-1}$ whereas the specific activity for limonene synthesis from [1−^{3}H]−GPP is 0.28 $\mu M\ min^{-1}\ mg^{-1}$. The interpretation of these findings is not clear. TPP can be either a good substrate analogue of carbocyclase or structurally similar to a catalytic intermediate. Besides, the possibility exists that TPP has a role as monoterpene precursor *in vivo* although it has not been detected in plants and no proposals concerning its biosynthesis have been made.

b) *Model reaction*

When [3−^{3}H]−TPP is incubated at pH 7.0 and 40°, it is transformed into α-TOH, limonene and terpinolene (Table I). Sixty two percent of the total products correspond to hydrocarbons, in contrast with the negligible amount of elimination products generated from the primary allylic diphosphates (NPP and GPP). In an aqueous media, alcohols are almost the only products formed from primary substrates in which substitution is not hindered (GPP, NPP). On the other hand, elimination competes efficiently with substitution on tertiary centers in which the

TABLE I

Hydrolysis products and reactivities of monoterpene diphosphates[a]

Substrate	Products (%)					k(s^{-1})
	LOH	TOH	NOH	GOH	Hydrocarbons	
TPP.....	0	38	0	0	62[b]	$0{,}5 \times 10^{-6}$
LPP[c].....	37	38	1,8	1,9	21	$2{,}4 \times 10^{-4}$
GPP.....	74	3	0	23	0	9×10^{-7}
NPP.....	16	71	5	0	7	$1{,}2 \times 10^{-6}$

a) 0.1M TES at pH 7.0 and 40°C.
b) limonene (41%) and terpinolene (21%).
c) From ref. 17.

ABBREVIATIONS

GPP: Geranyl diphosphate; TES: N-[Tris (hidroxymethyl)]-2-aminoetanesulfonic acid; ROH: Monoterpene alcohols; RN_3: Monoterpene azide derivatives; GP: Geranyl phosphomonoester; TPP: Terpinyl diphosphate; NPP: Neryl diphosphate; LPP: Linalyl diphosphate; PPi: Inorganic pyrophosphate; $RPPM_2$: Bismetallic complex of monoterpene diphosphate.

leaving group produces a considerable steric hindrance, as is the case for the tertiary derivative TPP.

Additionally, the great amount of hydrocarbons provides an evidence for heterolytic C-O fission as opposed to O-P bond cleavage that would give rise to α-TOH but not to limonene and terpinolene.

The four isomeric diphosphates GPP, NPP, TPP and linalyl diphosphate (LPP), exhibit relative reactivities according to the stability of their corresponding carbocations. The positive charge is better stabilized by allylic delocalization in a geranyl or neryl cation than in the tertiary non allylic terpinyl center. Both factors are present in the tertiary allylic diphosphate LPP, compound that has a rate constant several orders of magnitude higher than that of NPP, GPP, or TPP.

Chromatographic analysis of the products formed form TPP in the presence of 1M NaN_3 shows the appearence of a product with a longer retention time than standard geranyl azide (Fig. 4) which can be tentatively assigned to terpinyl azide, the only substitution product expected for this substrate. The reaction, carried out at constant ionic strength is zero order on the nucleophile.

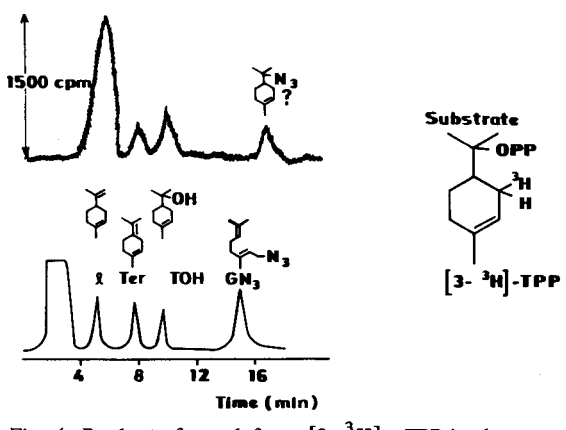

Fig. 4: Products formed from [3–^{3}H] –TPP in the presence of 1 M NaN_3 (radio-GLC analysis). Upper tracing: radioactivity. Lower tracing: carrier monoterpenes. 1 = limonene; ter = terpinolene.

III. *Effect of Mn^{+2} on terpinyl diphosphate transformations*

The rate of TPP transformation into α-TOH and hydrocarbons in the model reaction increases hyperbolically with added $MnSO_4$ (Fig. 5). The saturation kinetics can be explained as complexation of TPP into a more reactive species whose dissociation constant (K_d) can be obtained by linearization of these data (insert Fig. 5; K_d = 38 mM). This species probably is the bismetallic complex $TPPMn_2$ since $GPPMn_2$, $NPPMn_2$ and $LPPMn_2$ have been demonstrated to be the reactive species in analogous reactions (13, 14, 17).

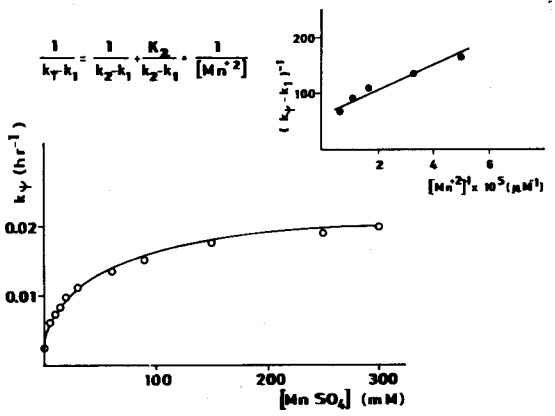

Fig. 5: Effect of $MnSO_4$ on TPP transformation in TES buffer at pH 7.0. The data fit the equation:

$$\frac{\text{rate}}{[TPP]} = k\psi = \frac{k_1K_2 + k_2[Mn^{+2}]}{K_2 + [Mn^{+2}]}$$ (see reference 17)

$k\psi$ = observed first order rate constant.

k_1 = rate constant for the TPPMn complex. ($k_1 = 10^{-6}s^{-1}$)

k_2 = rate constant for the $TPPMn_2$ complex. ($k_2 = 5{,}7 \times 10^{-6}s^{-1}$)

K_2 = diss. constant for $TPPMn_2$.

Insert: linearization of the data in the double reciprocal plot.

The kinetic rate constant of the complexed TPP species is obtained from rate data extrapolated to high Mn^{+2} concentrations at which all the substrate is metal-bound. A value of $5{,}7 \times 10^{-6}\,s^{-1}$, ten times higher than the kinetic constant for the free terpinyl diphosphate ester was obtained.

The demonstration that the reactive species on Fig. 5 is a bismetallic complex, can be accomplished by determining if its K_d agrees with that of the TPP complex

utilized by carbocyclase. This enzyme presents the peculiarity of recognizing as substrates only the bismetallic complexes of the monoterpene diphosphates GPP, NPP and LPP (13, 14, 17).

Enzymic rate data for the transformation of TPP into limonene are shown in Fig. 6 as a function of Mn^{+2} concentration. Calculated TPPMn and $TPPMn_2$ concentrations are plotted in the same figure. The dissociation constant of 38 mM (Fig. 5) was attributed to $TPPMn_2$ while a K_d of 40 μM was assumed for TPPMn, similar to the Kd value of LPPMn (17). The insert in Fig. 6 shows the rate data plotted as a function of the calculated $[TPPMn_2]$. Carbocyclase reaction rate correlates only with the concentration of this species, giving a hyperbolic curve, from which a K_m of 22 nM can be calculated. This value is similar to the K_m value already reported for carbocyclase with $LPPMn_2$ as substrate (17). Moreover the same K_m value for $TPPMn_2$ is obtained from enzymic rate data determined at variable TPP concentrations, supporting the proposal that $TPPMn_2$ is the complex evidenced in Figure 5.

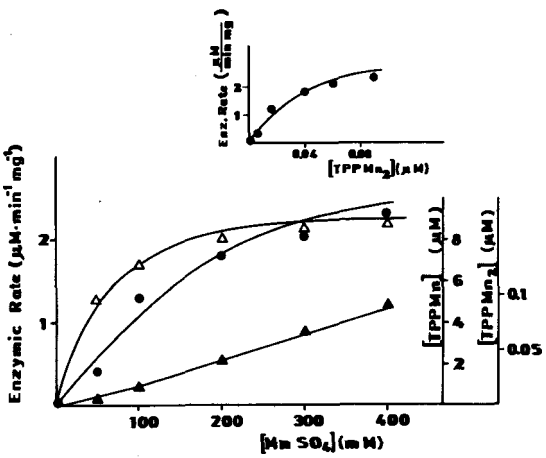

Fig. 6: $MnSO_4$ saturation curve for the enzymic transformation of TPP into limonene. TPP = 10 μM. ●—● observed rate (left ordinate); △—△ calculated [TPPMn] (right ordinate); ▲—▲ calculated $[TPPMn_2]$ (right ordinate).

DISCUSSION

Nucleophilic substitution on GPP by water or azide ion occurs through a stepwise mechanism in which the limiting step is the ionization of the substrate to give an ion pair intermediate. The positive charge is delocalized in the allylic portion of the ion pair since tertiary and primary alcohols are formed (Fig. 2). There is no certainty that geranyl azide separates from the isomeric linalyl azide under our chromatographic conditions, thus, the data presented did not let us find out the position of N_3^- attack on the intermediate.

The k_N/k_H ratio for geranyl derivatives, show that the intermediate has very low selectivity to reaction with different nucleophiles in constrast with k_N/k_H values of about 5×10^6 described for extremely stable free carbocations (18). The interpretation of k_N/k_H values in ion pair mechanisms is difficult, since many factors may influence both second order rate constants, such as stability of the cationic moiety, electrostatic repulsions and steric hindrance.

The cyclic monoterpene diphosphate TPP, has a structure much more appropriate than GPP for limonene synthesis in both enzymic and model reactions.

In the presence of Mn^{2+}, $TPPMn_2$ seems to be the reactive species for both processes. Relative utilization of $TPPMn_2$ and $GPPMn_2$ in the model reaction closely parallels the ratio of Vmax obtained with both substrates in the enzymic transformation (Table II). The E conformation of $GPPMn_2$ does not allow the formation of a C_1 - C_6 bond and thus, the allylic cation must rotate before cyclization. The energy

TABLE II

REACTION	ENZYMIC Vmax (μM $min^{-1}mg^{-1}$)	MODEL k (s^{-1})
$TPPMn_2$ → limonene	4	$1{,}68 \times 10^{-6}$
$GPPMn_2$ → limonene	0.28	$1{,}7 \times 10^{-7}$
$TPPMn_2/GPPMn_2$	14	10

barrier for this rotation has been reported to be between 11 and 29 Kcal · $mole^{-1}$ (19, 20), whereas limonene synthesis from $TPPMn_2$ is a facile reaction, that involves only proton elimination after the ionization step.

Finally, the great proportion of hydrocarbons formed from TPP in the model reaction, reflects leaving group protection from nucleophilic attack and gives additional evidence for the participation of ion pairs in the hydrolytic decomposition of monoterpene diphosphates. The findings reported here for an aqueous media, are not necessarily valid for enzymic reactions in which the limiting step as well as the mechanism can change. Thus, the role of ionic intermediates in monoterpene biosynthesis remains to be established.

ACKNOWLEDGMENTS

This work was supported by the Departamento Técnico de Investigación, Universidad de Chile (B-2661) and Fondo Nacional de Ciencia y Tecnología (Grant 0030). The participation of Miss V. Avalos and Miss M. Alarcón in the performance of some experiments presented in this report, is gratefully acknowledged. The authors are very indebted to Dr. Hernán Pavez, Facultad de Ciencias Químicas y Farmacéuticas, U. de Chile, for the synthesis of standard geranyl azide.

REFERENCES

1. CORI, O. (1976) En "Bases moleculares de la acción hormonal" Perreta, M.; Minguell, J. y Swaneck, G. (eds.). pp. 11-29. Ediciones Sede Santiago Sur. Universidad de Chile.
2. MANN, J. (1978) In "Secondary Metabolism". I Oxford Chem. Series O. Clarendon Press.
3. SMITH, L.E.H.; MOHR, L.H.; RAFTERY, M.A. (1973) *J. Am. Soc. 95:* 7497.
4. ROSENBERG, S.; KIRSCH, J. (1981) *Biochem. 20:* 3196-3204.
5. RITTERSDORF, W.; CRAMER, F. (1968) *Tetrahedron 24:* 43-52.
6. CORI, O.; CHAYET, L. and PEREZ, L.M. (1986) *J. Org. Chem. 51:* 1310-1316.
7. POULTER, C.D.; WIGGINS, P.L.; LE, A.T. (1981) *J. Am. Chem. Soc. 103:* 3926-3927.
8. CROTEAU, R.; WHEELER, C.J.; AKSELA, R.; OEHLSCHLAGER, A.C. (1986) *J. Biol. Chem. 261:* 7257-7263.
9. RAHIER, A.; GENOT, J.C.; SCHUBER, F.; BENVENISTE, P.; NARULA, A.S. (1984) *J. Biol. Chem. 259:* 15215-15223.
10. VIAL, M.V.; ROJAS, M.C.; PORTILLA, G.; CHAYET, L.; PEREZ, L.M. and CORI, O. (1981) *Tetrahedron 37:* 2351-2357.
11. CORI, O. and ROJAS, M.C. (1985) In "Methods in Enzymology". Law and Rilling Eds., Academic Press Vol. 110 part A 406-417.
12. VALENZUELA, P. and CORI, O. (1967) *Tetrahedron Letters* 3089-3094.
13. CHAYET, L.; ROJAS, M.C.; CORI, O.; BUNTON, C.A. and McKENZIE, D.C. (1984) *Bioorganic Chem. 12:* 329-338.
14. ROJAS, M.C.; CHAYET, L.; PORTILLA, G. and CORI, O. (1983) *Arch. Biochem. Biophys. 222:* 389-396.
15. RITCHIE, C.D.; VIRTANEN, P. (1972) *J. Am. Chem. Soc. 94:* 4966-4971.
16. BEYTIA, E.; VALENZUELA, P. and CORI, O. (1969) *Arch. Biochem. Biophys. 129:* 346-356.
17. PORTILLA, G.; ROJAS, M.C.; CHIONG, E. and CORI, O. (1987) *J. Chem. Soc.* Perkin Trans. I, in press.
18. BUNTON, C.A.; HUANG, S.K. (1972) *J. Am. Chem. Soc. 94:* 3536-3544.
19. ALLINGER, N.L.; SIEFERT, J.H. (1975) *J. Am. Chem. Soc. 97:* 752-760.
20. DENO, N.C.; HADDON, R.C.; NOWAK, E.N. (1970) *J. Am. Chem. Soc. 92:* 6691-6693.

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Initial stages of the oxidate-catabolism of monoterpenes and 2-phenylethanol in *Rosa damascena* Cultures.

Etapas iniciales del catabolismo oxidativo de monoterpenos y 2-feniletanol en cultivos de *Rosa damascena.*

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Tracer studies revealed that uptake of 2-phenylethanol, geraniol, nerol and citronellol of their β-D-glucosides into flowerheads of *Rosa damascena* led to rapid metabolic turn-over to produce water-soluble degradation products. Feeding experiments with mevalonate also demonstrated the instability of the monoterpene pool *in vivo* in this tissue. Use of microsomal preparations from tissue cultures of the rose indicated that the initial steps in the degradation of 2-phenylethanol involved the sequential formation of its β-glucoside and then styrene, styrene oxide and styrene diol. Geraniol and nerol were similarly monoepoxidised and the oxirane ring then was cleaved to give diols or triols as products. Citronellol was not metabolised by these preparations. The effect of specific inhibitors suggested that a cytochrome C-oxidoreductase linked to a terminal peroxidase was involved in the epoxidation system and that the subsequent hydrolytic or reductive ring opening was mediated by an enzyme with a thiol group at the active site.
A brief parallel study showed that geraniol and nerol were oxidatively metabolised by microsomes from pig liver by an overall similar route, although now a cytochrome P-450 system appeared to be implicated.

INTRODUCTION

Osvaldo Cori devoted the major part of his scientific career to the elucidation of the complicated enzymic pathways leading to the biosynthesis of the lower terpenoids in higher plants; and in this field he made numerous original theoretical and technical advances. The companion topic of the catabolism of these metabolites is however virtually uncharted. Shortly before his final illness, we planned a collaboration on such processes in the *Citrus* and *Pinus* species that he had studied so extensively; and we aimed to use tissue culture as our tool. The present paper outlines some exploratory work on related topics that we had in hand on a *Rosa* species, and is dedicated to the memory of a most distinguished biochemist, an eminent leader of Chilean science, and a much-valued friend.

Although it is generally appreciated that the pools of secondary metabolites such as monoterpenes are not static in higher plants, there is little evidence as to the mechanisms of the turn-over of these substances or of exogenously-supplied compounds such as pesticides. An obvious possible route for such compounds as possess alkenic unsaturation is *via* epoxidation, followed by hydration (to form hydroxy substituted compounds showing considerable water-solubility) and then glycollic cleavage to fragments capable of being β-oxidised to still smaller water-solubles. The required relatively or even virtually non-specific epoxidases and epoxide hydratases that could catalyse the initial steps have been characterised in many preparations from mammalian liver (Oesch, 1982; Hernández and Bend, 1982) but have much less frequently been demonstrated in plants. For the latter, most studies have probed the degradation of pesticides (Earl and Kennedy, 1975; Ross *et al.*, 1978a) but there is much contradiction in the details and the appropriate activities were not present in all of the (sometimes closely related)

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species tested (Mehendale *et al.*, 1972). In addition, cell-free extracts from whole plants and in a few cases from tissue cultures have been shown to epoxidise (and sometimes further metabolise) ethene (Dodds *et al.*, 1979), propene (Beyer, 1978), and stilbene (Ross *et al.*, 1978), and such an oxidative pathway is implicated in the biosynthesis of *inter alia* cutin (Kolattukudy and Walton, 1972). Monoterpene epoxides sometimes occur in the essential oils of higher plants (c.f. Hlubucket *et al.*, 1973) but the only enzymic studies on the formation and metabolism of these compounds consist of investigations of extracts of certain Compositae species (Banthorpe *et al.*, 1977) and brief reports of such activities in extracts of tissue cultures of *Jasminum* and *Rosa* species (Banthorpe and Osborne, 1984; Banthorpe *et al.*, 1986a). These extracts converted geraniol and nerol (together with linalool the generally-accepted parents of regular monoterpenes) as well as their C_5-precursors into mono- and sometimes di-oxides which were then converted into diols (*via* reductive cleavage of the oxirane ring) or triols (via analogous hydrolytic cleavage). These hydroxylated derivatives were then broken down *via* unspecified pathways to water-soluble fragments. The levels of such oxidative-degradation systems were seasonably dependent (Banthorpe *et al.*, 1983a) and their occurrence may mediate not only the seasonal but also the diurnal or even hourly fluctuations in the levels of monoterpenes *in vivo*. Such variations of oil content and components are well known to commercial growers (Guenther, 1954) although few quantitative investigations have been reported (c.f. Burbott & Loomis, 1967).

In the present paper, we extend our previous work of *Rosa damascena* to a preliminary study on the nature of the initial steps and of the oxidative enzyme systems implicated in the turn-over of the essential oil. It may be that such pathways of metabolism are ubiquitous in oil-producing plants: it would certainly be surprinsing if such detailed routes were restricted to one *Rosa* species chosen for study merely because of its commercial importance!

MATERIAL AND METHODS

Biological materials: substrates

Specimens of *R. damascena* were grown from cuttings provided by the Royal Botanic Gardens, Kew. Callus cultures from stem explants were initiated and propagated by conventional methods (passage time 7-8 weeks) on M and S medium at 27°C under natural illumination (max. ca. 400 lux) in a South-facing aspect. Full details of the growth patterns and morphology of similar callus lines have been published (Banthorpe *et al.*, 1986a, 1987). The callus line used in the present work was stable as regards morphology, ploidy and examined biochemical properties over the period of the experiments (ca. 2 yrs). Pig liver was obtained (within 3 hr. of slaughter) from an abbatoir.

[2–^{14}C]-Labelled geraniol, nerol and citronellol (all 4-9mCi mmole^{-1}) were prepared by standard methods (Banthorpe *et al.*, 1983b). 2-Phenylethanol (0.1 mCi mmole^{-1}) was extracted from the petals of a potted *Rosa* specimen that had been exposed to [^{14}C]–CO_2 (8 mCi; 3% CO_2 in air) in a growth chamber for 3 months. P-Glucosides of these alcohols were prepared (yields 5-20%) by the Koenig-Knorr method, and 2-phenylethyl pyrophosphate was obtained (23%) by a method adapted for the preparation of terpenyl pyrophosphates (Banthorpe *et al.*, 1983c). All the possible epoxides and hydroxylated products had been prepared for use as standards, and their chromatographic and spectroscopic parameters have been documented (Barrow, 1978; Osborne, 1979).

Turnover in vivo

Samples of the metabolites (0.1 g; 1 μCi) were emulsified (Triton X-100 or Lulvol) in MES-HCl buffer (0.2 cm^3; pH 7.0; 0.1M) and taken up into flowerheads under forced transpiration by the inverted tube technique. After the appropriate metabolism time, the petals were extracted (hexane; 2 x 10 cm^3) and the extracts were separated on TLC (SiGel H) with hexane: ethyl acetate (1:1 v/v) and assayed for radioactivity.

Cell free extracts

Extracts from plants were prepared by a recipe developed for the Compositae (Banthorpe *et al.*, 1977). Aliquots (5 cm^3) were incubated at 27°C (2 hr; to plateau region) with the appropriate substrates (0.4 mg; 1 μCi) that were emulsified (2% Triton X-100, or lubrol) in MES-HCl (1 cm^3) at pH 7.2. The NADPH-generating system (when used) involved addition of NADP (2 μmole), glucose-6-phosphate (20 μmole) and glucose-6-phosphate dehydrogenase (1 unit). Fractionation using Sephadex G-100 followed a previous procedure (Earl & Kennedy, 1975), as did the microsomal preparation from pig liver (Maynert *et al.*,

1970). Each aliquot of the latter (5 cm^3) was supplemented with geraniol or nerol (1.5 mg; 1 μCi) in ethanol or dimethyl suphoxide (0.1 cm^3) and after addition of the various co-factors or inhibitors, the contents of each tube were made up (to 10 cm^3) with phosphate buffer (pH 7.4; 0.1M) and incubated for 1 hr at 37°C.

After incubation the water-soluble products were extracted by previously worked-out techniques (c.f. Banthorpe *et al.*, 1977) and separated and assayed by TLC on SiGel H_{60} using (a) ether and (b) hexane-ethyl acetate as eluants. In addition, identification and quantification was sometimes made by GC on FFAP (10%; 5 m x 0.5 cm) or Carbowax 20 M (20%; 3 m x 0.5 cm) at 140-160° (programmed). The products from the high-yielding extracts (from liver) were routinely identified by GC-MS using authentic compounds as standards. Fractions were tested for chemical and radiochemical purity (> 98% capillary GC on Carbowax 20 M; 50 m x 0.02 mm; 80°C; 2π radioactive scanning) and assayed by LSC using Butyl-PBD (0.8% in toluene) as scintillant. 4×10^4 Disintegrations were accumulated so that 2σ was ± 1%.

RESULTS AND DISCUSSION

Our experimental material was *Rosa damascena* Mill. cv. trigintipetala Dieck. (damask rose), the source of the most expensive rose oils of commerce, e.g. attar of roses. The solvent-extractable oil (0.3% wt/wer wt.) from the flowerheads comprised 2-phenylethanol (65%) citronellol (25%), geraniol (7%) and nerol (3%) together with traces of 2(E), 6(E) - farnesol. A further quantity of the alcohols were bonded as the β-D-glucosides, mainly (> 90%) as the 2-phenylethanol derivative (Banthorpe *et al.*, 1987).

Turnover of oil components in vivo

Stem-feeding under forced transpiration of emulsions of ^{14}C-labelled 2-phenylethanol, geraniol, nerol or citronellol or of aqueous solutions of their β-D-glucosides to flowerheads in different states of development, and extraction after 1 to 24 hours metabolism periods gave erratic results. Up to 30% of the applied tracer was located in non-terpenoid water-solubles, but no epoxidised or hydroxylated monoterpenes could be detected. Feeding of [^{14}C]-mevalonate to flower buds at the state of initial splitting of the calyx led to rapid synthesis of monoterpenes and the passage of tracer into water solubles and evolved CO_2 in a more reproducible manner (Table I). The profiles closely resembled those in the only previous relevant study carried out on buds of *Rosa dilecta* (Francis and Allcock, 1969; Francis and O'Connell, 1969). It was checked that negligible amounts of monoterpenes were lost from the flowerheads by volatilisation. Stem-feeding of [^{14}C]-phenylalanine gave a similar time-profile with a maximum (ca. 6%) of incorporation of tracer at 10 hr, and passage of considerable amounts (ca. 4%) of applied tracer into water-solubles (excluding the substrate amino acid) and CO_2 at this time. These incorporation patterns may mirror the operation of salvage mechanisms that remove the unphysiologically-large quantities of exogenously-supplied metabolites which flood the storage sites and could prove

TABLE 1

Formation of monoterpenes in flowerheads of *R. damascena* stem-fed with [2-^{14}C]-mevalonate

Time, hr.(a)	% Incorporation(b)				
	Geraniol	Nerol	Citronellol	Water-solubles(c)	CO_2
2	5	2	0	1	0.1
3	19	3	1	2	1
5	25	5	3	2	1
6	12	6	5	4	3
10	6	1	5	6	4
12	3	1	4	10	5

(a) Time = 0 when aliquot was completely absorbed (time of uptake ~5 mins.).
(b) % incorporation of 3R-mevalonate. Monoterpenes were assayed as combined (free + glucosides). Standard errors ±20% recorded values.
(c) Extraction, see Experimental.

toxic unless eliminated. They clearly are consistent with the occurrence *in vivo* of the enzyme systems mentioned in the Introduction that mediate rapid turnover of the components of the essential oil.

Turnover of oil components in vitro

Attempts to establish suitably active cell-free extracts from petals of flowerheads capable of sustaining the presumed oxidative processes revealed in the previous subsection failed, and consequently we investigated callus tissue cultures as a prospective source of the enzyme systems. We have previously shown that the enzymes responsible for the formation of *inter alia* geraniol, nerol and farnesol can be extracted from callus of *Rosa* an other species with levels of activity up to 400-fold those in extracts from the parent plants (Banthorpe *et al.*, 1986a, b). This is probably not the consequence of intrinsically higher enzyme levels in the callus, but is due to the less robust cell walls and lower levels of phenolics (which complex with and deactivate proteins) in the latter making the extraction procedures less destructive. The metabolic capabilities of extracts (the S_9 supernatant) form a callus line (that accumulated neither monoterpenes nor 2-phenylethanol) towards the latter substrate and some possible alternative substrates are shown in Table II. The callus culture had been maintained for 5 to 7 passages under strictly defined conditions before harvesting (see Materials and Methods). Although the conversions recorded are low (see later) and no fragmentation or degradation to water-solubles could be detected (despite addition of a variety of possible cofactors and metal ions) the pattern is significant. All the enzymic machinery was demonstrated to be present whereby 2-phenyl ethanol is dehydrated *via* formation of its β-D-glucoside, and then the resulting styrene epoxidised and the product hydrated (Scheme 1). 2-Phenylethanol- β-D-glucoside was detected in the flowerheads, and control experiments demonstrated that extracts of callus could be obtained that could sustain its formation in adequate (ca. 8%) conversion from 2-phenylethyl pyrophosphate and UDP-glucose. The breakdown of 2-phenylethyl- β-D-glucoside (I; Scheme 1) to form styrene is unusual: β-D-glucosides usually cleave with sugar-oxygen fission, but the alternative breakage found here is presumably favoured by the presence of the phenylethyl group which can readily lose a proton concommitant with formation of the incipient carbocation to yield the alkene in one E2-like step. The only other known example of alkyl-oxygen fission in glycosides appears to be that of t-butyl- β-D-glucoside (Armour *et al.*, 1961): here the incipient carbocation is directly stabilised by the alkyl group. The occurrence and extension of the pathway of Scheme 1 *in vivo* could account for the formation of benzaldehyde and benzyl alcohol that have been recorded in several rose oils (Guenther, 1954;

TABLE 2

Oxidative modification of 2-phenylethyl derivatives by cell-free extracts from callus of *R. damascena*

Substrate[a]	% Conversions[b]			
	Styrene	Styrene oxide	Styrene-1,2-diol	Water-solubles
[^{14}C]-2-phenylethanol	0	0	0	0
[^{14}C]-2-pheylethyl-β-glucoside	5	2	2	0
Styrene	–	2	1	0
Styrene oxide	–	–	3	0
Styrene-1,2-diol	–	–	–	0

(a) Substrates solublised with Triton X-100 (1 cm^3 of 5% solution for 5 cm^3 incubation aliquot: see methods) or added in solution in dimethyl sulphoxide (20% v/v).
(b) Standard error ca. ±50% of recorded value; 0 signifies < 0.01% if any incorporation; – signifies assay inapplicable.

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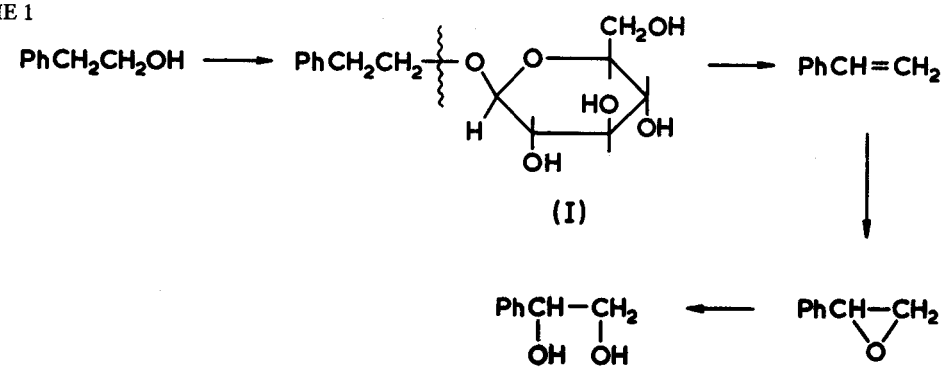
[^{14}C]-2-pheylethyl-β-glucoside	5	2	2	0
Styrene	–	2	1	0
Styrene oxide	–	–	3	0
Styrene-1,2-diol	–	–	–	0

(a) Substrates solublised with Triton X-100 (1 cm^3 of 5% solution for 5 cm^3 incubation aliquot: see methods) or added in solution in dimethyl sulphoxide (20% v/v).
(b) Standard error ca. ±50% of recorded value; 0 signifies < 0.01% if any incorporation; – signifies assay inapplicable.

SCHEME 1



Bugorskii *et al.*, 1978), although the latter compound could also directly result from β-oxidation of phenylpyruvic acid.

Unlike 2-phenylethanol, the monoterpene components of rose oil possess unsaturated sites susceptible to facile oxidative degradation and so may suffer such processes without preliminary derivatisation and modification. In the event, all of our further studies utilised these substrates. The crude cell-free extract (S_9) that was active towards 2-phenylethanol also exhibited epoxidase and epoxide hydratase activities towards geraniol and nerol to form the 2,3-oxides and diols and triols (Scheme 2; shown for geraniol) but no other products. A more detailed study showed that most (> 90%) of the total activity resided in the microsomes (pellet; 10^5 x g: 2 hr) although the balance was widespread across the lower sedimenting and supernatant fractions (owing to solubilisation with much deactivation during preparation?). A more efficient final preparation could be achieved if the crude homogenate was treated to remove polyphenolics and low molecular mass inhibitors by rapid anaerobic (under N_2) gel filtration through Sephadex G-100: this is a technique previously applied to the extraction of aldrin epoxidase (Earl and Kennedy, 1975). On this column the microsomal fraction containing the oxidation-hydration activities was completely excluded and the eluate could then be centrifuged to give the high-speed pellet which could be resuspended for enzyme assay. Such a procedure increased the measured epoxidase-epoxide hydratase activities by up to 8-fold compared with when the pellet was obtained from the crude homogenate. Unfortunately, it was not checked whether a similar procedure would have enhanced the similar enzyme activities towards 2-phenylethyl- β-glucoside, although the presumption is that it would for the steps involving the microsomal enzymes (The glucoside formation and elimination steps are probably mediated by soluble enzymes).

The results of the use of the 'improved' cell-free extract towards geraniol as substrate are in Table III: very similar values were obtained for nerol, although it is noteworthy that citronellol (no $\Delta^{2,3}$-bond) was uneffected by these systems. The β-glucosides of geraniol and nerol were also not accepted as substrates. Despite the amelioration produced by the gel filtration, the percentage conversions are

SCHEME 2

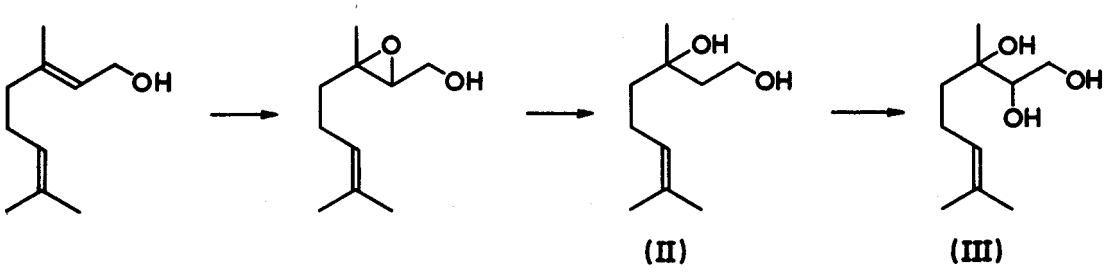


TABLE 3

Oxidative modification of [^{14}C]-geraniol by cell-free extracts from callus of *R. damascena*

Conditions [a]	Recovery [b]	% Conversions [c]		
		Epoxide	Diol	Triol
Standard ≡ RP + NADPH (ImM) [d]	90	2.4	1.0	1.3
RP (–NADPH)	96	0	0	0
Standard ($-O_2 + N_2$)	99	0.2	0	0
Standard + Cu^{++} (2mM)	98	0	0	0
Standard + Iodoacetamide (1mM)	94	2.8	0	0
Standard + SKF-525A (1mM) [e]	92	3.2	0	0
Standard + SKF-3301A (1mM) [f]	93	3.5	0	0
Standard + Cytochrome C (0.1mM)	90	0.3	0.1	0
Standard + KCN (1mM)	93	0.1	0	0
Standard, Boiled Control	97	0	0	0

(a) For preparation of extracts and incubation conditions, see Methods.
(b) Recovery (%) of unreacted substrate (± 5% of recorded value).
(c) Conversions into products in Scheme 2 (s.e. ± 30% of recorded values).
(d) RP ≡ resuspended pellet (10^5 x g, 2 hr). Use of a NADPH - generating system gave essentially the same results.
(e) β-Diethylaminoethyldiphenylpropylacetate hydrochloride.
(f) 2,2-Diphenyl-l-(β-dimethylaminoethoxy)pentane hydrochloride.

very low compared with the analogous reactions catalysed by liver extracts (next section): it is known that the endoplasmic reticulum of certain parenchyma cells are rapidly desintegrated on sectioning whereupon solubilisation of the microsomal components occurs, and it is likely that only a small proportion of the oxidate apparatus of plant cells is retained after the homogenisation and separation procedures (c.f. Earl and Kennedy, 1975).

Salient features of Table III are: (a) the requirement for NADPH and O_2; (b) the strict inhibition by Cu^{++}; (c) the powerful inhibition by KCN which suggests the implication of a terminal peroxidase; (d) the effect of cytochrome C (see later) and (e) the non-effect of the SKF drugs and iodoacetamide on epoxidation, but their inhibition of subsequent oxirane cleavage. SKF-525A, in particular, is known to effect epoxidation mediated by cytochrome P-450 systems, and all three reagents are known to inhibit enzymes with thiol groups at the active site (see later). There is no evidence that these additives ????? acting as competitive inhibitors of the epoxidases. Other findings *not* recorded in Table III are: (f) addition of NAD^+, $NADP^+$, or NADH (0.1 to 1 mM) to the NADPH-free system elicited no activity; (g) addition to the standard preparation of known inhibitors of liver epoxide hydratase such as octen-1,2-oxide, 3,3,3-trichloropropen-1,2-oxide, stilbene oxide or metyrapone (all 0.1 to 5 mM) was ineffective; (h) addition of FAD and FMN (0.1 to 1 mM) was ineffective; and (i) chelating and solubilising agents such as deoxycholate (1 mM), Triton X-100 (0.05%) and phenanthroline (1 mM) that are known to damage cytochrome P-450- dependent systems had no effect.

Observations (a), (c) and (d) closely resembled those obtained for aldrin epoxidase from pea roots from which it has been argued (Earl and Kennedy, 1975) that the epoxidation involved a NADPH-cytochrome C oxidoreductase bonded to peroxidase that acts as the terminal oxidase. Also liked the previous workers, we detected appreciable levels of soluble peroxidase in the various homogenates obtained after differential centrifugation, and these activities may represent disintegrated and solublised parts of the complex. We found no evidence (see (e), (i)) for the implication of cytochrome P-450 and none for the formation of hydroxylation products mediated by cytochrome P-450, such as the 10-hydroxy geraniol formed in cell-

free extracts of *Vinca rosea* (Madyastha *et al.*, 1976). This last compound is involved in the biosynthesis of iridoids and terpene alkaloids, and the oxidation system may well only occur in the relatively few genera of plants that produce such compounds.

Metabolism of geraniol by pig liver preparations

Mammalian liver is a known site for metabolism of pesticides and other xenobiotic compounds, and the sequence: alkene → epoxide → *cis*-diol: has been established for a variety of classes of substrates and detailed studies have been carried out on the monoxygenases involved, e.g. hepatic epoxidase (E.C. 1. 14. 1. 1), and on hepatic epoxide hydratase (E. C. 4. 2. 1. 63). These epoxidases are known to be cytochrome P-450 dependent (Oesch, 1982; Hernández and Bend, 1982). Recently, liver preparations have been reported to epoxidase and diepoxidase isoprene (Del Monte and Citti; 1985) and to hydroxylate geraniol and nerol at C-10 (Licht and Coscia, 1978). We here outline a brief study of the metabolism of geraniol by a fraction from pig liver for comparison with the plant enzyme preparation.

Table 4 records the effect of variation of incubation conditions utilising a S_9 supernatant prepared by a standard recipe (see Methods). This fraction gave essentially the same results as when a resuspended high-speed pellet (10^5 x g: 2 hr) of the microsomes was used, and no advantage was found in using the anaerobic filtration technique that was so efficacious for the plant systems: presumably phenolic-type inhibitors were not now present. Unlike the situation for the plant enzymes, now both the 2,3- and the 6,7- oxides together with some dioxide accompanied by the plethora of derived diols, triols and oligo-ols were found. But again there was no detectable cleavage to water solubles or other fission fragments. In the product assay (Table IV) the mono-oxides were individually characterised and determined but the total of the hydroxy- compounds is listed, although the product and isomer ratios were in fact elucidated with the aid of GC-MS and TLC and of authentic samples of all the putative products. Typically, the triols formed by cleavage of the 2,3- or 6,7- geraniol oxides were the predominant (ca. 90%) fractions of the total hydroxy compounds.

A salient difference between the liver and plant systems is the effect of the added epoxides and metyrapone as inhibitors of the epoxide hydratase (which allows

TABLE 4

Oxidative modification of [^{14}C]-geraniol by cells-free extracts of pig liver[a]

Conditions	% Conversions			
	2,3-oxide	6,7-oxide	Dioxide	OH compounds[b]
Standard ≡ S_9 fraction	0	0	0	10
+ NADPH (1mM)	0	0	0	40
+ NADPH gen. system.	trace	0	0	60
(−O_2, + N_2)	trace	trace	trace	trace
+ octen-1,2-oxide (0.1mM)	10	18	3	10
+ octen-1,2-oxide (1mM)	12	19	6	0
+ 3,3,3-trichloropropen-1-oxide (1mM)	10	15	3	0
+ metyrapone (1mM)	13	20	1	0
+ p-chloromercuribenzoate (0.1mM)	10	15	1	0
+ N-ethylmaleimide (0.1mM)	8	10	2	0
+ iodoacetamide (1mM)	21	10	1	0
Boiled control (std.)	0	0	0	0

(a) Most headings refer to the legends to Table 3.
(b) For composition, see text. Total recoveries (products + unreacted substrate) were 88-96%.

the epoxides to be identified as the precursors of the hydroxy compounds in the liver system). This inhibition is characteristic of the liver oxidation-degradation systems dependent on cytochrome P-450 that have been previously characterised. In addition, the detergent additives that did not affect the plant enzyme systems very much often reduced (to 10-50%) the activities of the liver systems. Such damage is characteristic of cytochrome P-450-mediated systems (Gunsalus *et al.*, 1975). However, Lubrol was one solubliser that did not appreciably affect the activity of the liver systems, and so this could be used to emulsify additives (as an alternative to the use of dimethyl sulphoxide or ethanol as a solvent for such putative substrates).

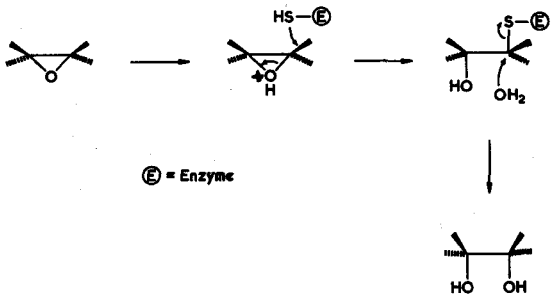
General

The main conclusions from this work are: (a) that the type of enzymes involved in the initial steps of oxidative degradation of endogenous monoterpenes (and perhaps of other terpenoids) in the *Rosa* species have been identified; and (b) that these enzymes are quite different from the corresponding systems in liver in not being dependent on cytochrome P-450. This is not unexpected as the latter pathway is rare in higher plants.

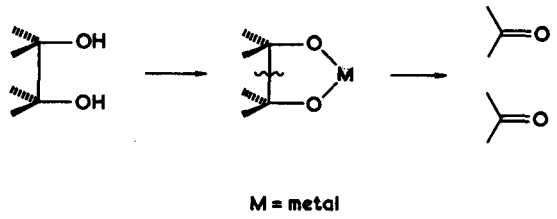
The epoxide hydratases from both plant and liver show all the characteristics, with regard to their susceptibility towards inhibitors, of enzymes with a thiol group at the active site. No cofactor requirements nor metal requirements save that of Mg^{++} have been found for this enzyme system (unpublished observations) and if the product from the *Rosa* preparations resulted from *cis* additon [as has been shown for appropriate substrates in liver systems (Oesch, 1982), and for the epoxide hydratase from *Fusarium* species (Kolattukudy and Brown, 1975)] then a mechanism involving double inversion in the ring-opening process, such as in Scheme 3 may occur.

Our *in vivo* studies indicated that exogenously-supplied or endogenously-formed monoterpenes were rapidly turned-over to give water solubles. These products have not been isolated in our *in vitro* systems which concentrated on the microsomal membrane-bonded enzymes involved with the initial water-insoluble substrates. We are currently investigating the later stages of the pathway. Here water-soluble substrates (triols, etc.) would be expected to be accepted by enzymes from the supernatant fractions of the processed homogenate, and the reactions would be anticipated to be dependent on specific metal cofactors if the cleavage occurred as in Scheme 4, analogous to that promoted in *cis*-1,2-diols by lead tetracetate, potassium permanganate, or osmium tetroxide. We are also studying the cleavage of the aromatic ring of 2-phenylethanol: this may be mediated by dioxygenases as is the degradation of catechol by extracts of a variety of plant tissue cultures (Ellis and Towers, 1970; Ellis, 1971).

SCHEME 3



SCHEME 4



ACKNOWLEDGMENTS

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REFERENCES

ARMOUR, C.A.; BUNTON, C.A.; PATAI, S.; SELMAN, L.H.; VERNON, C.A. (1961) Acid-catalysed hydrolysis of t-butyl- β-D-glycopyranoside and other glycosides. *J. Chem. Soc.*: 412-417.

BANTHORPE, D.V.; BUCKNALL, G.A.; GUTOWSKI, J.G.; ROWAN, M.G. (1977) Epoxidation and hydration of prenyl pyrophosphates by plant extracts. *Phytochemistry 16:* 355-358.

BANTHORPE, D.V.; BARROW, S.E.; OSBORNE, M.J. (1983a) Seasonal dependence of oxidation of terpenoids in *Tanacetum vulgare Z. Pflanzenphysiol. 111:* 175-177.

BANTHORPE, D.V.; LONG, D.R.S.; PINK, C.R. (1983b) Biosynthesis of geraniol and related monoterpenes in *Pelargonium graveolens. Phytochemistry 22:* 2459-2464.

BANTHORPE, D.V.; CHRISTIE, P.N.; PINK, C.R.; WATSON, D.G. (1983c) Metabolism of linaloyl, neryl, and geranyl pyrophosphates in *Artemisia annua. Phytochemistry 22:* 2465-2468.

BANTHORPE, D.V.; OSBORNE, M.G. (1984) Terpene epoxidases and epoxide hydratases from cultures of *Jasminum officinale. Phytochemistry 23:* 905-907.

BANTHORPE, D.V.; GREY, T.J.; POOTS, I.; FORDHAM, W.D. (1986a) Monoterpene metabolism in cultures of *Rosa* species. *Phytochemistry 25:* 2321-2326.

BANTHORPE, D.V.; BRANCH, S.A.; NJAR, V.C.O.; OSBORNE, M.G.; WATSON, D.G. (1986b) Ability of plant callus cultures to synthesize and accumulate lower terpenoids. *Phytochemistry 25:* 629-636.

BANTHORPE, D.V.; BRANCH, S.A.; POOTS, I.; FORDHAM, V.D. (1987) Accumulation of 2-phenylethanol by callus derwed from leaf-bred of *Rosa damascena. Phytochemistry;* submitted for publication.

BARROW, S.E. (1978) Preparation of terpene epoxides. *Ph.D. Thesis University of London:* 96-111.

BEYER, E. (1978) Rapid metabolism of propilene by pea seedlings. *Plant Physiol. 61:* 893-895.

BUGORSKII, P.S.; ZAPROMETOV, M.N. (1978) Biosynthesis of β-phenylethyl alcohol in rose petals. *Biokhimiya 43:* 2038-2042.

BURBOTT, A.J.; LOOMIS, W.D. (1967) Effect of light and temperature on the monoterpenes of peppermint. *Plant Physiol. 42:* 20-28.

DEL MONTE, M.; CITTI, L.; GERVASI, P.G. (1985) Isoprene metabolism by liver microsomal monooxygenases. *Xenobiotica 15:* 591-595.

DODDS, J.H.; MUSA, F.; JERIE, P.H.; HALL, M.A. (1979) Metabolism of ethylene to ethylene oxide by cell free preparations from *Vicia faba L. Plant Science Letters 17:* 109-114.

EARL, J.W.; KENNEDY, I.R. (1975) Aldrin epoxidase from pea roots. *Phytochemistry 14:* 1507-1512.

ELLIS, B.E.; TOWERS, G.N.H. (1970) Degradation of aromatic compounds by sterile plant tissues. *Phytochemistry 9:* 1457-1461.

ELLIS, B.E. (1971) A survey of catechol ring cleavage by sterile plant tissues. *FEBS Letters 18:* 228-230.

FRANCIS, M.J.O.; ALLCOCK, C. (1969) Geranyl-β-glucoside: occurrence and synthesis in rose flowers. *Phytochemistry 8:* 1339-1347.

FRANCIS, M.J.O.; O'CONNELL, M. (1969) Incorporation of mevalonic acid into rose petals monoteepenes. *Phytochemistry 8:* 1705-1708.

GUENTHER, E. (1952) *The essential oils,* 6 vols. Van Nostrand, N.Y.

GUNSALUS, I.C.; PEDERSEN, T.C.; SLIGER, S.G. (1975) Oxygenase-catalysed biological hydroxylations. *Ann. Rev. Biochem. 44:* 377-407.

HERNANDEZ, O.; BEND, J.R. (1982) Metabolism of epoxides in *Metabolism and basic detoxification* Ed. Jacoby, W.B.; Bend, J.R.; Caldwell, J.). Academic Press, New York: 207-208.

HLUBUCKEK, J.R.; AASEN, A.J.; ALMQUIST, S.; ENZELL, C.R. (1973) Volatile tobacco constituents of probable isoprenoid origin. *Acta Chem. Scand. 27:* 2232-2234.

KOLATTUKUDY, P.E.; WALTON, T.J. (1973) The biochemistry of plant cuticular lipids. *Prog. Chem. Fats Lipids 13:* 119-175.

KOLATTUKUDY, P.E.; BROWN, L. (1975) A soluble hydrase which catalyses *cis*-hydration from *Fusarium solani. Arch. Biochem. Biophys. 166:* 599-609.

LICHT, H.J.; COSCIA, C.J. (1978) Cytochrome P-450 LM_2 -mediated hydroxylation of monoterpene alcohols. *Biochemistry 17:* 5638-5646.

MADYASTHA, K.M.; MEEHAM, T.D.; COSCIA, C.J. (1976) Characterisation of a cutochrome P-450 dependent monoterpene hydroxylase from the higher plant *Vinca rosea. Biochemistry 15:* 1097-1102.

MAYNERT, E.W.; FOREMAN, R.L.; WATABA, T. (1970) Epoxides as obligatory intermediates in the metabolism of olefins to glycols. *J. Biol. Chem. 245:* 5234-5238.

MEHERDALE, H.M.; SKRENTY, R.F.; DOROUGH, H.W. (1972) Oxidative metabolism of aldrin by subcellular root fractions of several plant species. *J. Agric. Food Chem. 20:* 398-405.

OESCH, F. (1982) Fate of epoxides. *Adv. Exp. Med. Biol. 136A:* 39-52.

OSBORNE, M.G. (1979) Terpenoid metabolism in tissue cultures of *Jasminum officinale. Ph.D. Thesis,* University of London: 84-92.

ROSS, M.S.F.; LINES, D.S.; STEVENS, R.G.; BRAIN, K.R. (1978) Epoxidase and epoxide hydrase activity in cell cultures of *Phaseolus vulgaris. Phytochemistry 18:* 45-48.

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Avian liver mevalonate 5-diphosphate decarboxylase: substrate an inhibitor activities of the phosphorothioate analogues of adenosine triphosphate[1]

Descarboxilasa difosfomevalónica de hígado de ave: actividades como sustratos e inhibidores de los análogos fosforotioatos de adenosina trifosfato.

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The diastereoisomers of ATPαS and of ATPβS have been used as substrate analogues for avian liver mevalonate 5-diphosphate decarboxylase. When the diastereoisomers of ATPαS were used, no reversal of the stereospecificity was seen upon changing Mg^{2+} for Cd^{2+}, thus suggesting that the metal ion does not coordinate through the α-phosphoryl group of the nucleotide. Reversal of the stereospecificity, however, was observed when using the diastereoisomers of ATPβS and upon changing Mg^{2+} by Zn^{2+} as the activating metal ion. Similar competitive inhibition constants for the diastereoisomers of MgATPβS against MgATP were found. It is proposed that the active metal-nucleotide complex in catalysis is the Λ, β-γ MgATP complex.

Mevalonate 5-diphosphate (MVAPP)[2] decarboxylase (ATP : 5-diphosphomevalonate carboxyl-lyase (dehydrating) EC 4.1.1.33) catalyses the ATP-dependent decarboxylation of MVAPP in the presence of a bivalent metal cation:

$$\text{MVAPP} + \text{ATP} \xrightarrow{Me^{2+}} \text{isopentenyldiphosphate} + P_i + CO_2 + \text{ADP} \quad (1)$$

The enzyme from avian liver is composed of two subunits of 43,500 daltons each (Alvear *et al.,* 1982) and, as most nucleotide-utilizing enzymes, the true nucleotide substrate is the metal-nucleotide complex $MgATP^{2-}$ (Jabalquinto and Cardemil, 1987). Steady-state kinetic experiments suggest a sequential ordered mechanism for substrate binding, with MVAPP adding first to the enzyme (Jabalquinto, 1987), in agreement with the stereochemical course of the reaction with respect to phosphorus (Iyengar *et al.,* 1986; Jabalquinto *et al.,* 1988).

Stereochemical and structural aspects of metal-nucleotide-protein interactions have been extensively studied for several enzymes with the use of phosphorothioate analogues of the nucleotide (Eckstein, 1983; Eckstein, 1985) and inert chromium and cobalt - nucleotide complexes (Dunaway-Mariano and Cleland, 1980; Cleland, 1982). Substitution of a nonbridging oxygen by a sulfur atom in the α- and β- phosphoryl groups generates chirality at the phosphorus center, giving rise to a pair of diastereomer, R_p and S_p. Coordination of the β- and γ- phosphoryl groups in the bidentate complex of CrATP creates a chiral center at the β-phosphorus, and two screw sence isomers[3] exist, Λ, or left-handed, and Δ, or right-handed (Cleland, 1982) (Figure 1). For a given diastereomer, the metal chelates differ in their geometric

[1] This work is dedicated to the memory of Professor Osvaldo Cori, as a small recognition to his enormous contribution to the development of Biochemistry in Chile.

[2] Abbreviations: MVAPP, mevalonate 5-diphosphate; ATPαS, adenosine 5'-0-(1-thiotriphosphate); ATPβS, adenosine 5'-0-(2-thiotriphosphate); ATPγS, adenosine 5'-0-(3-thiotriphosphate); ADPβS, adenosine 5'-0-(2-thiodiphosphate); HPLC, high pressure liquid chromatography.

[3] The screw sence nomenclature is that of Cleland (1982).

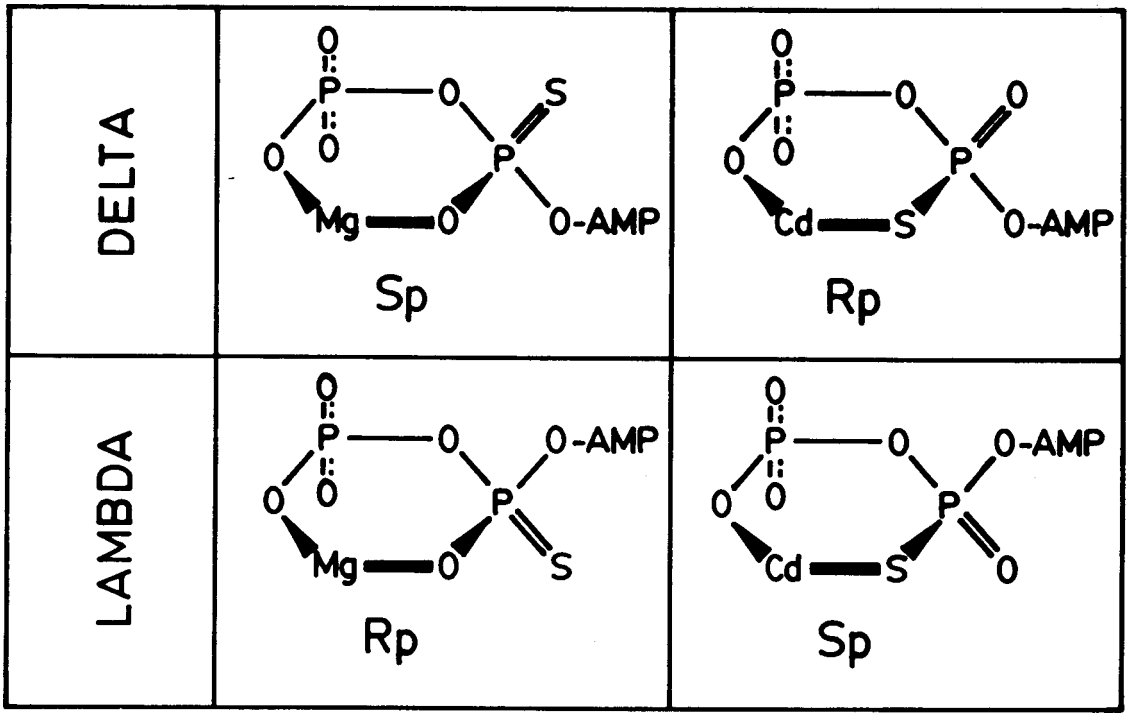

Fig. 1: Structure of β, γ-bidentate metal-ATP analogues.

configuration about the phosphorus, depending on the metal ions's preference for oxygen or sulfur ligands. From ^{31}P NMR studies, Jaffe & Cohn (1978) have shown that Mg^{2+} coordinates preferentially to the phosphoryl oxygen whereas Cd^{2+} prefers to bind to sulfur. As a consequence of this binding preference, any particular diastereomer of ATPβS or ATPαS complexes with Mg^{2+} will have the opposite stereochemical configuration of the metal chelate, Λ or Δ, compared to that with Cd^{2+}, as seen in Fig. 1. Thus, the reversal of isomer selectivity by an enzyme when Mg^{2+} is replaced by Cd^{2+} indicates that the metal ion is bound to that particular phosphorothioate group (Cohn, 1982; Jaffe & Cohn, 1979). In cases when the enzyme under study shows no activity with Cd^{2+}, this approach can also be employed by replacing Cd^{2+} for other preferentially sulfur-interacting metal ion like Zn^{2+} (Smith and Cohn, 1982; Jaffe *et al.*, 1982).

In this communication, we analyse the preference of avian liver MVAPP decarboxylase for sulfur-substituted ATP diastereomers as substrates or inhibitors for the reaction. The approach of the substitution-inert chromium- or cobalt-nucleotide complexes (Cleland, 1982) could not be employed since we had previously seen that the monodentate or bidentate complexes of CrATP are not inhibitors for the enzyme (Cardemil and Jabalquinto, 1985). A preliminary account of this work has been presented earlier (Jabalquinto *et al.*, 1986).

EXPERIMENTAL PROCEDURES

Materials. The purification of chicken liver MVAPP decarboxylase and the enzymic synthesis of MVAPP were as previously described (Cardemil and Jabalquinto, 1985). Phosphoenolpyruvate, lactate dehydrogenase, pyruvate kinase, NADH, acetylphosphate and β-mercaptoethanol were from Sigma. Aldolase, triosephosphate-isomerase, gliceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, acetate kinase and myokinase were obtained from Boehringer Mannheim. (R_p) ATPαS and (S_p)ATPαS were kindly provided to us by Dr. Fritz Eckstein. Thiophos-

phate was a gift of Dr. Radha Iyengar. All other reagents were of analytical grade.

ATPγS was synthesized enzymatically from thiophosphate and ADP, as described by Trentham and Webb (1980); ADPβS was obtained from ATPγS in the reaction of myokinase, as described by Shew *et al.* (1984); (R_p)ATPβS was synthesized from ADPβS and acetylphosphate using acetate kinase, as described by Shew *et al.* (1984); (S_p)ATPβS was synthesized from ADPβS by means of the phosphoglycerate kinase reaction, using the system described by Webb and Trentham (1980). The phosphorothioate analogues were purified by DEAE Sephadex A-25 column chromatography using triethylammonium bicarbonate gradients. The purity of the ATPβS analogues was examined by HPLC, using a Waters Novapack C_{18} column (3.9 mm x 15 cm).

Kinetic studies. Initial velocities using ATP, ATPαS or ATPβS as substrates in the presence of Mg^{2+}, Zn^{2+} or Cd^{2+} were determined by measuring the amount of the inorganic phosphate produced, according to Cardemil and Jabalquinto (1985). The reaction mixture contained 0.30 mM MVAPP, 100 mM KCl, 100 mM Tris-HCl buffer pH 7.0 at 3.0° in a total volume of 0.50 mL for the experiments using ATP or 0.10 mL for the experiments with the thioanalogues. The metal to nucleotide ratio was maintained at 2:1 for Mg^{2+}, 0.75:1 for Zn^{2+} or 1:1 for Cd^{2+}, in order to avoid the inhibitory effects of Zn^{2+} and Cd^{2+} (Jabalquinto and Cardemil, 1987). Apparent kinetic constants were determined by varying the corresponding metal-nucleotide complex between 0.5 to 3-4 times Km. Because of the slower reaction rates obtained with the analogues, the enzyme concentration was higher than used in experiments with ATP (2.5 to 10-fold for ATPαS and 25- to 70-fold for ATPβS). In preliminary experiments it was determined that the amount of inorganic phosphate produced was a linear function of time.

Inhibitions studies with the ATPβS isomers were carried out with the spectrophotometric assay of the enzyme that employs pyruvate kinase and lactate dehydrogenase as auxiliary enzymes (Cardemil and Jabalquinto, 1985), and keeping the concentration of Mg^{2+} 1 mM above that of the concentrations of ATP plus the corresponding ATPβS isomer. The concentrations of the metal-nucleotide or metal-thionucleotide complexes were calculated (Storer and Cornish-Bowden, 1976) by using the stability constants reported by Pecoraro *et al.* (1984) for the corresponding Mg^{2+} and Cd^{2+} complexes. For the Zn^{2+} complexes, the value given by Hubbard *et al.* (1985) for the ZnATP was used, and considered to be the same as for the Zn^{2+}-thionucleotide complexes.

Data analysis. The obtainment of the kinetic and inhibition constants (Km, V and K_i) and their standard errors were done by fitting the initial velocity data in the absence or presence of a competitive inhibitor to eq. 2 or eq. 3, respectively, with the NATO-1986 program vritten by Dr. Athel Cornish-Bowden.

$$v = VA/K + A \quad (2)$$

$$v = VA/K(I + I/K_i) + A \quad (3)$$

RESULTS

ATPαS diastereomers as substrate analogues of MVAPP decarboxylase

The apparent kinetic parameters of the MVAPP decarboxylase reaction with the Mg^{2+} or Cd^{2+} metal ion chelates are presented in Table I. It is clear that Cd^{2+} can effectively replace Mg^{2+} in the reaction with ATP as the nucleotide substrate, as judged by the higher value of the first-order rate constant V/Km obtained when Cd^{2+} is employed. For both metal ions, higher V/Km ratios are obtained when the (Rp) ATPαS stereoisomers are used, thus implying that no reversal of the stereochemical preference upon changing the metal ion occurs, which suggests absence of coordination of the metal ion through the α-phosphoryl group of the nucleotide.

TABLE I

Kinetic parameters of MVAPP decarboxylase with diastereomers of ATPαS

Metal nucleotide complex	Apparent Km (mM)	Apparent V (μmoles/mg of protein per min)	App V / App Km
MgATP	0.18 ± 0.02	3.70 ± 0.08	20.6 ± 2.7
CdATP	0.05 ± 0.01	2.30 ± 0.10	46.0 ± 11.2
(Rp)MgATPαS	0.35 ± 0.03	1.61 ± 0.06	4.6 ± 0.6
(Sp)MgATPαS	4.95 ± 0.06	2.00 ± 0.13	0.4 ± 0.03
(Rp)CdATPαS	0.11 ± 0.03	1.58 ± 0.01	14.4 ± 4.0
(Sp)CdATPαS	1.51 ± 0.16	7.44 ± 0.31	4.9 ± 0.7

The apparent kinetic constante were determined as described in the Experimental Procedures. All values are informed ± their standard errors.

ATPβS diastereomers as substrate analogues and as inhibitors of MVAPP decarboxylase

To study the possible interaction of the metal through the β-phosphoryl group of the nucleotide in the active metal-nucleotide complex, the ZnATPβS diastereomers were used instead of the CdATPβS complexes, since it was found that the non-enzymic rate of hydrolisis of the CdATPβS diastereomers were too high to allow reliable determinations of the enzymic reaction, which itself appeared to be very low in the presence of the CdATPβS diastereomers (results not shown).

Apparent Km and V values for the ATPβS diastereomers are shown in Table II. Kinetic constants could not be obtained for the (S_p) ZnATPβS complex since it followed non-michaelian kinetic behaviour (not shown), and no enzyme activity could be detected when (S_p) MgATPβS or (R_p) ZnATPβS were tested as substrates. This is in fact similar to the observation of Chiew *et al.* (1987), who have found no activity of hog liver MVAPP decarboxylase with either Mg^{2+} or Cd^{2+} as activating metal ions when the diastereomers of ATPβS were used as substrates.

For the reasons given above, a comparison of the relative efficiencies of the Mg^{2+} and Zn^{2+} complexes of the diastereomerers of ATPβS as substrates for the MVAPP decarboxylase can only be based on the apparent V of the enzyme reaction. When this is done, it is clearly seen that only the (R_p) isomer of MgATPβS and the (S_p) isomer of ZnATPβS are active as substrates for the decarboxylase, thus implying that the metal ion probably coordinates through the β-phosphoryl group of ATP in catalysis.

Since the diastereomerers of ATPβS elicit a low V compared to ATP, inhibition kinetics could also be studied. The inhibition kinetics were measured in the presence of Mg^{2+} as the activating metal ion. Both diastereomers of ATPβS showed linear competitive inhibition with similar K_i values, as seen in Table II, suggesting that both bind with near equal affinity. Thus, it appears that although the Δ screw sense isomer is much less active as a substrate, it binds as well as or better than the Λ screw sense isomer.

Effect of position of sulfur substitution on the kinetic parameters

The steady state kinetic parameters of the Mg^{2+} activated reactions of ATP and the more active isomers ATPβS and ATPαS are compared in Table III. The maximum velocities decrease in the order ATP > ATPαS >> ATPβS, fitting the general pattern (Ngoc *et al.*, 1979) that upon substitution of 0 with S in ATP, the rate decreases progressively as the substitution approaches the site of bond cleavage. The Km increase is in the same order.

TABLE II

Kinetic parameters and inhibition constants of the diastereomers of ATPβS in the MVAPP descarboxylase reaction

Metal nucleotide complex	Apparent Km (mM)	Apparent V (μmoles/mg of protein per min)	Ki (mM)
MgATP	0.18 ± 0.01	3.70 ± 0.08	
ZnATP	1.05 ± 0.05	2.18 ± 0.11	
(Rp)MgATPβS	7.43 ± 0.07	0.03 ± 0.002	0.61 ± 0.03
(Sp)MgATPβS		ns[a]	0.42 ± 0.03
(Rp)ZnATPβS		ns[a]	nd[b]
(Sp)ZnATPβS		0.08[c]	nd[b]

[a] ns, not a substrate. [b] nd, not determined. [c] non-hyperbolic kinetics. The value of V given corresponds to the initial velocity of the reaction at a concentration of the metal nucleotide complex of 5.9 mM.
For details see Experimental Procedures. All values are informed ± their standard errors.

TABLE III

Effect of position of sulfur substitution on the kinetic parameters of Mg^{2+}-activated mevalonate 5-diphosphate decarboxylase reaction

Nucleotide	Km (mM)	V_{max} (μmoles/mg of protein per min)
MgATP	0.18 ± 0.02	3.70 ± 0.08
(Rp)MgATPαS	0.35 ± 0.03	1.61 ± 0.06
(Rp)MgATPβS	7.43 ± 0.07	0.03 ± 0.002

Data taken from Tables I and II.

DISCUSSION

The purpose of the present study was to establish the absolute stereochemistry of the metal-nucleotide substrate at the active site of MVAPP decarboxylase, by using the approach suggested by Jaffe and Cohn (1978) that makes use of the diastereomeric phosphorothioate analogues of ATP.

The data in Table I show that with either Mg^{2+} or Cd^{2+} the V/Km ratio is higher with the R_p isomer of ATPαS, although it is 12 with Mg^{2+} and 3 with Cd^{2+}. This lack of reversal suggests that the α-phosphoryl group of the nucleotide is not coordinated to the metal in the chemical reaction on the enzyme but that the reaction is more efficient with the R_p isomer. This may be due to specific hydrogen-bonding interactions with the oxygen of the α-phosphoryl group of this isomer, which may not be possible when sulfur occupies this position. Both isomers bind with different affinities, and from the Km values it can be inferred that the R_p isomer binds about 14-fold more tightly with either Mg^{2+} or Cd^{2+}. This higher bonding affinity, however, is not matched with higher values of apparent V, which in fact are of the same order for all ATP and ATPαS complexes.

The possibility that the lack of reversal with the α-phosphoryl group could also be the result of some constraint imposed by the enzyme on the metal nucleotide complex, forcing it to form the same screw-sense isomer on the enzyme, even at the expense of an unfavorable Mg-S or Cd-O coordination (Jaffe and Cohn, 1978) can not be discarded.

Our results with the ATPβS diastereomers indicate that avian liver MVAPP decarboxylase is stereoselective for the Λ screw sense (righ-handed) isomer of the metal-nucleotide complex (Table II). For phosphoenolpyruvate carboxykinase, which

catalyses the somewhat chemically similar decarboxylation reaction of oxaloacetate, Lee *et al.* (1985) have shown that the preferred screw sense isomer of the metal-ATP complex is the Δ (left-handed). Other nucleotide-utilizing enzymes are about equally divided in their preference for the Δ or Λ isomers (for a review see Eckstein, 1985) and it is not uncommon to find that enzymes that catalyse similar reactions show different stereoselectivities for the isomer screw sence (Mejillano *et al.*, 1986). As opposed to the high preference for the Λ screw sense isomer for catalysis by MVAPP decarboxylase, our inhibition studies with the ATPβS isomers indicate that the Δ screw sense isomer binds equally well or better that the Λ isomer. A situation similar to this has also been reported for liver fructokinase (Pecoraro *et al.*, 1985) and bacterial formyltetrahydrofolate synthetase (Mejillano *et al.*, 1986), and the suggestion has been made by Mejillano *et al.* (1986) that this effect could be explained by assuming that the AMP portion of the nucleotide substrate fits into a specific site, thus placing the γ-phosphoryl group of the correct screw sence isomer at the appropriate distance from the catalytic center. For MVAPP decarboxylase this would mean that the phosphoryl group of the Δ isomer may be somewhat removed from the catalytic center where the chemical reaction takes place.

The results presented in Table II indicate a clear-cut reversal of stereospecificity for the β-phosphoryl group upon changing the activating cation from Mg^{2+} to Zn^{2+}, and this reversal of stereospecificity provides strong evidence that the metal is chelated to this phosphoryl group (Cohn, 1982; Jaffe *et al.*, 1982). This fact, together with the data of Table I which do not support a cation-dependent reversal of stereospecificity for the α-phosphoryl group of the ATPαS steroisomers, lead us to propose that the metal must be coordinated to the β-group but probably not to the α-phosphoryl in the active substrate. Further, taking into account the lesser thermodynamic stability of a β-monodentate complex as compared to a six-membered cyclic β, γ-bidentate complex (Huang and Tsai, 1982), we propose that $MgATP^{2-}$ is bound as the Λ, β-γ-bidentate complex in the MVAPP decarboxylase reaction. Similar arguments have been employed before for suggesting β-γ bidentate active complexes for other enzymes (Eckstein, 1985).

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REFERENCES

ALVEAR, M.; JABALQUINTO, A.M.; EYZAGUIRRE, J.; CARDEMIL, E. (1982) Purification and characterization of avian liver mevalonate-5-pyrophosphate decarboxylase. *Biochemistry 21:* 4646-4650.

CARDEMIL, E.; JABALQUINTO, A.M. (1985) Mevalonate 5-pyrophosphate decarboxylase from chicken liver. *Methods Enzymol. 110:* 86-92.

CHIEW, Y.E.; O'SULLIVAN, W.J.; LEE, C.S. (1987) Studies on pig liver mevalonate 5-phosphate decarboxylase. *Biochim. Biophys. Acta. 916:* 271-278.

CLELAND, W.W. (1982) Preparation of chromium (III) and Cobalt (III) nucleotides as chirality probes and inhibitors. *Methods Enzymol. 87:* 159-179.

COHN, M. (1982) Some properties of the phosphorothioate analogues of adenosine triphosphate as substrates of enzymic reactions. *Acc. Chem. Res. 15:* 326-332.

DUNAWAY-MARIANO, D.; CLELAND, W.W. (1980) Investigations of substrate specificity and reaction mechanism of several kinases using chromium (III) adenosine 5'-triphosphate and chromium (III) adenosine 5'-diphosphate. *J. Biol. Chem., 19:* 1506-1515.

ECKSTEIN, F. (1983) Phosphorothioate analogues of nucleotides Tools for the investigation of biochemical processes. *Angew. Chem. Int. Ed. Engl. 22:* 423-439.

ECKSTEIN, F. (1985) Nucleoside phosphorothioates. *Annu. Rev. Biochem. 54:* 367-402.

HUANG, S.L.; TSAY, M.D. (1982) Does the magnesium (II) ion interact with the α-phosphate of adenosine triphosphate. An investigation by oxygen-17 nuclear magnetic resonance. *Biochemistry 21:* 951-959.

HUBBARD, M.J.; SULLIVAN, P.A.; SHEPHERD, M.G. (1985) The kinetics and divalent cation inhibition of plasma membrane ATP ase in the yeast *Candida albacans. J. Biol. Chem. 260:* 6782-6787.

IYENGAR, R.; CARDEMIL, E.; FREY, P.A. (1986) Mevalonate-5-diphosphate decarboxylase: stereochemical course of ATP-dependent phosphorylation of mevalonate 5-diphosphate. *Biochemistry 25:* 4693-4698.

JABALQUINTO, A.M. (1987) Descarboxilasa difosfomevalónica: el complejo metal-ATP activo en la catálisis y estudios cinéticos. Ph.D. Thesis. Universidad de Chile.

JABALQUINTO, A.M.; CARDEMIL, E. (1987) Kinetic effects of ATP, divalent metal ions and pH on chicken liver mevalonate 5-diphosphate decarboxylase. *Biochim. Biophys. Acta. 916:* 172-178.

JABALQUINTO, A.M.; CARDEMIL, E.; IYENGAR, R.; FREY, P.A. (1986) Análogos fosforotioatos del ATP en el estudio del mecanismo de acción de la descarboxilasa difosfomevalónica. *Arch. Biol. Med. Exp. 19:* R-215.

JABALQUINTO, A.M.; ALVEAR, M.; CARDEMIL, E. (1988) Physiological aspects and mechanism of action of mevalonate 5-diphosphate decarboxylase. *Comp. Biochem. Physiol.* In the press.

JAFFE, E.K.; COHN, M. (1978) Divalent cation-dependent stereospecificity of adenosine 5'-0-(2-thiotriphosphate) in the hexokinase and pyruvate kinase reactions. *J. Biol. Chem. 253:* 4823-4825.

JAFFE, E.K.; COHN, M. (1979) Diastereomers of the nucleoside phosphorothioates as probes of the structure of the metal nucleotide substrates and of the nucleotide binding site of yeast hexokinase. *J. Biol. Chem. 254:* 10839-10845.

JAFFE, E.K.; NICK, J.; COHN, M. (1982) Reactivity and metal-dependent stereospecificity of the phosphorothioate analogs of ADP and ATP and reactivity of Cr(III)ATP in the 3-phosphoglycerate kinase reaction. *J. Biol. Chem. 257:* 7650-7656.

LEE, M.H.; GOODY, R.S.; NOWAK, T. (1985) Guanosine thiophosphate derivaties as substrate analogues for phosphoenolpyruvate carboxykinase. *Biochemistry 24:* 7594-7602.

MEJILLANO, M.R.; WENDLAND, M.F.; EVERETT, G. W.; RABINOWITZ, J.C.; HIMES, R.H. (1986) Substrates and inhibitor activities of the screw sence isomers of metal-nucleotide complexes in the formyltetrahydrofolate synthetase reaction. *Biochemistry 25:* 1067-1072.

NGOC, L.F.; JEBELEANU, G.; BARZU, O. (1979) Interaction of sulfur-containing ATP analogs with rabbit muscle phosphofructokinase. *FEBS. Letters. 97:* 65-68.

PECORARO, V.L.; HERMES, J.D.; CLELAND, W.W. (1984) Stability constants of Mg^{2+} and Cd^{2+} complexes of adenosine nucleotides and thionucleotides and rate constants for formation and disosociation of MgATP and MgADP. *Biochemistry 23:* 52-62-5271.

SHEW, K.-F.; HO, H.-T.; NOLAN, L.D.; MARKOVITZ, P.; RICHARD, J.P.; UTTER, M.F.; FREY, P.A. (1984) Stereochemical course of thiophosphoryl group transfer catalyzed by mitochondrial phosphoenolpyruvate carboxykinase. *Biochemistry 23:* 1779-1783.

SMITH, L.T.; COHN, M. (1982) Reactions of the analogues of adenosine 5'-triphosphate catalyzed by methionyl-tRNA synthetase from *Escherichia coli* and metal dependence of stereospecificity. *Biochemistry 21:* 1530-1534.

STORER, A.C.; CORNISH-BOWDEN, A. (1976) Concentration of $MgATP^{2-}$ and other ions in solution. *Biochem. J. 159:* 1-5.

WEBB, M.R. (1982) The stereochemical course of nucleoside triphosphatase reactions. *Methods Enzymol. 87:* 301-316.

WEBB, M.R.; TRENTHAM, D.R. (1980) Analysis of chiral inorganic [^{16}O, ^{17}O, ^{18}O] thiophosphate and the stereochemistry of the 3-phosphoglycerate kinase reaction. *J. Biol. Chem. 255:* 1775-1779.

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Subcellular distribution of enzymes of mono– and sesquiterpene biosynthesis in plant tissues

Distribución subcelular de las enzimas de la biosíntesis de mono– y sesquiterpenos en tejidos vegetales

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The biosynthesis of mono– and sesquiterpene precursors and components of essential oils, has been studied isolating enzyme systems from soluble fractions.

Improved methods for preparing homogenates from plant tissues, together with a knowledge of its ultrastructure, have allowed to determine that most of the processes of biosynthesis of terpenes, are associated to membranous organelles.

The results obtained by different groups are discussed, in order to give an overview of the subcellular distribution of enzymes and specialized organelles, that participate in the biosynthetic processes mentioned above.

Terpenes belong to a family of compounds commonly known as "secondary metabolites" (Mann, 1978). They derive from isoprene, C_5H_8, and their chain length depends on the number of isoprene units incorporated into the molecule. Isoprenoids are present in most living systems; the variety of molecules found in plants, which is much larger than those found in animal tissues, shows that there is no limitation for structural complications of the carbon skeleton, or presence of different chemical functional groups in them (Cori, 1983).

Their function inside the plant has not been completely understood, except in an ecological context or as intermediates in other metabolic processes (Seigler and Price, 1976). Some of them have been described as insect atractors or deterrents, or as phytoalexins (Harborne, 1982); while others carry out well known functions: abscisic acid and gibberelins as plant hormones; phytol as the side chain of chlorophyll and steroids and carotenoids, as structural components of membrane systems, etc.

Mono– and sesquiterpene hydrocarbons, alcohols, ketones and aldehydes, are commonly found in essential oils from higher plants. They are formed from mevalonic acid (MVA*) (George-Nascimento and Cori, 1971), through several steps (Figure 1) that include the biosynthesis of phosphorylated intermediates (Bruemmer, 1975; Cori, 1983), which are the precursors of the essential oils mentioned above.

The enzyme systems related to each of the steps of biosynthesis of mono– and sesquiterpenes, have been studied in different plant species, as well as in animal tissues and microorganisms. Their properties and methods of purification have appeared in several reviews (Beytía and Porter, 1976; Croteau, 1981; Poulter and Rilling, 1981; Satter-white, 1985; Rilling 1985; Ogura *et al.*, 1985; Croteau and Cane, 1985; Cori and Rojas, 1985).

However, there are few studies about subcellular localization of these enzymes. This fact can be due to some problems that arise from working with plant tissues: most of membranous systems are destroyed during homogenization, which is made in similar conditions to those used with animal tissues. These procedures make difficult the obtainment of clearly identified subcellular fractions upon differential centrifugation or density gradients. Other problem has been an apparently lack of in-

Dedicatory: "It is more blessed to give than to receive" (New Testament: Acts, XX, 35) Thanks Osvaldo.

* *Abbreviations:* DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; GPP, geranyl pyrophosphate; GGPP, geranyl-geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; MVA, mevalonic acid; NPP, neryl pyrophosphate.

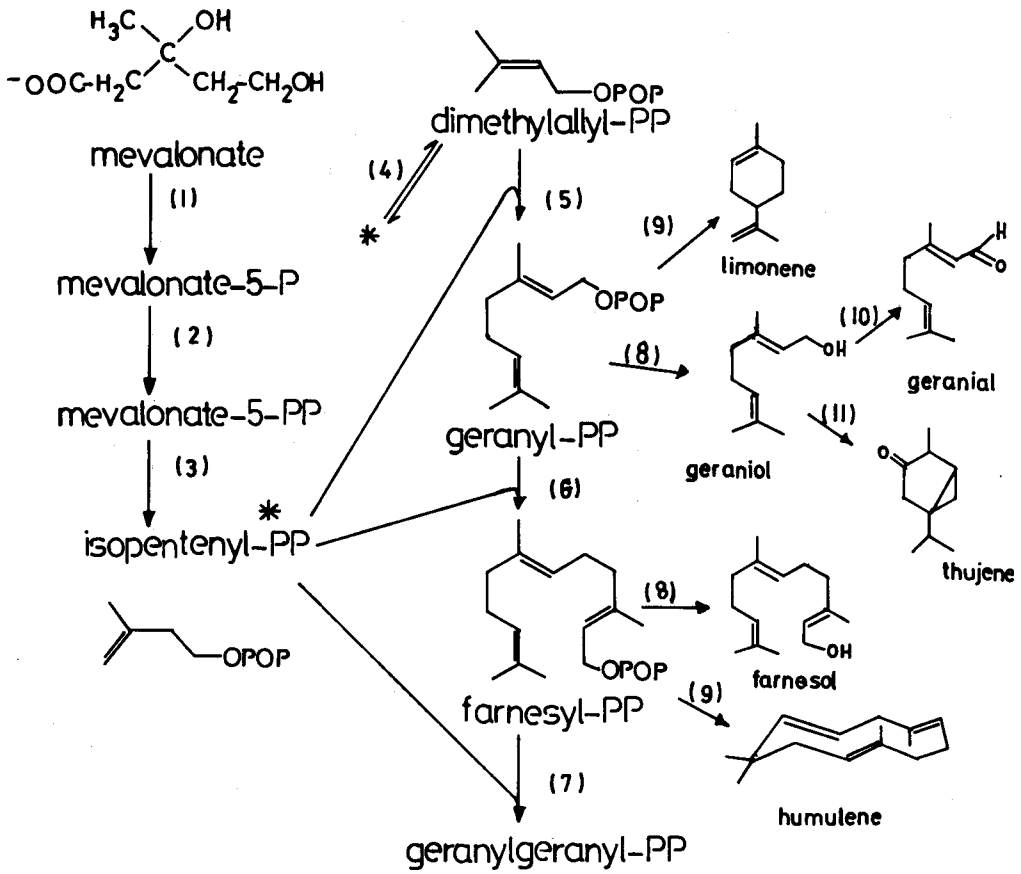

Fig. 1: 1 to 3: mevalonate kinase, mevalonate phosphate kinase and mevalonate diphosphate decarboxylase; 4: IPP isomerase; 5: C_5 prenyltransferase; 6: C_{10} prenyltransferase; 7: C_{15} prenyltransferase; 8: Phosphatase; 9: Cyclase; 10: Redox system; 11: Ketone synthetases.
Depending on the plant species 5, 6 and 7 could represent one or more enzyme species and their products could correspond only to E-conformers or to E and Z conformers.
Monoterpene and sesquiterpene fractions contain examples of the chemical functions found in them, which are formed by a large variety of isoprenoids differing mainly in their carbon skeleton.
(Cori, 1983; Croteau, 1981; Croteau and Cane, 1985; Poulter and Rilling, 1981; Rilling, 1985).

formation about the ultrastructure of the tissues used to isolate plant enzymes. This knowledge could improve homogenization techniques to avoid the damage of organelles thus obtaining better enzyme preparations.

Electron microscopy

Electron microscope studies on plant secretory structures, began to identify the sites of essential oil formation in higher plants. These observations, along with tracer studies of terpenoid biosynthesis, were reviewed by Loomis and Croteau (1973), where they indicate that glandular structures that have been associated with biosynthesis of mono– and sesquiterpenes, could be recognized as oil cells, glandular hairs, oil or resin ducts, or glandular epidermis.

Heinrich and coworkers, interested in the ultrastructure of *Citrus limonum* and *Poncirus trifoliata* (Heinrich, 1969, 1970; Heinrich *et al.*, 1980), reported a compartimentation of the biosynthesis of essential oils in *Poncirus* and *Monarda* (Heinrich *et al.*, 1982). These systems produced an essential oil rich in monoterpenes in the exocarp, while the endocarp mainly produced sesquiterpenes and oxigenated compounds. They also assumed that plastids were the site of their biosynthesis, on the basis of appearance of osmiophilic material inside these organelles.

A detailed ultrastructural study was also made in *Citrus deliciosa Ten* (Bosabalidis and Tsekos, 1982 a, b), to describe cell differentiation and formation of the central space that accumulates essential oils; but the authors only gave information about the presence of osmiophilic material inside the tissue.

Biosynthesis of terpene hydrocarbons

Conversely, the biosynthesis of mono– and sesquiterpenes precursors or components of essential oils have been demonstrated in different plant systems (Stanley, 1958; Beytía *et al.*, 1969; Cori, 1969; Banthorpe and Le Patourel, 1972; Croteau and Loomis, 1972; Jedlicki *et al.*, 1972; Chayet *et al.*, 1973, 1977; Croteau and Karp, 1976, 1977; Pérez *et al.*, 1980, 1983; De la Fuente *et al.*, 1981 Banthorpe *et al.*, 1983). Whereas, in these reports there is no conclusive evidence for the subcellular localization of most of the enzymes described, nor a simultaneous study of the ultrastructure of the plant systems used for the isolation of enzymes.

Studies on *Pinus pinaster* showed that monoterpene hydrocarbon biosynthesis was photodependent, and occurred inside leucoplasts from young epitelial cells of the resin ducts. Sesquiterpene hydrocarbons were formed in the endoplasmic reticulum of non specialized cells (Carde and Bernard-Dagan, 1982). Ultrastructural results (Bernard-Dagan *et al.*, 1982) agree with studies of incorporation of ^{14}C-MVA into sesquiterpene hydrocarbons in this plant species (Gleizes *et al.*, 1980).

Monoterpene alcohols (Pérez *et al.*, 1980) and hydrocarbons (George-Nascimento and Cori, 1971) are synthetized by cell free extracts from *Citrus sinensis* flavedo, where the enzymes appeared to be in a soluble fraction.

Improved methods of homogenation and centrifugation of *Citrus* flavedo, allowed the obtention of intact chromoplasts that formed limonene from ^{14}C-IPP (Pérez *et al.*, 1986, which agrees with the presence of osmiophilic material inside these organelles in intact tissue (Pérez and Garrido, 1985). These results indicate that intact chromoplasts from *Citrus sinensis* contain the activities of IPP isomerase, prenyltransferase and cyclase. Similar results were obtained with leucoplasts of *Citrofortunella mitis,* where there was also agreement between ultrastructural and biosynthetic studies (Gleizes *et al.*, 1983; Pauly *et al.*, 1986).

Leaf epidermis, which constitutes the major site of monoterpene biosynthesis in herbaceous plants (Croteau, 1977), has been a source for monoterpene cyclase isolation (Croteau and Cane, 1985). This enzymes has also been isolated from *Citrus limonum* (Chayet *et al.*, 1977), *Pinus radiata* (Cori, 1969); *Pinus* species (Banthorpe and Le Patourel, 1972; Banthorpe and Ekundayo, 1976) and *Tanacetum vulgare* (Banthorpe *et al.*, 1976); and similarly to other plant systems (Cori and Rojas, 1985), cyclases have been assayed and purified from soluble fractions, regardless of the subcellular distribution they could have inside the cell. This fact can be explained due to the low amounts of cyclase present in plant tissues, and therefore isolation of the enzymes from one subcellular compartment, would probably have resulted in a low yield of cyclase activity. Nevertheless, when specific activity of cyclase in crude homogenates from *Citrus sinensis,* is compared with that obtained in isolated chromoplasts, it is obvious that these organelles provide a better source of cyclase because of its higher specific activity (Pérez *et al.*, 1986).

Citrus sinensis homogenates form α-pinene and limonene from NPP or GPP (George-Nascimento and Cori, 1971), whereas isolated chromoplasts only form limonene from the same substrates (Pérez *et al.*, 1986). Limonene is the only monoterpene hydrocarbon found in the essential oil from orange (Kefford and Chandler, 1970), and therefore, the results obtained in crude homogenates could corresponded to an altered activity cyclase, because of its release from chromoplasts.

Changes in cyclising properties have been observed in *Pinus pinaster*, where soluble fractions could only form acyclic sesquiterpene hydrocarbons from FPP; while cyclic hydrocarbons were synthetized by a

38.000 x g pellet from the same substrate (Bernard-Dagan *et al.*, 1982).

It cannot be discarded that many enzymes involved in cyclic terpene synthesis may be solubilized during preparation of membranes, changing some of their properties.

IPP Isomerase and Prenyltransferase

Further studies on the capacity of leucoplasts from *Citrofortunella mitis* (Gleizes *et al.*, 1987), showed that these organelles contained IPP isomerase and prenyltransferase activities. The latter could form GPP, FPP and GGPP; and together with IPP isomerase, it was associated to the stroma of the plastid. Chromoplasts from *Citrus sinensis* contained stromal IPP isomerase and prenyltransferases, which formed allylic pyrophosphates up to FPP, but in contrast with leucoplasts from calamondins, they could synthetize carotenes (Bravo and Pérez, 1987).

The presence of IPP isomerase and prenyltransferase has been described in other plastids. Chromoplasts from *Narcissus pseudonarcissus* incorporate ^{14}C-IPP into GGPP and β-carotene (Beyer *et al.*, 1980; Kleining and Beyer, 1985). IPP isomerase and prenyltransferase were present in the stroma (Kreuz *et al.*, 1982), while the enzymes for β-carotene biosynthesis were associated with membranes, as has been demonstrated in reconstituted systems (Beyer *et al.*, 1985).

Chromoplasts from *Capsicum anuum* (Camara *et al.*, 1983; Camara 1985) are able to synthetize MVA, to activate MVA to MVAP and MVAPP and to form IPP. When these chromoplasts are incubated with ^{14}C-IPP, most of the radiactivity is found in carotenes, indicating that IPP isomerase and prenyltransferase are very active providing the precursors of carotenes. As in *Citrus sinensis* and *Citrofortunella mitis*, these enzymes are found in the stroma of plastids (Gleizes *et al.*, 1987). This same intraplastid distribution has been described in *Sinapis alba L.* (Lütke-Brinkhaus and Kleining, 1987), in *Spinacia oleracea L.* (Lütke-Brinkhaus and Kleinig, 1985; Kleinig and Beyer, 1985; Block *et al.*, 1980) and in tomato (Spurgeon *et al.*, 1984).

A special situation has been described in *Ricinus communis L.* seedlings, where IPP isomerase and prenyltransferase are associated to proplastids, the latter forming FPP as in chromoplasts from *Citrus sinensis.* Upon infection of the seedlings by the fungus *Rhizopus stolonifer,* a new prenyltransferase activity appears inside the proplastids, which forms GGPP that is the substrate for casbene synthetase, also induced after infection. Seedling response, also involves the appearance of IPP isomerase and prenyltransferase (forming FPP), inside the mitochondria. Both activities are also present in a soluble fraction, and the authors cannot rule out the cytosol as the source of these activities (Dudley *et al.*, 1986).

IPP isomerase and prenyltransferase have been found also in soluble fractions, obtained by differential centrifugation of *Citrus sinensis* flavedo homogenates (Pérez, 1985; Bravo and Pérez, 1986). They behave as associated enzyme activities, eluting from Sepharose 2-B as a protein of a relative molecular mass of 480.000 (Vial *et al.*, 1985). A soluble multiprotein complex containing both enzyme activities, was described in *Gossypium hirsutum* (Widmaier *et al.*, 1980); thus indicating that another form of compartmentation could exist, in the form of associated enzyme activities in a soluble fraction. These enzymes could provide the precursors for cyclic sesquiterpene hydrocarbons (Bernard-Dagan *et al.*, 1982) or for steroids, that are synthetized in the endoplasmic reticulum (Goodwin, 1981).

Prenyl alcohols, aldehydes and ketones

Prenols, wich are importants components of essential oils, are formed through the action of phosphatases, on mono– and sesquiterpene pyrophosphates (Banthorpe *et al.*, 1975; Pérez *et al.*, 1980). These enzymes have been studied in several plant systems as a consequence of their hydrolytic activity on substrates for prenyltransferase and cyclase. Most of them have been found in soluble fractions, although they have been described in every organelle, as a contaminant enzyme. No specific phos-

phatase has been found for any plant system. However there must be a certain degree of specificity, both for prenyltransferase and phosphatase (Banthorpe *et al.*, 1983), which must agree with the prenol composition of the essential oil of a given plant. Prenyltransferase from avian liver, which normally catalyzes the addition of IPP to an allylic pyrophosphate, has been found to catalyze the hydrolysis of its allylic substrate (Poulter and Rilling, 1976). This is a possibility that cannot be discarded for plant systems, where a phosphatase activity of a prenyltransferase has not been described yet.

Prenyl aldehydes and ketones are also found in essential oils. While prenyl aldehydes are believed to be formed by redox mechanisms of the corresponding alcohols, as has been described in soluble fractions from *Pinus radiata* (Jacob *et al.*, 1972), *Citrus sinensis* (Chayet, *et al.*, 1973) and in cell suspensions from *Lavandula angustifolia* (Lappin *et al.*, 1987) and *Vitis vinifera* (Ambid *et al.*, 1983); ketones are formed from the corresponding allylic pyrophosphates, through alcohols as intermediates. These enzymic transformations have been studied for thujane derivatives in *Thuja, Tanacetum* and *Juniperus* (Banthorpe *et al.*, 1970, 1978) and for fenchane derivatives in *Foenimulum vulgare* (Croteau *et al.*, 1980 a, b), always isolating the enzymes systems from soluble fractions.

CONCLUDING REMARKS

Most of the studies on mono– and sesquiterpene biosynthesis have been made using soluble fractions for the isolation of enzymes. Nevertheless, mono– and sesquiterpenes appear to be formed in specialized compartments, depending of the final products or intermediates formed in them.

Joint studies of ultrastructure of plant tissues, and enzyme activities of the isoprenoid pathway, indicate that mono– and sesquiterpenes can be formed in more than one compartment inside the cell. Chromoplasts, which from carotenes, have all the enzyme systems for the transformation of ^{14}C-MVA or ^{14}C-IPP to these tetraterpenes. Chloroplasts also behave in a similar way. Plastids from essential oil producing plants, form monoterpene hydrocarbons, as has been demosntrated in *Citrofortunella mitis* leucoplasts and *Citrus sinensis* chromoplasts. It is possible then, that plastids are always the site of biosynthesis of lipophilic components of essential oils along with other membranous systems as endoplasmic reticulum. These membranous organelles will provide then the compartmentation necessary to isolate these compounds, which otherwise produce a great damage inside the cell.

On the other hand, precursors of other terpenes found in plants, as phytoalexins, plant hormones, steroids, etc., must be formed in other compartments, and the soluble fraction cannot be completely excluded. Although all terpene molecules are highly hydrophobic, it is possible that the enzymes involved in their metabolism could be loosely bound to membranes, and be released to a soluble fraction during preparation of homogenates.

Further studies on the biosynthesis, metabolism and subcellular localization of enzymes related to terpenes, must bear in mind that preparation of homogenates is a crucial step to obtain perfectly identified fractions through differential centrifugation or gradient separations. The use of components that could trap essential oils during homogenation of plant tissues, is also advisable to avoid rupture of membranes systems that maintain integrity of organelles. (Pérez *et al.*, 1986).

The knowledge of the subcellular localization of the metabolic pathways of terpene biosynthesis and metabolism, will allow to obtain enzyme preparations without altered activities and with higher specific activities; and to understand the function of most of isoprenoids in the different tissues of higher plants.

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REFERENCES

AMBID, C.; MOISSEEFF, M.; FALLOT, J. (1983) Interconversion des aldéhydes et des alcools monoterpéniques par des cellules de Raisin muscat cultivées in vitro. *Physiol. Vég. 21:* 87-92.

BANTHORPE, D.V.; MANN, J.; TURNBULL, K.W. (1970) Terpene biosynthesis. Part II. Biosynthesis of thujane derivatives in *Thuja, Tanacetum* and *Juniperus* species. *J. Chem. Soc. C,* 2689-2693.

BANTHORPE, D.V.; LE PATOUREL, G.N.J. (1972). The biosynthesis of (+) − α-pinene in *Pinus* species. *Biochem. J. 130:* 1055-1061.

BANTHORPE, D.V.; CHAUDHRY, A.R.; DOONAN, S. (1975) Specificity and inhibition of phosphatases in *Tanacetum vulgare. Z. Pflanzenphysiol. Bd. 76:* 143-154.

BANTHORPE, D.V.; EKUNDAYO, O. (1976) Biosynthesis of (+)-caren-3ene in *Pinus* species. *Phytochemistry 15:* 109-112.

BANTHORPE, D.V.; BUCKNALL, G.A.; DOONAN, H.J.; DOONAN, S.; ROWAN, M.G. (1976) Biosynthesis of geraniol and nerol in cell free extracts of *Tanacetum vulgare. Phytochemistry 15:* 91-100.

BANTHORPE, D.V.; EKUNDAYO, O.; ROWAN, M.G. (1978) Evidence for geraniol as an obligatory precursor of isothujone. *Phytochemistry 17:* 1111-1114.

BANTHORPE, D.V.; LONG, D.R.S.; PINK, C.R. (1983) Biosynthesis of geraniol and related monoterpenes in *Pelargonium graveolens. Phytochemistry 22:* 2459-2463.

BERNARD-DAGAN, C.; PAULY, G.; MARPEAU, A.; GLEIZES, M.; CARDE, J.P.; BARADAT, P. (1982) Control and compartimentation of terpene biosynthesis in leaves of *Pinus pinaster. Physiol. Vég. 20:* 775-795.

BEYER, P.; KREUZ, K.; KLEINING, H. (1980) βcarotene synthesis in isolated chromoplasts from *Narcissus pseusonarcissus. Planta 150:* 435-438.

BEYER, P.; WEISS, G.; KLEINING, H. (1985) Solubilization and reconstitution of membrane bound carotenogenic enzymes from daffodil chromoplasts. *Eur. J. Biochem. 153:* 341-346.

BEYTIA, E.; VALENZUELA, P.; CORI, O. (1969) Terpene biosynthesis: Formation of Nerol, geraniol and other prenols by an enzyme system from *Pinus radiata* seedlings. *Arch. Biochem. Biophys. 129:* 346-356.

BEYTIA, E.; PORTER, J.W.; (1976) Biochemistry of polyisoprenoid biosynthesis. *Annu. Rev. Biochem. 45:* 113-142.

BLOCK, M.A.; JOYARD, J.; DOUCE, R. (1980) Site of synthesis of geranylgeraniol derivatives in intact spinach chloroplasts. *Biochim. Biophys. Acta 631:* 210-219.

BOSABALIDIS, A.; TSEKOS, I. (1982a) Ultrastructural studies on the secretory cavities of *Citrus deliciosa Ten.* I. Early stages of the gland cells differentiation. *Protoplasma 112:* 55-62.

BOSABALIDIS, A.; TSEKOS, I. (1982b) Ultrastructural studies on the secretory cavities of *Citrus deliciosa Ten.* II. Development of the essential oil accumulating central space of the gland and process of active secretion. *Protoplasma 112:* 63-70.

BRAVO, C.; PEREZ, L.M. (1986) Subcellular distribution of prenyltransferase in *Citrus sinensis* flavedo. *Arch. Biol. Med. Exp. 19:* R-183 (Abstract).

BRAVO, C.; PEREZ, L.M. (1987) Evidence for isoprenic biosynthetic cooperativity of chromoplast subfractions in *Citrus sinensis. Arch. Biol. Med. Exp. 20:* R-180 (Abstract).

BRUEMMER, J.H. (1975) Aroma substances of *Citrus* fruits and their biogenesis. In Internat. Symp. Geruch und Ge chmackstoffe pp. 167-176. (F. Drawer, Editor) Verlag, H. Clark, Nuremberg.

CAMARA, B.; BARDAT, F.; DOGBO, O.; BRANGEON, J.; MONEGER, R. (1983). Terpenoid metabolism in Plastids. Isolation and biochemical characteristic of *Capsicum anuum* chromoplasts. *Plant Physiol. 73:* 94-99.

CAMARA, B. (1985) Carotene synthesis in *Capsicum anuum* chromoplasts in Methods in *Enzymology 110:* 244-253 (Ed. J.H. Law and H.C. Rilling) Ac. Press.

CARDE, J.P.; BERNARD-DAGAN, C. (1982) Compartimentation de la synthèse terpénique chez le pin maritime. *Bull. Soc. Bot. Fr. 129,* Actual. bot., 53-70.

CHAYET, L.; PONT-LEZICA, R.; GEORGE-NASCIMENTO, C.; CORI, O. (1973) Biosynthesis of sesquiterpene alcohols and aldehydes by cell-free extracts from orange flavedo. *Phytochemistry 12:* 95-101.

CHAYET, L.; ROJAS, C.; CARDEMIL, E.; JABALQUINTO, A.M.; VICUÑA, R.; CORI, O. (1977). Biosynthesis of monoterpene hydrocarbons by soluble enzymes from *Citrus limonum. Arch. Biochem. Biophys. 180:* 318-327.

CORI, O. (1969) Terpene biosynthesis: utilization of neryl pyrophosphate by an enzyme system from *Pinus radiata* seedlings. *Arch. Biochem. Biophys. 135:* 416-418.

CORI, O. (1983) Enzymic aspects of the biosynthesis of monoterpenes in plants. *Phytochemistry 22:* 331-341.

CORI, O.; ROJAS, M.C. (1985) Carbocyclases from *Citrus limonum* in Methods in Enzymology *110:* 406-417. (Ed. J.H. Law and H.C. Rilling). Academic Press. Inc.

CROTEAU, R.; LOOMIS, W.D. (1972) Biosynthesis of mono- and sesquiterpenes in peppermint from mevalonate-2-^{14}C. *Phytochemistry 11:* 1055-1066.

CROTEAU, R. (1977) Site of monoterpene biosynthesis in *Majorana* hortensis leaves. *Plant Physiol. 59:* 519-520.

CROTEAU, R.; KARP, F. (1976) Biosynthesis of monoterpenes: enzymic conversion of neryl pyrophosphate to 1,8-cineole, α-terpineol and cyclic monoterpene hydrocarbons by a cell-free preparation from sage (*Salvia officinalis*). *Arch. Biochem. Biophys. 176:* 734-745.

CROTEAU, R.; KARP, F. (1977) Biosynthesis of monoterpenes. Preliminary characterization of bornyl pyrophosphate synthetase from Sage (*Salvia officinalis*) and demonstration that geranyl pyrophosphate is the preferred substrate for cyclization. *Arch. Biochem. Biophys 198:* 512-522.

CROTEAU, R.; FELTON, M.; RONALD, R.C. (1980a) Biosynthesis of monoterpenes: conversion of the acyclic precursors geranyl pyrophosphate and neryl pyrophosphate to the rearranged monoterpenes fenchol and fenchone by a soluble enzyme preparation from fennel (*Foeniculum vulgare*). *Arch. Biochem. Biophys. 200:* 524-533.

CROTEAU, R.; FELTON, M.; RONALD, R.C. (1980b) Biosynthesis of monoterpenes: preliminary characterization of l-endo-fenchol synthetase from fennel (*Foeniculum vulgare*) and evidence that no free intermediate is involved in the cyclization of geranyl pyrophosphate to the rearranged product. *Arch. Biochem. Biophys 200:* 534-546.

CROTEAU, R. (1981) Biosynthesis of monoterpenes in Biosynthesis of isoprenoid compounds. Vol. 1. pp. 226-282 (Ed. J.W. Porter and S.L. Spurgeon) John Wiley and Sons, Inc.

CROTEAU, R.; CANE, D. (1985) Monoterpene and sesquiterpene cyclases in "Methods in Enzymology". Vol *110:* 383-405 (Ed. J.H. Law and H.C. Rilling) Ac. Press.

DE LA FUENTE, M.; PEREZ, L.M.; HASHAGEN, U.; CHAYET, L.; ROJAS, M.C.; PORTILLA, G.; CORI, O. (1981) Prenyltransferases from the flavedo of *Citrus sinensis. Phytochemistry 20:* 1551-1557.

DUDLEY, M.W.; DUEBER, M.T.; WEST, C.A. (1986) Biosynthesis of the macrocyclic diterpene casbene in castor bean (*Ricinus communis L.*) seedlings: changes in enzyme levels induced by fungal infection and intracellular localization of the pathway. *Plant Physiol. 81:* 335-342.

GEORGE-NASCIMENTO, C.; CORI, O. (1971) Terpene biosynthesis from geranyl and neryl pyrophosphates by enzymes from orange flavedo. *Phytochemistry 10:* 1803-1810.

GLEIZES, M.; CARDE, J.P.; PAULY, G.; BERNARD-DAGAN, C. (1980) In vivo formation of sesquiterpene hydrocarbons in the endoplasmic reticulum of Pine. *Plant. Sci. Lett. 20:* 79-90.

GLEIZES, M.; PAULY, G.; CARDE, J-P.; MARPEAU, A.; BERNARD-DAGAN, C. (1983). Monoterpene hydrocarbon biosynthesis by isolated leucoplasts of *Citrofortunella mitis. Planta 159:* 373-381.

GLEIZES, M.; CAMARA, B.; WALTER, J. (1987) Some characteristics of terpenoid biosynthesis by leucoplasts of *Citrofortunella mitis. Planta 170:* 138-140.

GOODWIN, T.W. (1981) Biosynthesis of plant sterols and other triterpenoids in "Biosynthesis of Isoprenoid compounds". Vol. 1 pp. 443-480. (Ed. J.W. Poter and S.L. Spurgeon) John Wiley and Sons, Inc.

HARBORNE, J.B. (1982) Introduction to Ecological Biochemistry. Academic Press.

HEINRICH, G. (1969) Elektronenmikroskopische Beobachtungen zur Entstehungsweise der Exkretbehälter von *Ruta graeolens, Citrus limon* und *Poncirus trifoliata. Osterr. Bot. Z. 117:* 397-403.

HEINRICH, G. (1970) Elektronenmikroskopische Beochtungen an den Drüsenzellen von *Poncirus trifoliata;* zugleich ein Beitrag zur Wirking atherischer Ole auf Pflanzenzellen und eine Methode zur Unterscheidung fluchtiger von nichtflüchtigen lipophilen Komponenten. *Protoplasma 69:* 15-36.

HEINRICH, G.; ZCHULTZE, W.; WEGENER, R. (1980) Zur Kompartimentierung der Synthese von Mono–und Sesquiterpenen des ätherischen Ols bei *Poncirus trifoliata. Protoplama 103:* 115-129.

HEINRICH, G., PFAB, I.; PLAWER, J.; WEGENER, R.; SCHULTZE, W. (1982) Compartimentation of the biosynthesis of the essential oil in *Poncirus* and *Monarda* in Atherische Ole: Analytik, Physiologie, Zusammenstzung. (Ed. K.H. Kubeczka) Georg Thieme Verlag Stuttgart-New York. pp. 198-205.

JACOB, G.; CARDEMIL, E.; CHAYET, L.; TELLEZ, R.; PONT-LEZICA, R.; CORI, O. (1972) Synthesis of isomeric farnesols by soluble enzymes from *Pinus radiata* seedlings. *Phytochemistry 11:* 1683-1688.

JEDLICKI, E.; JACOB, G.; FAINI, F.; CORI, O.; BUNTON, C.A. (1972) Stereospecificity of Isopentenylpyrophosphate Isomerase and Prenyltransferase from *Pinus* and *Citrus. Arch. Biochem. Biophys. 152:* 590-596.

KEFFORD, J.F.; CHANDLER, B.V. (1970) The Chemical constituents of *Citrus fruits.* Advances in Food Research, supplement 2. (C.O. Chichester, E.M. Mrak, G.F. Stewart, Eds). Academic Press, New York and London, 246 pp.

KLEINIG, H.; BEYER, P. (1985) Carotene synthesis in Spinach (*Spinacia Oleracea L.*) chloroplasts and daffodil (*Narcissus pseudonarcissus L.*) chromoplasts in Methods in *Enzymology 110:* 267-273. (Ed. J.H. Law and H.C. Rilling). Ac. Press.

KREUZ, K.; BEYER, P. KLEINIG, H. (1982) The site of carotenogenic enzymes in chromoplasts from Narcissus pseudonarcissus L. *Planta 154:* 66-69.

LAPPIN, G.J.; STRIDE, J.D.; TAMPION, J. (1987) Biotransformation of monoterpenoids by suspension cultures of *Lavandula angustifolia. Phytochemistry 26:* 995-997.

LOOMIS, W.D.; CROTEAU, R. (1973) Biochemistry and Physiology of lower terpenoids in Terpenoids: structure, biogenesis and distribution. Recent Advances in *Phytochemistry 6:* 147-185.

LUTKE-BRINKHAUS, F.; WEISS, G.; KLEINIG, H. (1985) Prenyl lipid formation in spinach chloroplasts and in a cell-free system of *Synechococcus* (Cyanobacteria): polyprenols, chlorophylls and fatty acid prenyl esters. *Planta 163:* 68-74.

LUTKE-BRINKHAUS, F.; KLEINIG, H. (1987) Carotenoid and chlorophyll biosynthesis in isolated plastids from mustard seedling cotyledons (*Sinapis alba L.*) during etioplast-chloroplasts conversion. *Planta 170:* 121-129.

MANN, I. (1978) Secondary metabolism. Oxford, Clarendon Press.

OGURA, K.; NISHINO, T.; SHINKA, T.; SETO, S. (1985) Prenyltransferases of pumpkin fruit in Methods in *Enzymology 110:* 167–171 (Ed. J.H. Law and H.C. Rilling) Ac. Press.

PAULY, G.; BELINGHERI, L.; MARPEAU, A.; GLEIZES, M. (1986) Monoterpene formation by leucoplasts of *Citrofortunella mitis* and *Citrus unshiu.* Steps and conditions of biosynthesis. *Plant Cell Reports 5:* 19-22.

PEREZ, L.M.; TAUCHER, G.; CORI, O. (1980) Hydrolysis of allylic phosphates by enzymes from the flavedo of *Citrus sinensis. Phytochemistry 19:* 183-187.

PEREZ, L.M.; LOZADA, R.; CORI, O. (1983) Biosynthesis of allylic isoprenoid pyrophosphates by an enzyme preparation from the flavedo of *Citrus paradiss. Phytochemistry 22:* 431-433.

PEREZ, L.M., (1985) Ph. D. Thesis: "Study on the enzymic isomerization of sesquiterpenols in *Citrus sinensis* flavedo". Faculty of Sciences, University of Chile.

PEREZ, L.M.; GARRIDO, J. (1985) Ultrastructure of cells from *Citrus sinensis* flavedo. *Arch. Biol. Med. Exp. 18:* 41-56.

PEREZ, L.M.; CARDE, J.P.; PAULY, G. (1986) Biosynthesis of limonene by isolated chromoplasts from *Citrus sinensis.* Proceedings from the VI Meeting of Botany, Chile, p. 172 (Abstract).

POULTER, C.D.; Rilling, H.C. (1976) Prenyltransferase: the mechanism of the reaction. *Biochemistry 15:* 1079-1083.

POULTER, C.D.; RILLING, H.C. (1981) Prenyltransferases and isomerase in "Biosynthesis of isoprenoid compounds". Vol. 1 pp. 161-224. (Ed. J.W. Porter and S.L. Spurgeon) John Wiley and Sons, Inc.

RILLING, H.C. (1985) Eukaryotic prenyltransferases in Methods in *Enzymology 110:* 145-152 (Ed. J.H. Law and H.C. Rilling) Ac. Press.

SATTERWHITE, D.M. (1985) Isopentenyldiphosphate Δ-Isomerase in Methods in *Enzymology 110:* 92-99 (Ed. J.H. Law and H.C. Rilling) Ac. Press.

SEIGLER, D.; PRICE, P.W. (1976) Secondary compounds in plants: primary functions. *The American Naturalist 110:* 101-105.

SPURGEON, S.L.; SATHYAMOORTHY, N.; PORTER, J.W. (1984) Isopentenyl pyrophosphate Isomerase and Prenyltransferase from tomato fruit plastids. *Arch. Biochem. Biophys. 230:* 446-454.

STANLEY, R.G. (1958) Terpene formation in pine from mevalonic acid. *Nature* (London), *182,* 738-739.

VIAL, M.V.; QUAAS, A.; BRAVO C.; PEREZ, L.M. (1985) Prenyltransferase from *Citrus sinensis* flavedo: different forms of enzyme. *Arch. Biol. Med. Exp. 18:* R-372 (Abstract).

WIDMAIER, R.; HOWE, J.; HEINSTEIN, P. (1980) Prenyltransferase from *Gossypium hirsutum. Arch. Biochem. Biophys. 200:* 609-616.

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Neuroquímica de la substantia nigra mesencefálica: Interrelaciones entre aminoácidos excitatorios, receptores del tipo N-metil-D-aspartato y sistemas neuronales intrínsecos*

Neurochemistry of the substantia nigra: interrelations between excitatory amino acids, N-methyl-D-aspartate type receptors and intrinsic neuronal systems.

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Recent studies have demonstrated the existence of cortico-nigral fibers in the brain which may use L-glutamic or L-aspartic acid as their transmitters. Such cortico-nigral neuronal pathway seems to functionally interact with intrinsic neuronal systems in substantia nigra (SN) by first activating different subtypes of excitatory amino acid (EAA) receptors. Particulary interesting are results showing that activation of N-methyl-D-aspartate (NMDA) subtype receptor may evoke the Ca^{2+}-dependent release of dopamine from dendrites of nigro-striatal neurons. In addition, NMDA and other EAA receptors may influence the functional activity of gabaergic, glycinergic and peptidergic neurons located in SN. Acidic aminoacid-containing neurons in SN may thus play a pivotal role in determining the outflow information from SN to caudal motor-related as well as rostral limbic-related areas in the brain.

Hallazgos recientes, provenientes de nuestro laboratorio, sugieren la existencia en substantia nigra (SN) mesencefálica de terminales nerviosos que almacenan y liberan aminoácidos neuroexcitarios y que parte de estos terminales corresponden a una vía neuronal excitatoria que se origina en la corteza prefrontal (sistema o vía neuronal cortico-nigral) (1,2). Estos hallazgos resultan particularmente interesantes, ya que diversos estudios realizados los últimos 6 u 8 años son coincidentes en el sentido de que aminoácidos excitatorios, como el ácido glutámico (GLU) y el ácido aspártico (ASP), constituyen los principales neurotransmisores químicos a nivel de sinapsis excitatorias en el SNC de mamíferos (3, 4). Los aminoácidos parecen ejercer su función neurotransmisora al actuar, al menos, sobre 3 subtipos de receptores que, en función de la afinidad relativa que ellos presentan hacia diversos agonistas, han sido clasificados como receptores del subtipo quisqualato (Q), kainato (K) y N-metil-D-aspartato (NMDA). De tal forma que los aminoácidos excitatorios liberados desde terminales nerviosos cortico-nigrales debieran' interactuar funcionalmente con receptores para estos aminoácidos localizados en sistemas neuronales intrínsecos en la SN. De hecho, en nuestro laboratorio hemos detectado recientemente que aminoácidos excitatorios liberados desde aferentes nigrales parecen regular o modular la liberación de dopamina (DA) desde dendritas de neuronas dopaminérgicas nigro-estriatales, a través de un mecanismo que involucra receptores del subtipo NMDA (5, 6).

La presente revisión tiene por objeto discutir evidencia experimental referente a: 1) la naturaleza química del transmisor ex-

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citatorio proveniente de la vía cortico-nigral; 2) la regulación de su liberación; 3) las características bioquímicas y localización de receptores de aminoácidos excitatorios, a nivel nigral, especialmente los del subtipo NMDA, y finalmente, 4) las interacciones funcionales que los aminoácidos excitatorios y sus receptores ejercen con neuronas nigrales del tipo dopaminérgico y además con otros sistemas neuronales existentes a nivel nigral.

A. *La vía neuronal cortico-nigral*

A.1. *Su relación con aminoácidos neuroexcitatorios*

Investigaciones de naturaleza anatómica coinciden sobre la existencia de una vía neuronal que se origina en la corteza sensorial y motora y cuyos terminales nerviosos se localizan a nivel de las dendritas y somas de la SN mesencefálica (7-9). La existencia de esta vía cortico-nigral plantea la pregunta sobre su expresión funcional, la cual parece ser fundamentalmente de tipo neuroexcitatoria. Así, estudios de microscopia electrónica (ME), realizados luego de lesiones a nivel de corteza frontal, demuestran que los terminales nerviosos de la vía cortico-nigral exhiben características morfológicas propias de terminales del tipo excitatorio (10). Estudios electrofisiológicos, realizados por Nieoullon y cols. (1 1) también apoyan la existencia de una vía neuronal cortico-nigral exhiben características morfo-investigadores han informado que la liberación dendrítica de [^{3}H]dopamina, a nivel de SN, aumenta marcadamente en respuesta a estímulos eléctricos aplicados en la corteza motora del gato. Los experimentos de Nieoullon y cols. sugieren que la vía cortico-nigral se proyecta directamente a las células dopaminérgicas nigro-estriatales, lo que coincide con los trabajos anatómicos antes citados en el sentido de que terminales de la vía cortico-nigral forman sinapsis tanto a nivel de la zona compacta como reticulata de la SN (7, 8).

Tanto ablaciones corticales del tipo fronto-parietal como lesiones electrolíticas bilaterales a nivel de corteza prefrontal de rata resultan, respectivamente, en una marcada reducción en los niveles nigrales de L-GLU endógeno y en la captación de alta afinidad Na^+-dependiente de L-GLU-^{14}C, por sinaptosomas preparados a partir de SN (12-14). En base a estos resultados, Carter ha propuesto que la vía cortico-nigral corresponde a una vía excitatoria aminoacídica de tipo glutamatérgica, aunque no excluyó la posibilidad de que esta vía sea aspartérgica, ya que tanto el L-GLU como el L-aspartato (L-ASP) son aminoácidos que usan transportadores comunes para ser incorporados en terminales nerviosos (15, 16). Un criterio más estricto e importante para identificar a una substancia química como neurotransmisor es su liberación específica desde los terminales en presencia del estímulo nervioso o condiciones despolarizantes (17). Al respecto, estudios realizados en nuestro laboratorio indican que el ácido D-aspártico (D-ASP) tritiado (un análogo no-metabolizable del L-GLU) es captado por cortes nigrales y luego liberado desde ellos, en respuesta a despolarizaciones inducidas, ya sea por estímulo eléctrico o por Veratridina, y a través de un proceso que es dependiente del calcio extracelular (1). Además, la destrucción de la corteza frontoparietal reduce marcadamente la captación y liberación de D-ASP-[H^3] desde cortes nigrales, en respuesta a un estímulo despolarizante (1). Estos experimentos apoyan la proposición de que terminales cortico-nigrales almacenan, liberan y utilizan aminoácidos excitatorios del tipo L-GLU y/o L-ASP como el posible neurotransmisor. Sin embargo, el D-ASP-[^{3}H] no discrimina entre neuronas que almacenan y liberan L-GLU y/o L-ASP. De tal forma que persiste la pregunta si es que la vía cortico-nigral corresponde a una vía excitatoria del tipo glutamatérgica, aspartérgica o mixta.

A.2. *Regulación presináptica de la vía neuronal cortico-nigral*

Información existente a nivel del SNC indica que sistemas neuronales, principalmente del tipo catecolaminérgico, colinérgico y gabaérgico, poseen mecanismos presinápticos que regulan la liberación del

neurotransmisor. Tal regulación parece ejercerse a dos niveles: 1) el transmisor liberado es capaz de controlar su propia liberación actuando, ya sea en forma positiva o negativa, a través de "autorreceptores" localizados presinápticamente (18,19), y 2) los terminales nerviosos también poseen receptores presinápticos o "heterorreceptores", que son sensibles y responden ante la presencia de transmisores de naturaleza química diferente al liberado por el terminal nervioso (19). En general, existe escasa información sobre la presencia de tales mecanismos en sistemas neuronales excitatorios del tipo aminoacídico en el SNC de mamíferos. En estudios recientes de Bustos y cols. (20) se analizó la interesante posibilidad de que la liberación desde sinapsis CA_3-CA_1 de hipocampo, de aminoácidos endógenos de tipo excitatorio, como son el L-GLU y el L-ASP, estuviera regulada por la presencia de "autorreceptores" para esos aminoácidos. Se encontró que receptores de aminoácidos excitatorios del subtipo Q y K inhiben la liberación evocada de L-ASP y L-GLU; en cambio, receptores del subtipo NMDA sólo potencian la liberación de L-ASP. Información concerniente a la regulación de la liberación de estos aminoácidos por "heterorreceptores" también proviene fundamentalmente del hipocampo: en cortes del "área dentada" de la región hipocámpica de rata, agonistas de receptores β-adrenérgicos estimulan (21) mientras que agonistas de receptores de adenosina inhiben la liberación evocada por despolarización de L-GLU radiactivo (22). Con respecto a vías cortico-fugales aminoacídicas excitatorias, las principales evidencias provienen de estudios realizados en neuronas cortico-estriatales y que sugieren la existencia de receptores presinápticos o "heterorreceptores" dopaminérgicos localizados en los terminales de estas neuronas (23-25). Sin embargo, existe total desconocimiento sobre la regulación presináptica de la liberación del transmisor desde la vía cortico-nigral. Sólo cabría citar experimentos preliminares nuestros en los que hemos informado que la liberación evocada de D-ASP-H^3 desde terminales cortico-nigrales es potenciada por la presencia de agonistas de receptores dopaminérgicos como son la apomorfina y el ADTN (26). Estos hallazgos sugieren que la vía cortico-nigral muy posiblemente está sujeta a regulaciones medidas por otros neurotransmisores (dopamina, GABA, glicina, neuropéptidos, etc.) existentes a nivel de SN.

B. *Localización, características y regulación de receptores de aminoácidos excitatorios, especialmente del subtipo NMDA, a nivel de substantia nigra.*

Los receptores de aminoácidos excitatorios del subtipo Q y K median principalmente la respuesta excitatoria que evocan aminoácidos como el L-GLU y L-ASP a nivel postsináptico y que permite una rápida despolarización inducida por influjo de iones Na^+ (27). La activación de los receptores NMDA evoca una más compleja, pero muy interesante respuesta a nivel efector, respuesta que es básicamente voltaje-dependiente y bloqueada por concentraciones fisiológicas de iones Mg^{2+}. Así, en condiciones de potencial de reposo, los receptores NMDA no son activados funcionalmente por aminoácidos excitatorios: en cambio, en condiciones de ausencia de Mg^{2+} externo o en condiciones de despolarización neuronal, los receptores NMDA son activados por L-GLU y L-ASP, al desaparecer el bloqueo ejercido por Mg^{2+} a nivel del complejo receptor NMDA-ionóforo. La activación de los receptores NMDA se traduce entonces en una notable "amplificación" de la respuesta neuroexcitatoria, inducida previamente por receptores Q y K, permitiendo una entrada masiva a la célula efectora no sólo de iones Na^+, sino que también de Ca^{2+} (27). No es sorprendente entonces que los receptores NMDA jueguen una importante función en memoria y aprendizaje, y que además constituyan una importante etapa en los mecanismos involucrados en la generación y propagación de descargas epileptiformes en varios modelos experimentales de epilepsia (28-30).

En lo que se refiere a la SN, experimentos de tipo conductual, electrofisiológicos y con metodología de radioligandos,

sugieren la presencia, a nivel nigral, de uno o varios sitios sensibles a la acción de aminoácidos excitatorios y que pudieran corresponder a receptores para estos aminoácidos. En nuestro laboratorio, y usando L-GLU-^{3}H como radioligando, hemos establecido la presencia en este núcleo cerebral de, al menos, dos poblaciones de sitios de unión específica para L-GLU^{3}H, uno de alta afinidad (K_D, 139 nM; Bmax, 3,5 pmoles/mg proteína) y otra de menor afinidad (K_D, 667 nM; Bmax, 15,1 pmoles/mg proteína) (2). La primera población de sitios aparentemente corresponde a sitios receptores sensibles a quisqualato (Q). Sin embargo, en estos experimentos no fue posible identificar y, por lo tanto, caracterizar receptores del subtipo NMDA. Lo anterior debido al hecho de que en las condiciones experimentales usadas frecuentemente el L-GLU-^{3}H se une principalmente no a sitios receptores sino a sitios que corresponden a un nuevo, y previamente no descrito, sistema de captación celular para este aminoácido (31). Sin embargo, recientemente se han descrito condiciones experimentales simples y adecuadas para caracterizar, mediante metodología de radioligandos, el receptor de NMDA en membranas de cerebro anterior e hipocampo (Bustos y Nadler, manuscrito en preparación). Además, se ha descrito un nuevo antagonista del receptor NMDA denominado CPP [3(2-carboxipiperazin-4-il) propil-l-fosfonato] de alta afinidad y selectividad y que ha mostrado ser útil como marcador del receptor NMDA (32, 33). De tal forma, que es posible ahora realizar estudios bioquímicos con metodología de radioligandos, que permitan caracterizar y estudiar la regulación y la localización celular del receptor NMDA en estructuras neuronales de la SN. De particular importancia resulta analizar si el receptor de NMDA en SN está sujeto a regulaciones de tipo "alostérico", semejantes a las que la despolarización neuronal y ligandos como la glicina inducen sobre receptores de aminoácidos excitatorios subtipo Q y NMDA, en cuerpo estriado e hipocampo, respectivamente (34-36).

C. *Interacciones funcionales entre aminoácidos excitatorios y sistemas neuronales existentes en substantia nigra.*

Existe escasa información con respecto al papel funcional que aminoácidos excitatorios y sus receptores pudieran ejercer a nivel de la SN. En nuestro laboratorio hemos detectado recientemente que agonistas de receptores de NMDA estimulan la liberación de DA-^{3}H captada previamente por cortes nigrales (26, 5). A su vez, tal estimulación es bloqueada por D-2-amino-5-fosfono-valerato (APV) y Mg^{2+}, antagonistas del complejo receptor NMDA-ionóforo. En estos experimentos se usó un sistema de superfusión que esencialmente detecta liberación dendrítica de DA-^{3}H desde cortes de SN (37). De tal forma que los resultados sugieren que aminoácidos excitatorios, liberados desde aferentes nigrales, pueden regular la liberación de DA-^{3}H desde dendritas de neuronas dopaminérgicas nigrales, a través de un mecanismo mediado por receptores del subtipo NMDA. Esto plantea la posibilidad de que DA liberada desde dendritas dopaminérgicas represente un puente de unión entre aferentes excitatorios a nivel de SN y las vías neuronales de salida desde este núcleo cerebral. De hecho, y en apoyo a lo anterior, estudios electrofisiológicos sugieren que DA liberada dendríticamente, pudiera suprimir o atenuar el efecto inhibitorio que GABA produce sobre neuronas eferentes de la SN (38).

A nivel de la SN existe una gran cantidad de somas neuronales y terminales nerviosos ricos en mediadores químicos como GABA, glicina y varios neuropéptidos (substancia P, colecistokinina, dinorfinas, enkefalinas, etc.). De tal forma que se plantea la posibilidad de que la activación de receptores de aminoácidos excitatorios, especialmente del subtipo NMDA, modulen no sólo la expresión funcional de células dopaminérgicas, sino también la de otras neuronas existentes a nivel nigral. De hecho, en experimentos recientes hemos encontrado que el efecto potenciador que agonistas del receptor NMDA producen sobre la liberación de DA-H^3 nigral es abolido por la presencia de TTX y estricnina. Y, además,

que glicina estimula la liberación de DA-H^3 nigral en una forma revertida por estricnina (5). De tal forma que interneuronas glicinérgicas pudieran mediar una interacción funcional entre aminoácidos excitatorios y dendritas dopaminérgicas en SN. Sin embargo, es necesario suministrar mayor evidencia experimental para establecer interacciones, ya sea funcionales o farmacológicas, entre aminoácidos excitatorios y neuronas glicinérgicas y/o gabaérgicas en SN.

Referente a interacciones entre sistemas neuronales aminoacídicos y neuropeptidérgicos, a nivel nigral, la información también es muy escasa. Sólo cabría mencionar el trabajo de Torrens y cols. (39), quienes han informado que el L-GLU, pero no el D-GLU, inhibe la liberación, evocada por despolarización con K^+, de substancia P desde cortes de SN. Además, el dietil éster del L-GLU, un antagonista de receptores del subtipo Q, impidió la acción inhibitoria del L-GLU. Estos resultados sugieren que la regulación presináptica de la liberación de substancia P nigral pudiera estar mediada por receptores tipo glutamatérgico y/o aspartérgico. En trabajos recientes de otra índole, Meyer y cols. (40) han informado que la administración parenteral de ácido kaínico, un agonista de receptores de aminoácidos del subtipo K, produce un marcado aumento en los niveles endógenos de colecistokinina en SN, los cuales persisten hasta 10 días luego de la administración del agonista. Efectos similares se producen sobre los niveles endógenos de neurotensina en SN (41). De tal forma que tales cambios pudieran deberse a interacciones químicas en SN entre aferencias excitatorias cortico-nigrales, los diversos subtipos de receptores de aminoácidos excitatorios y sistemas neuronales ricos en neuropéptidos.

De las evidencias experimentales presentadas se desprenden las siguientes observaciones:

1. No se ha caracterizado adecuadamente la naturaleza química del transmisor excitatorio que se libera desde terminales nerviosos cortico-nigrales ¿corresponde este transmisor al L-GLU o L-ASP o a una mezcla de ambos?

2. Se desconocen los mecanismos presinápticos que regulan la liberación del transmisor desde la vía cortico-nigral. Interesa conocer si tales regulaciones se ejercen a través de "autorreceptores" y/o "heterorreceptores" presinápticos, considerando que en SN existen altas concentraciones no sólo de L-GLU y L-ASP, sino también de otros mediadores químicos como dopamina, GABA, glicina y varios neuropéptidos.

3. Aparentemente existen, a nivel de SN, varios sitios receptores sensibles a la acción de aminoácidos excitatorios. Sin embargo, los estudios realizados no permiten definir aún las características bioquímicas, localización celular y regulación de estos receptores. Se considera especialmente importante el receptor del subtipo NMDA, por su eventual participación en la generación de actividad convulsiva en el cerebro; actividad que confluye y es modulada, en parte, por un núcleo cerebral como la SN.

4. Aparentemente la actividad de neuronas intrínsecas nigrales, como es el caso de las dopaminérgicas, puede ser regulada a través de receptores de aminoácidos excitatorios, especialmente del subtipo NMDA. Es necesario confirmar y extender estas observaciones, especialmente en lo que se refiere a los mecanismos involucrados. También es necesario investigar sobre las eventuales interacciones, ya sea funcionales o farmacológicas, que aminoácidos excitatorios y sus receptores ejercen con neuronas nigrales de tipo gabaérgico, glicinérgico y neuropeptidérgico.

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REFERENCIAS

1. ABARCA, J.; BUSTOS, G.; (1985) *Neurochem. Int. 7:* 229-236.
2. FIEDLER, J.E.; ARQUEROS, L.; BUSTOS, G. (1986) *J. Recept Res. 6:* 339-360.
3. WATKINS, J.C.; EVANS, R.H. (1981) *Ann Rev. Pharmac. Toxic. 21:* 165-204.
4. FAGG, G.E.; FOSTER, A.C. (1983) *Neuroscience. 9:* 701-719.
5. ARANEDA, R.; BUSTOS, G. (1986) *Arch. Biol. Med. Exp. 19:* R-173.
6. ARANEDA, R. (1986) *Tesis de Bioquímico,* Universidad de Concepción.
7. AFIFI, A.K.; BAUTH, N.B.; KAELBER, W.W.; MIKHAEL, E.; NASSAR, A. (1974) *J. Anat. 118:* 469-476.
8. BECKSTEAD, R.M. (1979) *J. Comp. Neurol. 184:* 43-62.

9. KUNZLE, H. (1978) *Brain Behav. Evol. 15:* 185-234.
10. USUNOFF, K.G.; ROMANSKY, K.U.; MALINOV, G.B., IVANOV, D.P.; BLAGOV, Z.A.; GALABOV, G.P. (1982) *J. Hirnforsch. 23:* 23-29.
11. NIEOULLON, A.; CHERAMY, A.; GLOWINSKI, (1978) *J. Brain Res. 145:* 69-83.
12. KORNHUBER, J.; KIM, J.S.; KORNHUBER, M.E.; KORNHUBER, H.H. (1984) *Brain Res. 132:* 124-126.
13. CARTER, C.J. (1980) *Brit. J. Pharmacol. 70:* 50P.
14. KERKERIAN, E.; NIEOULLON, A.; DUSTICIER, N. (1983) *Exp. Neurol. 81:* 598-612.
15. WOFSAY, A.R.; KUHAR, M.J.; SNYDER, S.H. (1971) *Proc. Natl. Acad. Sci. 68:* 1102-1106.
16. BALCAR, V.J.; JOHNSON, G.A.R. (1972) *J. Neurochem. 19:* 2657-2666.
17. ORREGO, F. (1979) *Neuroscience 4:* 1037-1057.
18. LANGER, S. (1980) *Trends Neurol. Sci. 3:* 110-114.
19. CHESSELET, M.F. (1984) *Neuroscience 12:* 347-375.
20. BUSTOS, G.; BRAY, S.; NADLER, J.V. *XVII Ann. Meeting Soc. Neurosc.,* New Orleans, USA. *13,* 762 (1987) Abstract.
21. LYNCH, M.A.; BLISS, T.V. (1986) *Neuropharmacology 25:* 493-498.
22. DOLPHIN, A.C.; ARCHER, E.R. (1983) *Neurosc. Lett. 43:* 49-54.
23. MITCHELL, P.R.; DOGETT, M.S. (1980) *Life Sci. 26:* 2073-2077.
24. ROWLANDS, G.J.; ROBERTS, P.J. (1980) *Eur. J. Pharmacol. 62:* 241-246.
25. GODUKHIN, O.V.; ZHARIKOVA, A.D.; BUDANTSEV, A.Y. (1984) *Neuroscience 12:* 377-383.
26. BUSTOS, G.; ABARCA, J.; ARANEDA, J. (1985) *Arch. Biol. Med. Exp. 18:* R-120.
27. FAGG. G.E.; FOSTER, A.C.; GANONG, A.A. (1986) *Trends Pharmacol. Sci. 7:* 357-363.
28. MELDRUM, B.S. (1985) *Clin, Sci. 68:* 113-122.
29. HERRON, C.E.; WILLIAMSON, R.; COLLINGRIDGE, G.L. (1985) *Neurosci. Lett. 61:* 255-260.
30. MELDRUM, B.S. (1986) *Adv. Exp. Med. Biol. 203:* 321-329.
31. PIN, J.-P.; BOCKAERT, J.; RECASSENS, M. (1984) *FEBS Lett. 175:* 31-36.
32. DAVIES, J.; EVANS, R.F.; HERRLING, P.L.; JONES, A.W.; OLVERMAN, H.J.; POOK, P.J.; WATKIN, J.C. (1986) *Brain Res. 382:* 169-173.
33. MURPHY, D.E.; SCHNEIDER, J.; LEHMANN, J.; WILLIAMS, M. (1986) Abst. Soc. Neurosc. *XVI Ann. Meeting, Washington,* p. 59.
34. RUDOLPH, M.I.; BUSTOS, G. (1986) *Neurochem. Int. 8:* 481-492.
35. MONAGHAM, D.T.; COTMAN, C.W. (1986) *Proc. Natl. Acad. Sci. 83:* 7532-7536.
36. JOHNSON, J.W.; ASCHER, P. (1987) *Nature 325:* 529-533.
37. GEFFEN, L.B.; JESSELL, T.M.; CUELLO, A.C.; IVERSEN, L.L. (1976) *Nature 260:* 258-260.
38. WASZCZAK, B.L.; WALTERNS, J.R. (1986) *J. Neuroscience 6:* 120-126.
39. TORRENS, Y.; BEAUJOUAN, J.C.; BESSON, M.J.; MICHELOT, R.; GLOWINSKI, J. (1981) *Eur. J. Pharmacol. 71:* 383-392.
40. MEYER, D.K.; WIDMANN, R.; SPERK, G. (1986) *Neurosc. Lett. 69:* 208-211.
41. SPERK, G.; WIESER, R.; WIDMANN, R.; SINGER, E.A. (1986) *Neuroscience 17:* 1117-1126.

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Farmacología-bioquímica y fisiopatología de la disquinesia tardía: revisión de los progresos y controversias*

Biochemical pharmacology of tardive dyskinesia: review of progress and current controversies

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Se analiza la disquinesia tardía como patología neurológica yatrogénica y se discuten las controversias respecto a su etiología, fisiopatología y farmacología bioquímica.

Tardive dyskinesia is described as a distinct neurological entity. Its probable etiology, pathophysiology and biochemical pharmacology are reviewed and discussed in the light of the latest findings.

INTRODUCCION

La introducción de los neurolépticos en Francia en 1952, causante de uno de los mayores avances en la psiquiatría moderna, fue seguida, a fines de la década de los años 50, de una serie de trabajos sobre movimientos involuntarios en la esfera oro-linguo-facial, que se asociaban al uso prolongado de estos fármacos. Este cuadro, obviamente yatrogénico, fue llamado disquinesia tardía (1), precisamente porque se manifestaba largo tiempo después de disminuir o suspender la administración del antisicótico.

La descripción clínica actual de este síndrome comprende movimientos involuntarios de masticación, inflado de mejillas, muecas, chasquido de labios, succión, lamido, protrusión de lengua confinados al área oro-linguo-facial en adición a movimientos coreoatetoídeos de dedos, manos, brazos, pies; movimientos balísticos, en especial de los brazos e, incluso, hiperquinesias axiales y movimientos involuntarios diafragmáticos (2, 3). Tanto el tipo de los movimientos involuntarios como su gran diversidad entre paciente y paciente, asemeja el cuadro clínico a las disquinesias inducidas por L–DOPA y otros cuadros coreicos.

Actualmente, esta riqueza de signos se resume en cuatro tipos de movimientos involuntarios: temblor, corea, tics y distonías (4). Se reconoce también que los pacientes más en riesgo son de sexo femenino, mayores de 70 años, que han recibido neurolépticos "incisivos" de mayor potencia antisicótica y capaces de inducir parkisonismo agudo (butirofenomas, fenotiazinas piperazínicas, tioxantenos, etc., (4, 5).

El cuadro de la disquinesia tardía se ha unido en forma clásica a una hipersensibilidad de receptores dopaminérgicos estriatales (6, 7, 8) inducida por el bloqueo prolongado que ejercen sobre ellos los agentes antisicóticos (9). Sin embargo, una serie de preguntas y controversias parecen indicar que esta teoría no siempre es válida o bien es condición necesaria, pero no suficiente, para la expresión de la disquinesia tardía.

Estas controversias se podrían resumir en la siguiente forma:

1. ¿Debe o no existir otra vulnerabilidad neurológica en el paciente que hace una disquinesia tardía?

* Se dedica este trabajo a la memoria del profesor doctor Osvaldo Cori Moully, formador y apreciado amigo del autor. El mejor tributo de sus discípulos es que su ética, rigor científico, humanismo y calidad humana puedan perdurar en nosotros.

2. ¿Es la disquinesia tardía parte de un cierto tipo de esquizofrenia?
3. ¿Se puede explicar el efecto diferencial de algunos neurolépticos atípicos sobre la producción de disquinesia tardía por su mecanismo de acción a nivel de distintos subtipos de receptores dopaminérgicos estriatales o límbicos?
4. La ubicación anatómica estriatal intrínseca y el papel funcional atribuido a los subtipos de receptores dopaminérgicos, ¿puede explicar las diferentes respuestas conductuales, farmacológicas y bioquímicas a los agentes antisicóticos?
5. ¿Depende la aparición de la disquinesia tardía sólo de la alteración de sensibilidad de un cierto tipo de receptor dopaminérgico o hay otros sistemas estriatales de neurorreguladores involucrados?
6. Si la disquinesia tardía involucra varios de estos factores, ¿qué hipótesis se puede plantear para unificarlos?

En la próxima sección se intentará desarrollar y responder a estas interrogantes.

FISIOPATOLOGIA Y FARMACOLOGIA BIOQUIMICA

El neoestriado, formado por el conjunto del núcleo caudado y el putamen es el centro modulador del circuito reverberante llamado de los ganglios basales que desempeña un papel clave en el control de la motricidad y que funciona en base, fundamentalmente, a la inhibición.

Si bien aún persiste la visión tradicional de considerar el neoestriado, el globus pallidus y los núcleos subtalámicos con aferentes estriatales provenientes de la corteza, los núcleos talámicos intralaminares y la sustancia nigra, y salidas de señal a partir del globus pallidus interno y la sustancia nigra pars reticular como "el sistema de *ganglios basales*", *evidencias recientes implican a estructuras límbicas como la sustancia innominata*, el núcleo accumbens y el tubérculo olfatorio dentro también de los ganglios basales. De esta forma, se considera que los ganglios basales se dividen en un sistema *dorsal* y un sistema *ventral*. El sistema dorsal es el tradicional, mientras el sistema ventral que existe en paralelo incluye el n. accumbens, la parte externa del tubérculo olfatorio, parte del globus pálido ventral, la sustancia innominata y núcleos preópticos. Los aferentes y eferentes de ambos sistemas existen en paralelo con diferencias sutiles en su origen. También los sistemas nigroestriatal y mesolímbico-mesocortical poseen innervación cruzada. Esta anatomía particular puede explicar hechos de la clínica en la relación entre control motor y actividad emocional, así como la acción de fármacos sobre ambas funciones (10).

La clave de la acción de drogas sobre el estriado y los resultados característicos del bloqueo dopaminérgico estriatal (Parkinsonismo); la estimulación dopaminérgica estriatal (Corea, disquinesia); el bloqueo colinérgico (antiparkinsonismo; agrava Corea y disquinesia) indican la estrecha relación entre acetilcolina, dopamina y GABA (ácido gama-amino-butírico), este último mediador de los circuitos de retroalimentación estrionigral y de las salidas de señal del neoestriado. Aunque ésta es una sobresimplificación, pues no toma en cuenta el papel de numerosos otros neurorreguladores, como colecistokinina (CCK), serotonina, encefalinas, sustancia P, probablemente neurotensina, etc., provee una base para probar la teoría de la hipersensibilidad dopaminérgica a la luz de los hechos experimentales.

Entrando a intentar responder las controversias enumeradas en la sección precedente, tenemos:

1. La edad es el único factor (envejecimiento neurológico) que predispone a la aparición de la disquinesia tardía (11, 12). Este hecho ha sido confirmado, comparando grupos de animales senescentes con animales jóvenes, ambos expuestos a neurolépticos por períodos de hasta tres meses. *Los animales viejos desarrollaron significativamente más movimientos estereotipados* oro-linguo-faciales. En ambos grupos se estimaron parámetros cinéticos de receptores D_1 estriatales (^{3}H-piflutixol) y D_2 (H^3-espiroperidol). Los animales viejos mostraron niveles normales de receptores D_1 en densidad y afinidad: los D_2 aparecían con

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FISIOPATOLOGIA Y FARMACOLOGIA BIOQUIMICA

El neoestriado, formado por el conjunto del núcleo caudado y el putamen es el centro modulador del circuito reverberante llamado de los ganglios basales que desempeña un papel clave en el control de la motricidad y que funciona en base, fundamentalmente, a la inhibición.

Si bien aún persiste la visión tradicional de considerar el neoestriado, el globus pallidus y los núcleos subtalámicos con aferentes estriatales provenientes de la corteza, los núcleos talámicos intralaminares y la sustancia nigra, y salidas de señal a partir del globus pallidus interno y la sustancia nigra pars reticular como "el sistema de ganglios basales", evidencias recientes implican a estructuras límbicas como la sustancia innominata, el núcleo accumbens y el tubérculo olfatorio dentro también de los ganglios basales. De esta forma, se considera que los ganglios basales se dividen en un sistema *dorsal* y un sistema *ventral.* El sistema dorsal es el tradicional, mientras el sistema ventral que existe en paralelo incluye el n. accumbens, la parte externa del tubérculo olfatorio, parte del globus pálido ventral, la sustancia innominata y núcleos preópticos. Los aferentes y eferentes de ambos sistemas existen en paralelo con diferencias sutiles en su origen. También los sistemas nigroestriatal y mesolímbico-mesocortical poseen innervación cruzada. Esta anatomía particular puede explicar hechos de la clínica en la relación entre control motor y actividad emocional, así como la acción de fármacos sobre ambas funciones (10).

La clave de la acción de drogas sobre el estriado y los resultados característicos del bloqueo dopaminérgico estriatal (Parkinsonismo); la estimulación dopaminérgica estriatal (Corea, disquinesia); el bloqueo colinérgico (antiparkinsonismo; agrava Corea y disquinesia) indican la estrecha relación entre acetilcolina, dopamina y GABA (ácido gama-amino-butírico), este último mediador de los circuitos de retroalimentación estrionigral y de las salidas de señal del neoestriado. Aunque ésta es una sobresimplificación, pues no toma en cuenta el papel de numerosos otros neurorreguladores, como colecistokinina (CCK), serotonina, encefalinas, sustancia P, probablemente neurotensina, etc., provee una base para probar la teoría de la hipersensibilidad dopaminérgica a la luz de los hechos experimentales.

Entrando a intentar responder las controversias enumeradas en la sección precedente, tenemos:

1. La edad es el único factor (envejecimiento neurológico) que predispone a la aparición de la disquinesia tardía (11, 12). Este hecho ha sido confirmado, comparando grupos de animales senescentes con animales jóvenes, ambos expuestos a neurolépticos por períodos de hasta tres meses. Los animales viejos desarrollaron significativamente más movimientos estereotipados oro-linguo-faciales. En ambos grupos se estimaron parámetros cinéticos de receptores D_1 estriatales (^{3}H-piflutixol) y D_2 (H^3-espiroperidol). Los animales viejos mostraron niveles normales de receptores D_1 en densidad y afinidad: los D_2 aparecían con

densidad significativamente disminuida y el ligamen no específico aumentado (gliosis-pérdida neuronal) (13, 14, 15).

2. Se ha observado mayor disquinesia tardía en enfermos esquizofrénicos crónicos, de mala respuesta a neurolépticos, donde prima lo "defectual" y "negativo", con daño cerebral (hiperdilatación ventricular, pérdida neuronal), que corresponde a la esquizofrenia tipo II de Crow (11) (16). Este tipo de proceso se asemeja más a un cuadro demenciante, con compromiso global cerebral que al tipo I, sicosis, donde prima la "hiperfunción dopaminérgica límbica". (17, 18).

3. Algunos neurolépticos, precisamente no aquellos de mayor poder antisicótico, presentan nula o muy baja incidencia en producir disquinesia tardía u otros cuadros extrapiramidales. En efecto, clozapina y tioridazina han sido llamados "neurolépticos atípicos" por no producir la clásica rigidez parkinsoniana de estos compuestos. Durante un tiempo, al observar su gran potencia anticolinérgica, se explicó que a esto debían su falta de inducción de extrapiramidalismo (19), ya que su efecto bloqueador dopaminérgico estriatal era enmascarado por el bloqueo del receptor muscarínico de las interneuronas estriatales. Sin embargo, se demostró posteriormente que la adición de un anticolinérgico central a un neuroléptico muy inductor de efectos extrapiramidales no asemejaba el efecto al de un neuroléptico atípico.

La explicación estaría entonces en que los neurolépticos atípicos bloquean diferencialmente los receptores dopaminérgicos límbicos que los estriatales. Si bien al principio hubo discrepancias entre varios autores, la uniformidad de las técnicas de ligamen-droga-receptor in vitro ha permitido demostrar que los neurolépticos "atípicos" clozapina y tioridazina son equipotentes con haloperidol y flufenazina sobre los receptores dopaminérgicos límbicos, pero son mucho menos bloqueadores a nivel de receptores dopaminérgicos estriatales, lo que explica su falta de toxicidad extrapiramidal (21, 22).

Se ha podido demostrar, además, bioquímicamente que los neurolépticos atípicos producen un mayor aumento del recambio de dopamina a nivel límbico que a nivel estriatal, mientras que los neurolépticos clásicos no discriminan (23, 24).

4. Una explicación alternativa y/o complementaria para la indemnidad del sistema motor frente a los neurolépticos atípicos en cuanto a la aparición de disquinesia tardía puede estar en la comprensión de los subtipos de receptores dopaminérgicos, su respuesta frente a agonistas y antagonistas, su relación con distintas vías neuronales y sus distintos papeles funcionales.

En experimentos con ratas lesionadas unilateralmente con 6-OH-Dopamina (que mostraban hipersensibilidad por denervación), para demostrar qué mecanismos dopaminérgicos distintos (*receptores*) estaban asociados a la inervación de *interneuronas GABA* e *interneuronas colinérgicas,* se inhibió la transmisión GABAérgica con picrotoxina y la colinérgica con escopolamina.

La picrotoxina potenció la estimulación con pergolide (agonista D_2 preferente) (25), que produce rotación contralateral al lado lesionado, pero inhibió la rotación por apomorfina. Exactamente al revés sucedió con escopolamina (26). Esto muestra que la apomorfina y el pergolide afectan diferentemente a neuronas colinérgicas sensibles a dopamina y a neuronas GABA sensibles a dopamina. Aún más, los receptores D_1 estarían preferentemente en neuronas colinérgicas y los D_2 en neuronas GABA. Posteriormente, al elaborar una hipótesis unitaria, se verá la importancia que los *receptores* D_2 *estriatales*, sensibles a concentraciones nanomolares de neurolépticos aparezcan asociados a neuronas GABA.

5. Compromiso de otros neurotransmisores en ganglios basales en la disquinesia tardía.

Monos (Cebus apella) tratados crónicamente, ya sea con haloperidol decanoato o flufenazina decanoato por un período de 3-6 años, fueron sacrificados y sus cerebros analizados en zonas discretas anatómicas, encontrándose una alteración (descenso) de la actividad de la enzima glutámico descarboxilasa (GAD) en tres áreas cerebrales: globus pálido medial, núcleo subtalámico y sustancia nigra, sólo en aquellos animales

que desarrollaron espontáneamente disquinesia tardía después del tratamiento (27).

Este hallazgo muestra que la enzima que cataliza la formación de GABA estaba disminuida sólo en aquellos animales que desarrollaron una disquinesia tardía persistente.

La baja actividad de GAD en globus pálido y la sustancia nigra puede relacionarse con una disfunción dentro de los sistemas neuronales estrionigrales y acumbens-palidal. Estos sistema GABAérgicos se afectan profundamente con la administración prolongada de neurolépticos. Cuando las neuronas GABA estriatales intrínsecas se desinhiben por el bloqueo de la transmisión dopaminérgica, debida a los neurolépticos, las vías internucleares se inhiben (28). Hasta dos meses posteriores a la suspensión del tratamiento, la supresión de las neuronas nigrales y palidales se manifestó en los animales disquinéticos, pero no en los controles (que no hicieron disquinesia, a pesar de haber recibido neurolépticos de igual manera). Es probable, y hay antecedentes, que vías GABA inhibidas por largo tiempo pueden degenerar. Así, un mecanismo neuroquímico importante en la génesis de la disquinesia tardía sería la desinhibición de fibras nigrofugales (de nigra reticular) por baja actividad inhibitoria (GAD baja). En apoyo a esto, la inyección intranigral de antagonistas GABA causa disquinesia oral (29).

Tanto en los animales de estos experimentos como en pacientes, los agonistas GABA tienden a reducir la sintomatología, aunque por desgracia se requieren dosis muy altas que producen ataxia (30, 31).

6. Resumen de contradicciones, coincidencias e intento de hipótesis unitaria.

Los contradictores principales de la teoría dopaminérgica única de la disquinesia tardía argumentan (18) (32, 33):

a) La disquinesia tardía clínica aparece tarde; la hipersensibilización de receptores dopaminérgicos estriatales es muy rápida.
b) Sólo el 20 por ciento de los pacientes **hacen** disquinesia tardía; el 100 por ciento de los animales muestran hipersensibilidad dominérgica.
c) La hipersensibilidad dopaminérgica es fugaz en los animales; en el hombre persiste en un 40 por ciento aprox.
d) Los receptores D_2 están hipersensibles en cerebros de esquizofrénicos con o sin disquinesia tardía al momento de fallecer
e) Los estudios en monos, que muestran baja de la GAD, podrían traducir la hipersensibilidad dopaminérgica, pero puede que la baja actividad de GAD sea sólo reflejo funcional de hiperactividad dopaminérgica estriatal.

Los autores que postulan la hipersensibilidad dopaminérgica como condición *necesaria*, pero no suficiente, para la disquinesia tardía (34), postulan lo incontrovertible de la farmacología de la disquinesia tardía:

a) Siempre asociada a uso crónico de neurolépticos.
b) Agravada por agonistas dopaminérgicos.
c) Aliviada por neurolépticos o depletadores de dopamina.
d) Agravada por bloqueadores muscarínicos.

Como hipótesis unitaria de estas controversias, se puede mencionar el mecanismo de radicales libres (35). Se postula que el desbalance de neurotransmisores no es suficiente para provocar una alteración tan persistente como la disquinesia tardía, que el aumento del recambio (y la oxidación por MAO-B) de catecolaminas producidas por el feedback del bloqueo de neurolépticos lleva al aumento de la producción de radicales libres, como superóxido, peróxido de hidrógeno, radical oxhidrilo, además que tanto la L-DOPA como la dopamina pueden autooxidarse a quinonas. Así, la generación de radicales libres causaría un *daño estructural estriatal* que se reflejaría en baja de GAD en individuos que hacen disquinesias. Los mecanismos específicos protectores de radicales libres, como superóxido dismutasa, catalasa, peroxidasa, glutation peroxidasa y los inespecíficos, antioxidantes, como vitamina E, ascorbato, disminuyen con la edad y quizás por ello los ancianos son más lábiles.

Finalmente, se podría postular que la validación de esta atractiva teoría de unifi-

cación podría ser puesta a prueba administrando antioxidantes a los pacientes que reciben neurolépticos.

REFERENCIAS

1. UHRBRAND, L. and FARBYE, A. (1960) Reversible and irreversible dyskinesia after treatment with perphenazine, chlorpromazine, reserpine, ECT therapy. Psychopharmacologia *1:* 408-418.
2. KLAWANS, H.; GOETZ, C. G.; PERLIK, S. (1980) Tardive Dyskinesia: Review and Update. Am. J. Psychiatry, *137:* (8) 900-908.
3. Task Force Report 18 (1980) Tardive Dyskinesia, Ed. by American Psychiatric Association, Washington D.C., USA, pp. 23-24.
4. KLAWANS, H. (1985) Recognition and Diagnosis of Tardive Dyskinesia. J. Clin. Psychiatry *46:* (4, Sec. 2), 3-7.
5. BALDESSARINI, R. (1985) Clinical and Epidemiologic Aspects of Tardive Dyskinesia. J. Clin. Psychiatry *46:* (4, sec. 2) 8-13.
6. GOMEZ, A.; TRONCOSO, E.; PESENTI, L. (1982) Hipersensibilidad a la apomorfina en ratas tratadas con neurolépticos: un modelo de disquinesia tardía. Rev. Méd. Chile *110:* 319-322.
7. GOMEZ, A. y TRONCOSO, E. (1984) Modelo animal de disquinesia tardía: hipersensibilidad a la apomorfina y unión de H^3-espiroperidol a membranas de estriado de ratas tratadas con haloperidol. Rev. Chil. Neuropsiquiat., *22:* 55-59.
8. HITRI, A. (1980) Biochemical and behavioural studies of neuroleptic-induced behavioural supersensitivity. In Fann W.E.; Smith R.C., Davis J.M. et al. Eds.: Tardive Dyskinesia. Research and treatment. New York, Spectrum Publ.
9. GOMEZ, A.; WYNEKEN, U. (1986) Mecanismos moleculares de acción de fármacos antisicóticos. Rev. Chil. Neuropsiquiat. *24:* 176-181.
10. MARSDEN, C. D. (1985) The enigma of the basal ganglia and movement. In The motor system in neurobiology, Ed. by E. V. Evarts, S. P. Wise and D. Bousfield, Elsevier Biomedical Press. Amsterdam, pp. 277-283.
11. CROW, T. J. (1982) Schizophrenia. In Crow, T. J. ed. Disorders of Neurohumoral transmission. London. Academic Press.
12. SMITH, J. M.; BALDESSARINI, R. J. (1980) Changes in the prevalence, severity and recovery in tardive dyskinesia with age. Arch. Gen. Psychiat. *37:* 1368-1373.
13. WADDINGTON, J. L.; CROSS, A. J.; GAMBLE, S. J.; BOURNE, R. C. (1983) Spontaneous orofacial dyskinesia and dopaminergic function in rats after 6 months of neuroleptic treatment. Science *220:* 230-232.
14. WADDINGTON, J. L. (1983) Dopamine receptor function and spontaneous orofacial dyskinesia in rats during 6-month neuroleptic treatment. Adv. Biochem. Psychopharmacol. *37:* 299-308.
15. O'BOYLE and WADDINGTON, J. L. (1984) Selective and stereospecific interaction of R- SKF 38393 with H^3-piflutixol but not H^3-spiperone binding to D_1 and D_2 dopamine receptors. Comparison with SCH23390. Eur. J. Pharmacol. *98*:433-436.
16. CROW, T. J. (1980) Molecular pathology of schizophrenia: More than one disease process? Brit. Med. J. *280:* 66-68.
17. WADDINGTON, J. L. (1984) Tardive Dyskinesia: A critical reevaluation of the causal role of neuroleptics and of the dopamine receptor hypothesis. In Callaghan N., Galvin R. eds. Recent Research in Neurology. London. Pitman Books.
18. WADDINGTON, J. L. (1986) Is the dopamine hypothesis of tardive dyskinesia completely wrong? - Reply from Waddington - Trends in Neurosci *9*:260.
19. SNYDER, S. H.; GREENBERG, D.; YAMAMURA, H. I. (1974) Antischizophrenic drugs and brain cholinergic receptors: Affinity for muscarinic sites predicts estrapyramidal effects. Arch. Gen. Psychiat. *31:* 58-61.
20. LJUNGBERG, T.; UNGERSTEDT, U. (1978) Classification of neuroleptic drugs according to their ability to inhibit apomorphine-induced locomotion and gnawing: Evidence for two different mechanisms of action. Psychopharmacology *56:* 239-247.
21. BORISON, R. L., FIELDS, J. Z., DIAMOND, B. I. (1981) Site specific blockade of dopamine receptors by neuroleptic agents in human brain. Neuropharmacology *20:* 1321-1323.
22. BORISON, R. L. (1983) Antipsychotic drugs action: Clinical, biochemical and pharmacological evidence for site specificity of action. Clin. Neuropharmacol. *6:* 137-150.
23. BARTHOLINI, G. (1976) Differential effect of neuroleptic drugs on dopamine turnover in the extrapyramidal and limbic system. J. Pharm. Pharmacol. *28:* 429-433.
24. ZIVKOVIC, B.; GUIDOTTI, A.; REVUELTA, A.; COSTA, E. (1975) Effect of thioridazine, clozapine and other antipsichotics on the kinetic state of tyrosine hydroxilase and on the turnover rate of dopamine in striatum and nucleus accumbens. J. Pharmacol. Exp. Ther. *194:* 37-46.
25. UNGERSTEDT, U.; HERRERA-MARSCHITZ, M. (1981) Tardive Dyskinesia: an attempt to bring together data from the clinic and the animal laboratory. In Perris C., Struwe G., Jansson B. eds. Biological Psychiatry. Amsterdam, Elsevier Biomedical Press.
26. HERRERA-MARSCHITZ, M.; HYTTEL, J.; UNGERSTEDT, U. (1984) The dopamine D-1 antagonist SCH23390 inhibits apomorphine but not pergolide induced rotation. Acta Physiol. Scand. *122:* 427-428.
27. GUNNE, L. M. and HAGGSTROM, J. E. (1985) Experimental tardive dyskinesia. J. Clin. Psychiatry *46:* (4, sec. 2), 48-49.
28. GALE, K.; CASU, M. (1981) Dynamic utilization of GABA in substancia nigra: regulation by dopamine and GABA in the striatum and its clinical and behavioural implication. Mol Cell Biochem. *39:* 369-405.
29. ARNT, J.; SCHEEL-KRUGER, J. (1980) Intranigral GABA antagonists produce dopamine independent biting in rats. Eur. J. Pharmacol. *62:* 51-61.
30. GUNNE, L. M.; GROWDON, J.; GLAESER, B. (1982) Oral dyskinesia in rats following brain lessions and neuroleptic drug administration. Psychopharmacology *77:* 134-139.
31. BARANY, S.; GUNNE, L. M. (1976) Pharmacological modification of experimental tardive dyskinesia. Acta Pharmacol. Toxycol. *45:* 107-111.
32. FIBIGER, H. C. and LLOYD, K. G. (1984) Trends in Neurosci *7:* 462-464.
33. FIBIGER, H. C. and LLOYD, K. G. (1986) Trends in Neurosci *9:* 259-260.
34. JENNER, P. and MARSDEN, C. D. (1986) Is the dopamine hypothesis of tardive Dyskinesia completely wrong? Trends in Neurosci *9:* 259.
35. CADET, J. L.; LOHR, J. B.; JESTE, D. V. (1986) Free radicals and Tardive Dyskinesia. Trends in Neurosci. *9:* 107-108.

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Induction of an allergic reaction to alcohol metabolites by immunization

Inducción de una reacción alérgica a metabolitos del alcohol, por inmunización

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Acetaldehyde, a product of alcohol metabolism, is known to bind covalently to plasma and red cell proteins, yielding stable adducts which have recently shown are recognized as foreign by the immune system. The present study demonstrates that immunization of mice with protein-acetaldehyde adducts in aluminum hydroxide gel results in the production of reaginic antibodies that recognize the adducts and trigger an allergic-anaphylatic reaction. These findings may lead to new approaches in the treatment of excessive alcohol consumption in humans.

INTRODUCTION

Ethanol is metabolized in the liver to acetaldehyde and further to acetate (1). In most individuals, the oxidation of acetaldehyde is very efficient, such that only small levels of acetaldehyde appear in the circulation (1). However, if aldehyde dehydrogenase enzyme is inhibited, acetaldehyde levels are markedly increased, leading to a number of aversive reactions which include marked flushing, dyspnea, nausea, hypotension and tachycardia (2, 3). These reactions, simulating in many ways the reactions which characterize severe allergic-anaphylatic reactions, are also observed in individuals who lack the high affinity aldehyde dehydrogenase (4, 6). Individuals with such a genetic trait appear to be effectively deterred from excessive alcohol consumption. For example in Japan, where 40-50% of the general population show the aldehyde dehydrogenase deficiency (6, 7), only 2% of alcoholics in treatment centres show the enzyme deficiency (6, 8).

Disulfiram and calcium carbimide, drugs which are widely used in the treatment of alcohol addiction, are both known to inhibit aldehyde dehydrogenase (2, 3), causing acetaldehyde accumulation and aversive reactions if alcohol is consumed during treatment. However, as these drugs must be taken daily or twice in order to be effective, their potential clinical utility is severely curtailed by lack of compliance with this dosage regimen by the patients (9, 10). Subcutaneous implantation of disulfiram has been attempted, but the amounts that must be implanted are too high to be acceptable and the aversive reaction is too variable (11), and thus this practice has generally been discontinued.

Acetaldehyde, in the concentrations that occur in the blood of alcoholics actively binds to plasma, red cell and liver proteins to form stable adducts (12, 14). We have recently shown that these adducts are recognized as foreign by the immune system (15); antibodies against the acetaldehyde protein-adducts can be raised in laboratory animals by immunization with such adducts, as well as by chronic alcohol consumption (15). These antibodies are able to recognize the acetaldehyde containing epitopes in the adducts, independently of the nature of the protein carrier, and moreover they also appear in the sera of human alcoholics (16, 17).

The techniques employed earlier (15, 17) to measure antibody levels in both animal

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and human studies detect primarily circulating IgGs, IgMs, and IgAs, but not reaginic IgEs which are involved in allergic-anaphylatic reactions. The latter are present in serum at 100,000 fold lower concentrations than those of other immunoglobulins (18). However, IgEs strongly bind to mast cells for prolonged periods up to 12 weeks (18). Upon appropriate antigen recognition, bound IgEs trigger a series of cascade reactions in the mast cells which ultimately lead to mast cell degranulation, including histamine release and the allergic-anaphylatic reaction (19).

Studies in the past decade have shown that IgEs can be induced preferentially over other types of immunoglobulins upon presentation of the antigens in gel microparticles, whether by injection or by inhalation in aerosols (19). Recent studies have shown that such IgE response can be amplified by X-ray irradiation and by adoptive transfer of spleen cells from a previously immunized animal into a naive animal which receives the gel-protein antigens at the time of the adoptive transfer (19, 22). In the present studies, we hypothesized that immunization with acetaldehyde protein-adducts can result in the induction of reaginic which recognize the acetaldehyde adducts triggering an allergic-anaphylatic response.

METHODS

Mice (C57BL/6J) were immunized with acetaldehy-keyhole limpet hemocyanin (Ach-KLH) conjugate (2 ug) prepared as described previously (15) and absorbed on aged aluminum hydroxide gel in a ratio of 2 ug acetaldehyde adduct to 4 mg of gel (20). Booster injections were given on days 7 and 14, and serum samples were collected on days 21 to 28 (series N° 1). To amplify any reaginic antibody responses, the techniques of X-irradiation (series N° 2) and adoptive transfer of spleen cells (series N° 3) were applied (20, 22). Mice in series N° 2 received 250 R on day 8, while mice in series N° 3 were treated as in series N° 2 until day 14. On day 15, their spleen cells were transferred intravenously into naive C57BL/6J mice irradiated with 700 R, followed by an injection of 2 ug of Ach-KLH in aluminium hydroxide within two to three hours. Recipient animals were sacrificed on day 28 and sera were collected for the determination of the acetaldehyde addcut specific reaginic antibodies by the passive cutaneous anaphylaxis (PCA) test in rats (23).

RESULTS AND DISCUSSION

Table I shows that sensitization of rats with sera from mice immunized with acetaldehyde-KLH condensates led to a marked allergic reaction when the rats were challenged with acetaldehyde-human plasma protein condensates, whereas no reaction was observed towards unmodified human plasma proteins. Immunization with carrier KLH did not produce a positive cutaneous anaphylatic reaction either for the acetaldehyde-human plasma protein adducts or for the corresponding unmodified proteins. Treatment of mice with X-rays or adoptive transfer led to two –and 16– fold amplifications of the reaginic antibody titers, respectively.

Data presented indicate that an allergic reaction can be generated against products of ethanol metabolism by specific immunization techniques. It should be noted that the animals were challenged with pre-formed acetaldehyde-protein condensates. It has been previously shown, however, that

TABLE I

Determination of reaginic antibodies against the acetaldehyde-conjugated proteins by the passive cutaneous anaphylaxis (PCA) test.

Immunization	PCA-test*	
A. Acetaldehyde-protein conjugate	Ach-HPP**	Control HPP
Ach-KLH**	80	NIL***
Ach-KLH+250 R	160	NIL
Ach-KLH+adoptive transfer	640-2560	NIL
B. Control protein		
KLH	NIL	NIL
KLH+250 R	NIL	NIL
KLH+adoptive transfer	NIL	NIL

* Male Sprague-Dawley rats (250-350 g) were injected intradermally with 0,1 ml of mouse sera at dilutions from 1:10 to 1:2560. After 24-48 hours, the rats received 1,5 mg of acetaldehyde-human plasma protein intravenously in 1,5 ml of phosphate buffered saline containing 0,5%. Evans Blue. After 30 minutes, the animals were sacrificed and the blue extravasation reactions were examined from the dissected skins. The titres are expressed as reciprocals of the highest dilutions yielding a minimal of 5 mm blueing reaction.

** Preparation of the acetaldehyde-conjugates to keyhole limpet hemocyanin (Ach-KLH) and to human plasma protein (Ach-HPP) has been previously described (15).

*** No blueing at 1:10.

acetaldehyde binds to blood proteins (12, 14) at concentrations which occur *in vivo* in the blood of human alcoholics. Consequently, a similar anaphylatic reaction should occur following alcohol consumption, provided that high reaginic antibody titers have been induced. It should be noted that in atopic individuals with very high natural titers of reaginic antibodies, minute levels of allergens can lead to mast cell degranulation and anaphylaxis (24). Such a level of immunization against acetaldehyde adducts should also be attainable either by passive immunization with specific IgEs or perhaps by active immunization or by potentiating IgE antibody formation (21, 25). Using either approach, a controllable aversive reaction against alcohol could be induced. Since IgEs remain bound to mast cells for prolonged periods of up to three months, the deterrent effect would be tantamount to those produced by well-established agents such as Antabuse. However, a vaccine approach offers the critically important advantage that it would not be subject to the vagaries of compliance to a self-dosing schedule with a drug that produces a variety of aversive side-effects of its own. If its effectiveness is indeed borne out by human studies, a vaccine approach might provide a new approach in the treatment of alcoholism.

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REFERENCES

1. KHANNA, J.M.; ISRAEL Y., in Liver and Biliary Tract Physiology; JAVITT, N.B. Ed. (University Park Press, Baltimore, 1980) Vol. 21, pp. 275-315.
2. KITSON, T.M. (1977) *J. Stud. Alcohol.* 38, 96.
3. SALASPURO, M.; LINDROS, K. in *Alcohol Related Diseases in Gastroenterology,* SEITZ, H.K.; KOMMERELL, B. Eds. (Springer-Verlag, Berlin, 1985) pp. 106-123.
4. MIZOI, Y.; IJIRI, I.; TATSUNO, Y.; KIJIMA, T.; FUJIWARA, S.; ADACHI, J. and HISHIDA, S. (1979) *Pharmacol. Biochem. Behav.* 10, 303-312.
5. IKAWA, M.; IMPRAIN, C.C.; WANG, G.; YOSHIDA, A. (1983) *J. Biol. Chem.* 258, 6282-6287.
6. GOEDDE, H.W.; AGARWAL, D.P., in *Ethnic Differences in Reactions to Drugs and Xenobiotics* (Alan R. Liss, Inc., 1986) pp. 113-138.
7. WOLFF, P.H. (1972) *Science.* 175, 449-450.
8. HARADA' S.; AGARWAL D.P.; GOEDDE, H.W.; TAKAGI, S.; ISHIKAWA, B. (1982) *Lancet ii,* 827.
9. PEACHEY, J.E. (1981) *J. Clin. Psychopharmacol.* 1, 6.
10. SELLERS, E.M.; NARANJO, C.A.; PEACHEY, J.E. (1981) *N. Engl. J. Med.* 305, 1255-1262.
11. WILSON, A. (1975) *J. Stud. Alcohol.* 36, 555.
12. STEVENS, V.J.; FANTL, W.J.; NEWMAN, C.B.; SIMS, R.V.; CERAMI, A.; PETERSON, C.M. (1981) *J. Clin. Invest.* 67, 361-369.
13. DONOHUE, T.M.; TUMA D.J.; SORRELL, M.F. (1983) *Arch. Biochem. Biophys.* 220, 239-246.
14. DONOHUE, T.M.; TUMA, D.J.; SORRELL M.F. (1983) *Lab. Invest.* 49, 226-229.
15. ISRAEL, Y.; HURWITZ, E.; NIEMELÄ, O.; ARNON, R. (1986) *Proc. Natl. Acad. Sci. U.S.A.* 83, 7923-7927.
16. NIEMELÄ, O.; ORREGO, H.; ISRAEL, Y. (1986) *Hepatology.* 6, 1105.
17. HOERNER, M.; BEHRENS, U.J.; WORNER, T.; LIEBER, C.S. (1986) *Res. Comm. Chem. Pathol. Pharmacol.* 54, 3.
18. PAUL, W.E. (Raven Press, New York, 1984) *Fundamental Immunology.*
19. ISHIZAKA, K.; ISHIZAKA, T., in *Allergy, Principles and Practice,* MIDDLETON, E. REED C.E., ELLIS, E.F. Eds. (C.V. Mosby, St. Louis, 1983) Vol. 1, pp. 43-73.
20. ESHHAR, Z.; OFARIM, M. WAKS, T. (1980) *J. Immunol.* 124, 775-780.
21. ISHIZAKA, K. (1984) *Ann. Rev. Immunol.* 2, 159.
22. BARGATZE, R.F.; KATZ, D.H. (1980) *J. Immunol.* 125, 2306-2310.
23. OVARY, Z. in *Immunological Methods,* ACKROYD, J. Ed. (Blackwell, Oxford, 1964) pp. 259-283.
24. SMITH, J.M. in *Allergy, Principles and Practice,* MIDDLETON, E.; REED, C.E. ELLIS, E.F. Eds. (C.V. Mosby, St. Louis, 1983) Vol. 2, pp. 771-803.
25. KATZ, D.H.; MARCELLETTI, J.F. (1983) *Progr. Immunol.* 5, 465.

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Estudios sobre el mecanismo bioquímico de acción del flavonoide silybina: Relación con sus propiedades terapéuticas

Studies on the biochemical mechanism of action of the flavonoid silybin: relationship with its therapeutic properties

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Este estudio presenta en forma sucinta los aspectos mas relevantes de nuestro trabajo de investigación con el flavonoide silybina. Su mecanismo de acción como citoprotector se relacionaría con una acción a tres niveles: como antioxidante, evitando la lipoperoxidación celular inducida por xenobióticos; aumentando la concentración intracelular de glutatión, permitiendo mejorar la función protectora y de desintoxicación de este tripéptido, y regulando la permeabilidad de las membranas celulares en forma relativamente específica a la entrada o salida de metabolitos. Se discuten las proyecciones terapéuticas del flavonoide, así como su efecto protector específico en la toxicidad hepática de la fenilhidrazina, el etanol y el acetaminofeno.

INTRODUCCION

Los flavonoides pertenecen a la familia de los derivados de las benzo-γ-pironas y son muy abundantes en las células fotosintéticas de los vegetales. En la actualidad se conocen más de 500 variedades diferentes y su existencia en la naturaleza está muy difundida, ya que al incorporarse a la cadena alimentaria están presentes en los insectos, moluscos, reptiles e incluso mamíferos. Desde tiempos inmemoriales, se ha atribuido a muchos flavonoides propiedades terapéuticas y curativas de variada índole, aunque existen muy pocos estudios científicos sobre estas propiedades y mucho menos sobre el mecanismo de acción de estas estructuras. En la actualidad algunos flavonoides como la quercetina, la taxifolina y la silybina, entre otros, son utilizados como principios farmacológicos como tales, o como componentes de formulaciones más complejas (1). De ellos, quizás la silybina es el más conocido y el que presenta propiedades terapéuticas más definidas.

La silybina es uno de los isómeros que conforman la silymarina, complejo estructural que se extrae de las semillas del cardo Mariano o cardo lechero, muy abundante en los países con clima mediterráneo (2). Los otros dos isómeros que componen la silymarina, la silydianina y la silycristina, se encuentran en menor proporción y son mucho menos activos farmacológicamente, Fig. 1. La silybina ha sido definida desde el punto de vista médico como un citoprotector y más específicamente como un hepato protector (3). Este flavonoide es, prácticamente, el único antídoto contra la intoxicación por el hongo amanitas faloides (4) y en la actualidad es utilizado en el tratamiento de numerosas hepatopatías que involucran necrosis degenerativas con compromiso de la función hepática. A pesar de su uso en diferentes formas y preparados farmacéuticos en muchos países, incluido el nuestro, es muy poco lo que se sabe sobre su mecanismo de acción a nivel celular.

Nuestro grupo ha dedicado estos últimos cuatro años a caracterizar el mecanismo bioquímico involucrado en la acción de la silybina, Aquí presentamos una reseña de nuestros principales resultados como un modesto homenaje al Dr. Osvaldo Cori, formador de muchas generaciones de bioquímicos y de quien nos honramos en haber sido sus alumnos.

[1] A quien se debe dirigir la correspondencia.

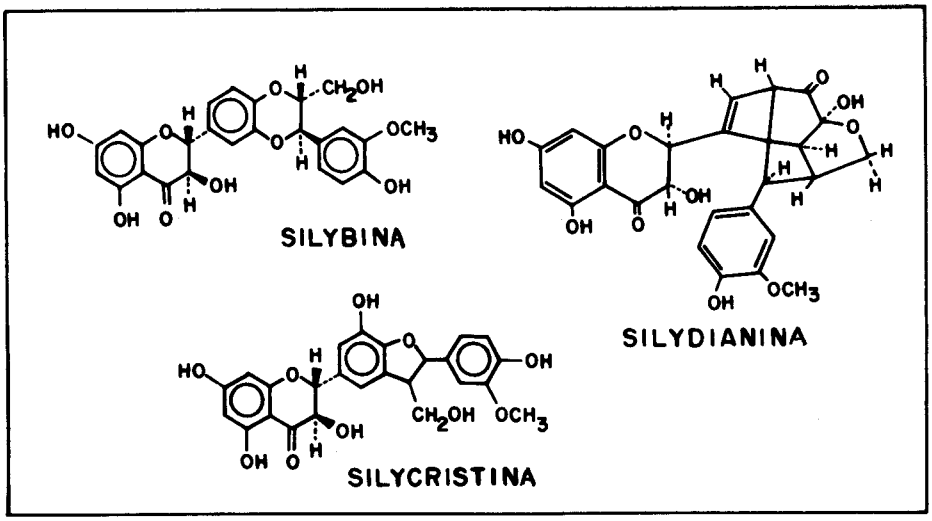

Fig. 1: Estructura química de los tres isómeros del flavonoide silymarina.

I. *Propiedades antioxidantes del flavonoide silybina*

Los flavonoides en general son buenos antioxidantes, ya que la presencia de grupos hidroxilos como sustituyentes de los núcleos bencénicos que conforman su estructura, hace posible su acción como donantes de hidrógenos capaces de neutralizar los radicales libres involucrados en los fenómenos peroxidativos (1). La silybina no escapa a esta propiedad, ya que un derivado hidrosoluble de ésta, en la forma dihemisuccinato, presenta un poderoso efecto antioxidante cuando es ensayada en sistemas modelos de peroxidación, como es el formado por una emulsión de linoleato de potasio cuya oxidación es catalizada por Fe^{+2} (5). Su efecto antioxidante es incluso más eficiente que el obtenido en este mismo sistema con antioxidantes como el butil hidroxianisol (BHA) o el butil hidroxitolueno (BHT). La Figura 2 resume estos resultados.

Este efecto antioxidante se expresa incluso en sistemas más complejos, ya que si se cataliza la peroxidación de microsomas obtenidos de hígados de rata mediante la adición de NADPH-Fe^{+2}-ADP al medio de incubación es posible obtener un efecto antilipoperoxidativo que es dependiente de la concentración del flavonoide (6). En este caso, la lipoperoxidación fue evaluada mediante dos criterios diferentes. Medición de la formación de malondialdehído (MDA), técnica que mide productos finales del proceso lipoperoxidativo y medición de la quimioluminiscencia espontánea (QL) que permite cuantificar el decaimiento de formas inestables del oxígeno que emiten

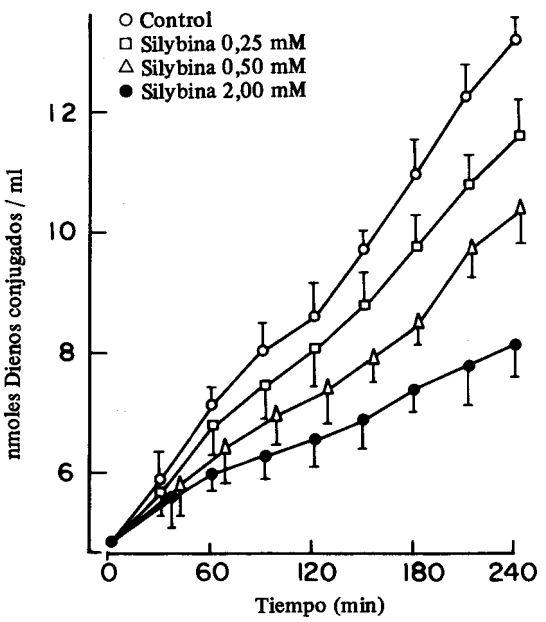

Fig. 2: Efecto antioxidante de diferentes concentraciones de silybina en la peroxidación de una emulsión de linoleato de potasio catalizada por Fe^{+2} (500 uM). La peroxidación se midió a través de la formación de dienos conjugados. Cada punto representa el promedio de seis experimentos ± E.S.M. Adaptado de Valenzuela y col. (5).

luz. Estos resultados se muestran en la Figura 3. El efecto antilipoperoxidativo de silybina no es general, ya que si la peroxidación de los microsomas es catalizada por otro iniciador, como es el terbutil hidroperóxido (TBH), el efecto es nulo (6). Esta observación nos permitió plantear una selectividad por parte del flavonoide, en su capacidad para atrapar y anular radicales libres. En efecto, el sistema NADPH-Fe^{+2}-ADP es un potente generador de radicales libres hidroxilos (·OH) (7); en cambio, la estructura involucrada en el efecto prooxidante del TBH es el radical libre terbutoxi. Este último es un radical voluminoso, desde el punto de vista molecular, siendo el radical hidroxilo mucho más pequeño. Nuestro grupo plantea que la selectividad del flavonoide frente al efecto de ambos iniciadores estaría determinada por la posibilidad de interacción de éste con el radical libre hidroxilo y terbutoxi, al ser este último más voluminoso, la interacción se dificultaría, probablemente debido a problemas de tipo estéricos.

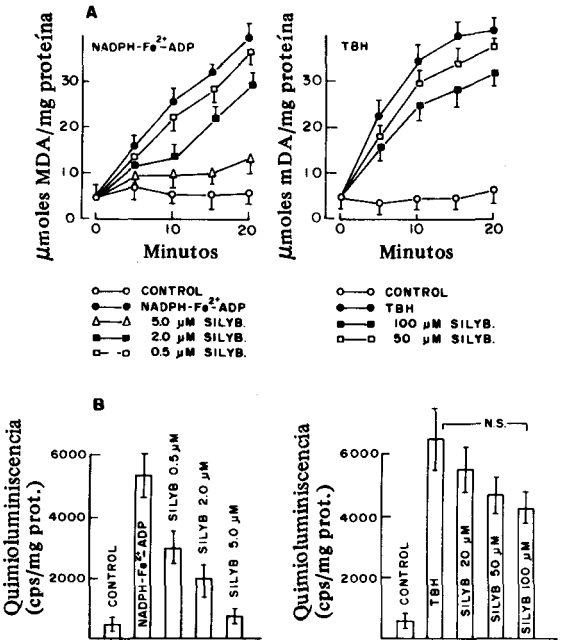

Fig. 3: Efecto de silybina en la peroxidación de microsomas de hígado de rata catalizada por NADPH-Fe^{2+}-ADP y terbutil hidroperóxido (TBH). A: Formación de malondialdehído (MDA), B: Emisión de quimioluminiscencia (QL) después de 10 minutos de exposición al catalizador. Adaptado de Valenzuela y col. (6).

II. *Efecto protector de silybina en modelos biológicos donde se induce estrés oxidativo*

A. Estudios con fenilhidrazina

Conceptualmente el estrés oxidativo es la respuesta de un tejido, órgano u organismo a la generación no controlada de formas prooxidantes del oxígeno (8). Generalmente se produce frente a inductores, cuya actividad prooxidante sobrepasa la capacidad de defensa del organismo alterando la homeostasis del sistema. Puesto que las propiedades antioxidantes observadas en la silybina se relacionarían con su capacidad para atrapar ciertos radicales libres (probablemente del tipo hidroxilo), nos propusimos utilizar algunos modelos experimentales utilizando drogas cuya citotoxicidad se relaciona con la inducción de estrés oxidativo.

La técnica del hígado perfundido es especialmente adecuada para la realización de estudios de estrés oxidativo, ya que en estas condiciones es posible medir muchos parámetros metabólicos en este órgano, tales como consumo de oxígeno tisular, liberación de metabolitos al líquido de perfusión, variación de la concentración de metabolitos en el órgano durante la perfusión, etc. Como agente prooxidante elegimos a la fenilhidrazina, cuya toxicidad celular ha sido relacionada por nuestro grupo con la generación de radicales libres de tipo hidroxilo (9).

En la Figura 4 se puede observar la respuesta de hígados de rata que son perfundidos con fenilhidrazina, siendo previamente tratados los animales con silybina. La perfusión con fenilhidrazina produce un brusco aumento del consumo de oxígeno del tejido que se correlaciona muy bien con el aumento de la liberación de MDA al líquido de perfusión (10). Estos resultados son una expresión del estrés oxidativo que induce la fenilhidrazina en el tejido hepático. Cuando los animales son previamente tratados con silybina (16 horas antes de la perfusión, inyección intraperitoneal), la respuesta producida por la fenilhidrazina, tanto en el consumo de oxíge-

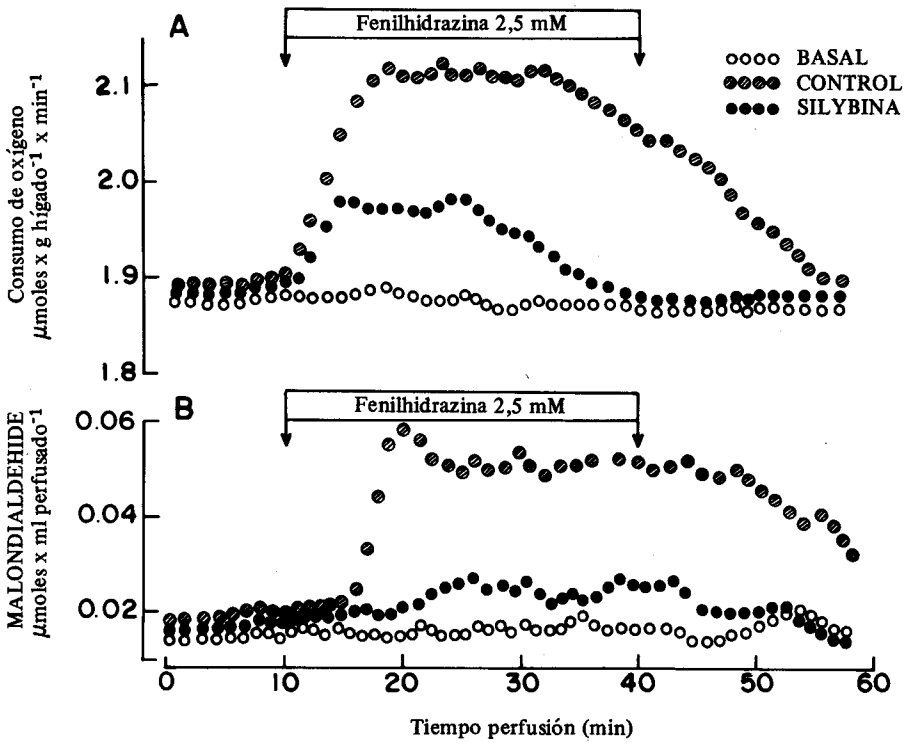

Fig. 4: Efecto protector de silybina en el aumento del consumo de oxígeno hepático (A) y en la liberación de malondialdehído al medio (B) al perfundir con fenilhidrazina. Adaptado de Valenzuela y col. (10).

no hepático como en el MDA liberado por el tejido, es considerablemente menor. En esta situación, el flavonoide está ejerciendo un efecto hepatoprotector probablemente derivado de su actividad antioxidante.

El glutatión reducido (GSH) es considerado como una de las biomoléculas más importantes en la protección celular contra el estrés oxidativo (11). De hecho, situaciones metabólicas que implican estrés oxidativo, acompañadas por aumento del consumo de oxígeno de un tejido y aumento de la formación de MDA, se acompañan también por disminuciones del contenido intracelular de GSH, que generalmente guarda relación con el nivel de daño oxidativo producido. En el caso de la intoxicación con fenilhidrazina, ésta produce una disminución del contenido hepático de GSH (Figura 5). Si los animales son tratados previamente con el flavonoide, se produce una importante protección de la disminución del tripéptido (10). Más aún, un aspecto que es intere-

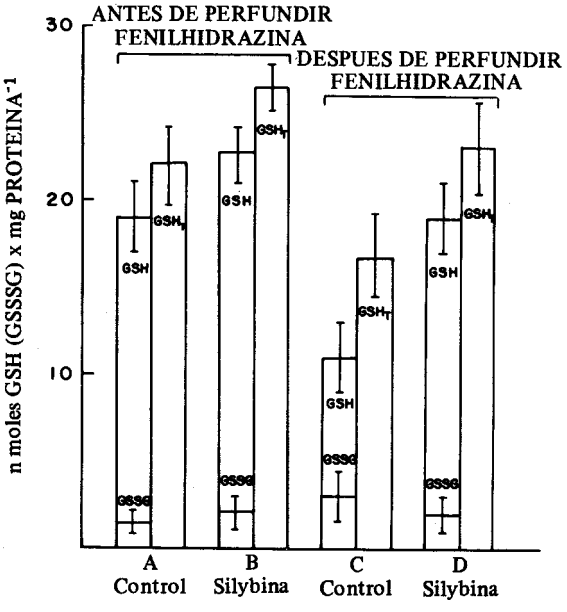

Fig. 5: Depleción de GSH hepático producido por la perfusión con fenilhidrazina (C). Protección obtenida al pretratar los animales con silybina (D). El tratamiento con el flavonoide antes de perfundir con fenilhidrazina produce un aumento en el GSH hepático (B). Adaptado de Valenzuela y col. (10).

sante destacar es el aumento en los niveles de glutatión que se observa en los hígados de ratas tratadas con el flavonoide antes de ser perfundidos con fenilhidrazina. Aunque no tenemos una explicación clara sobre el mecanismo de este efecto, más adelante se aventuran algunas hipótesis.

El estudio del efecto oxidativo y hemolítico de la fenilhidrazina en eritrocitos obtenidos de ratas tratadas con el flavonoide tiene características similares a aquellas obtenidas en la perfusión hepática. La formación de MDA, así como el aumento de la quimioluminiscencia espontánea que produce la fenilhidrazina en los eritrocitos, es inhibida por el flavonoide (Fig. 6) (12); del mismo modo, la silybina produce una inhibición del consumo de oxígeno de los eritrocitos, que es estimulado por fenilhidrazina (12). Cabe destacar que, a diferencia del hígado, el eritrocito no tiene respiración metabólica, de modo que el consumo de oxígeno que estimula la fenilhidrazina es principalmente derivado de la lipoperoxidación celular.

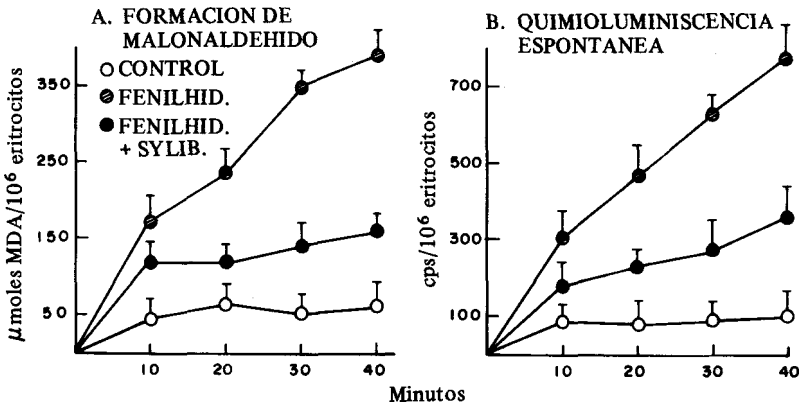

Fig. 6: Efecto inhibidor de silybina en la formación de malondialdehído (MDA) (A) y en la emisión de quimioluminiscencia (B) producidas por eritrocitos de ratas incubados con fenilhidrazina. Adaptado de Valenzuela y col. (12).

B. Estudios con etanol

El etanol no sólo es un inductor de lipoperoxidación en algunos tejidos como el hígado y pulmón (13) sino que es un importante depletador de GSH (14). Se ha relacionado la caída del GSH hepático como una consecuencia del estrés oxidativo (15), de modo que tejidos con bajos niveles de GSH serían más susceptibles al daño lipoperoxidativo (16). La silybina inhibe la lipoperoxidación hepática inducida por una intoxicación aguda con etanol (5 g/kg) en ratas y también protege de la disminución que produce este tóxico en los niveles hepáticos de GSH (17). (Fig. 7). Es interesante destacar que en los animales controles, inyectados con el flavonoide, los niveles de GSH son considerablemente más altos que en los controles no tratados. Este efecto, que también fue observado en el caso de la perfusión con fenilhidrazina, puede ser consecuencia de una acción del flavonoide diferente a su acción como atrapador de radicales li-

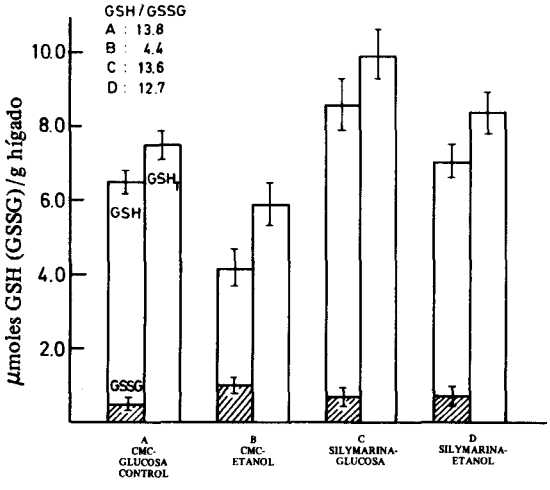

Fig. 7: Efecto protector de silybina en la depleción del GSH hepático producida por una intoxicación aguda con etanol en la rata. Adaptado de Valenzuela y col. (17).

bres, esto es, un efecto directo en el metabolismo del GSH hepático. En la actualidad estamos estudiando este aspecto utilizando hepatocitos aislados e inhibidores de la síntesis de GSH como la butionina sulfoximida (18) con el propósito de observar un posible efecto estimulador del flavonoide en alguna enzima clave involucrada en la biosíntesis del tripéptido.

III. *Efecto de silybina en la permeabilidad celular y en la resistencia de los eritrocitos al shock osmótico*

El carácter lipofílico de la silybina ha permitido postular a algunos autores (2, 3) que su mecanismo de acción, o, al menos, algunos de sus efectos fisiológicos, se relacionarían con un efecto de control sobre la permeabilidad celular. En efecto, la permeabilidad de células de médula ósea de rata frente a un precursor radiactivo (uridina-H^3) aumenta considerablemente cuando los animales, antes de la extracción del tejido, son tratados con silybina (19). (Fig. 8). El aumento de la concentración intracelular de este precursor, tanto en el nucleoplasma como en el citoplasma, se refleja en un aumento de la síntesis de RNA (uridina-H^3 es un precursor específico). Este aumento de la síntesis de RNA no obedece a un estímulo en la actividad de alguna de las polimerasas por parte del flavonoide (19), por lo cual es interpretado por nuestro grupo como una consecuencia del aumento del precursor radiactivo en el medio intracelular. Estos resultados estarían indicando que el flavonoide ejercería un efecto, que aún no sabemos si es específico o inespecífico, a nivel de la permeabilidad celular.

Esta idea es reforzada por los resultados que hemos obtenido con eritrocitos de rata. La resistencia de estas células al shock osmótico aumenta considerablemente cuando los animales son previamente tratados con el flavonoide (Fig. 9) (20). Más aún, el efecto metahemoglobinizante de la fenilhidrazina, que no es inhibido por el flavonoide en preparaciones de hemoglobina libre, es impedido si los eritrocitos expuestos a la fenilhidrazina provienen de ratas tratadas con el flavonoide (12). Estos resultados, en conjunto con aquellos obtenidos con células de médula ósea, refuerzan la idea que el flavonoide actuaría modulando la permeabilidad celular e incluso modificaría algunas propiedades físico-químicas de la membrana (cambiando su resistencia). Nuestros resultados estarían de acuerdo con lo postulado por algunos autores que proponen un efecto estabilizador de membranas (2, 4).

IV. *Efecto hepatoprotector de la silybina contra la toxicidad de xenobióticos*

Nuestros resultados nos han permitido plantear que en aquellas situaciones patológicas que involucren depleción intra-

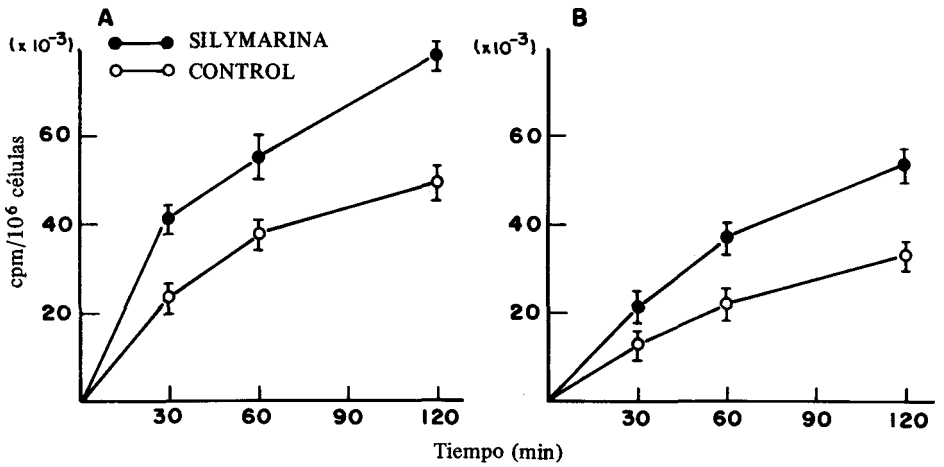

Fig. 8: Efecto estimulador de silybina en la incorporación de uridina-H^3 a la fracción ácido soluble (A) y ácido insoluble (RNA) (B) en células aisladas de médula ósea de rata. Adaptado de Garrido y col. (19).

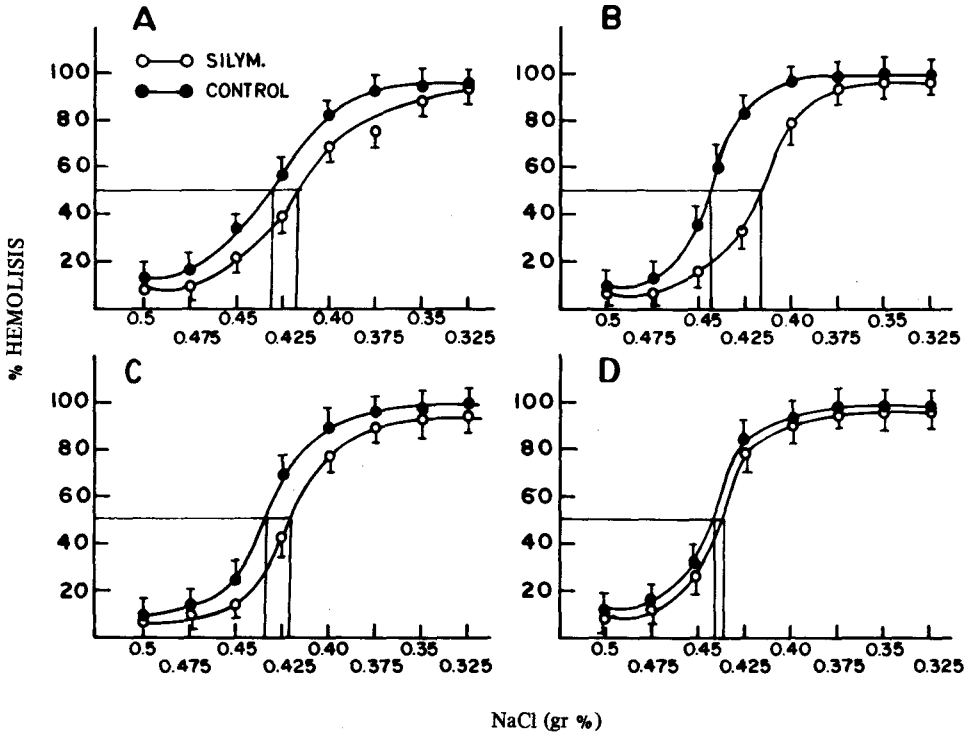

Fig. 9: Efecto de silybina en el aumento de la resistencia al shock osmótico de eritrocitos de rata incubados 1 hora (A), 2 horas (B) y sensibilizados por fenilhidrazina (C y D). Adaptado de Valenzuela y col. (20).

celular de GSH y/o inducción de lipoperoxidación, el flavonoide podría tener efectos beneficiosos. Como modelo de estudio elegimos el acetaminofeno (N-acetil-p-aminofenol) cuya toxicidad se relaciona con una marcada depleción de GSH y con la inducción de lipoperoxidación cuando es administrado en forma aguda en dosis superiores a 300 mg/kg (21). La silybina protege al hígado de rata contra ambos efectos cuando es administrada hasta una hora después de la intoxicación con acetaminofeno (350 mg/kg) (22) (Fig. 10). Del mismo modo, otro parámetro indicador de hepatotoxicidad, como es el aumento sérico de la actividad de las transaminasas glutámico-oxaloacética (GOT) y glutámico-pirúvica (GPT), es inhibido en forma importante al tratar a los animales intoxicados con el flavonoide (22). Estos resultados, que son muy similares a los obtenidos con etanol, abren una interesante perspectiva de estudio en la posible aplicación terapéutica del flavonoide en el caso de la intoxicación con acetaminofeno. Cabe destacar que este fármaco, que es un muy buen analgésico y antipirético, es utilizado en muchos países, incluido el nuestro, con fines suicidas (10 a 15 g son suficientes para producir una hepatotoxicidad mortal). En la actualidad, las intoxicaciones con acetaminofeno son tratadas con precursores de la síntesis de GSH o con sustancias que pueden reemplazarlo en su actividad citoprotectora como es el caso de la N-acetil cisteína, la cisteamina o la cisteína como tal. Puesto que el mecanismo citoprotector de la silybina no estaría relacionado con el de los antídotos actualmente utilizados, es posible vislumbrar una importante nueva aplicación terapéutica para este flavonoide como coadyuvante de los antídotos mencionados. Es indudable que este aspecto requiere de un estudio muy amplio, ya que hay que tener presente que la rata no es un buen modelo para el estudio de hepatotóxicos, debido a su extraordinaria resistencia a los fármacos y drogas de estas características.

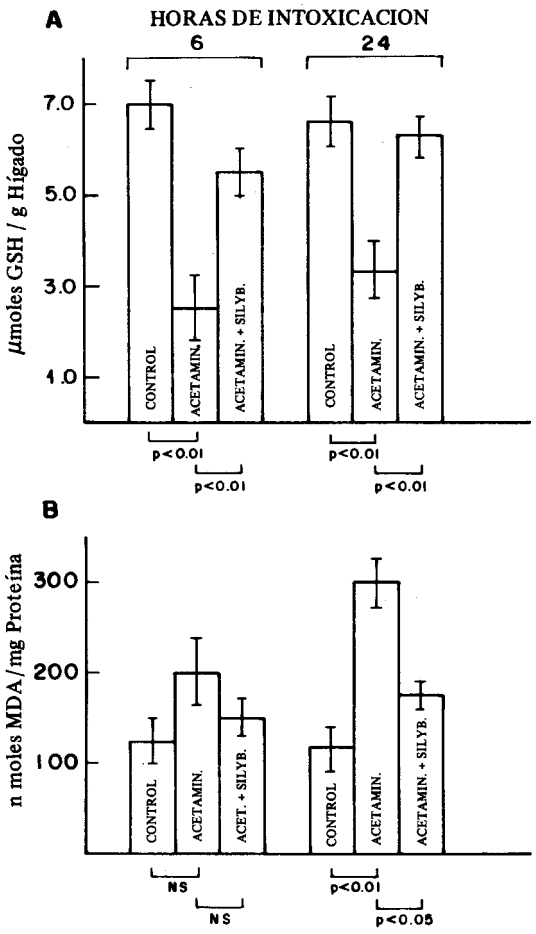

Fig. 10: Efecto protector de silybina sobre la depleción hepática de GSH (A) y sobre la formación de malondialdehído (MDA) (B) producida por una intoxicación aguda con acetaminofeno en la rata. Adaptado de Campos y col. (22).

En la actualidad nuestro grupo está estudiando el efecto del flavonoide en la hepatotoxicidad del acetaminofeno potenciada por el etanol, tanto en modelos *in vivo* como *in vitro* utilizando, en este último caso, las técnicas de perfusión hepática y la preparación de hepatocitos aislados. Cabe destacar que en nuestro país esta patología es de frecuencia cada vez más creciente, ya que muchos bebedores en exceso buscan aliviar las molestias ocasionadas por el alcohol consumiendo analgésicos como la aspirina y el acetaminofeno (paracetamol^{R}).

V. *Apreciaciones finales y perspectivas futuras*

Nuestros resultados, aunque si bien no son de carácter preliminar, son aún muy básicos, nos permiten clarificar algunos aspectos sobre el mecanismo de acción del flavonoide silybina. Su acción citoprotectora se podría centrar en tres aspectos: A) Su actividad antioxidante, que le permitiría controlar y detener los procesos lipoperoxidativos inducidos por xenobióticos en el medio intracelular. Esta actividad protectora se expresaría principalmente a nivel de las membranas celulares (citoplasmática, microsomal, mitocondrial, etc.) y estaría favorecida por el carácter lipofílico del flavonoide. B) Efecto a nivel de los niveles intracelulares de GSH. Si bien el mecanismo de este efecto no ha sido aclarado aún por nuestro grupo, es claro que el aumento de la disponibilidad intracelular del tripéptido puede tener importantes repercusiones en el estado de citoprotección celular. La mayor disponibilidad del GSH puede favorecer sus funciones como antioxidante, como agente de conjugación de metabolitos celulares y de xenobióticos y como cofactor de numerosas enzimas involucradas en la desintoxicación celular, como es el caso de la enzima glutatión peroxidasa, entre otras. C) Su función a nivel de la permeabilidad celular, que requiere de mayores estudios, puede relacionarse con los dos efectos ya comentados. Silybina se fija a nivel de la membrana citoplasmática, modulando probablemente la entrada y salida de metabolitos, dentro de los cuales estaría el GSH, quien en forma continua y más marcadamente en situaciones de estrés abandona la célula (en su forma oxidada principalmente). No sabemos aún si los mayores niveles intracelulares de GSH que se observan en los animales tratados con el flavonoide, especialmente en los órganos digestivos (23), sean consecuencia de una regulación en el eflujo de GSH, de una regulación a nivel de su síntesis y/o degradación o de ambos efectos en conjunto.

Queda mucho aún por investigar sobre el mecanismo de acción y las propiedades terapéuticas de este flavonoide, así como también de muchos otros flavonoides de similares características. La zona norte de Chile es rica en especies vegetales silvestres con algún contenido de flavonoides simi-

lares a la silybina (que se obtiene de plantaciones en Alemania, la India y Argentina), lo cual nos permite vislumbrar una interesante perspectiva de investigación farmacológica con productos de nuestra flora vegetal.

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REFERENCIAS

1. HAVSTEEN, B. (1983) Flavonoids, a class of natural products of high pharmacological potency. *Biochem. Pharmacol. 32:* 1141-1145.
2. LECOMTE, J. (1975) Les propiétés pharmacologiques de la silybine et de la silymarine. *Rev. Med. Liege. 30:* 110-114.
3. VOGEL, G. (1976) Pharmacological properties of silymarin, the antihepatotoxic agent from the seeds of the milk thistle, silybum marianum (L) Gaerth. En Symposium on the Pharmacodynamics of Silymarin. R. Braatz and L. Schneider (eds.). Urban & Schwarzenberg. Munchen, p. 1.
4. VOGEL, G. (1968) Silymarin, das antihepatotoxische Wirskprinzip aus silybum marianum (L). Gaerth. Als antagonist der phalloidin-wirkung. *Arzneim-Forsch (Drug. Res) 18:* 1063-1067.
5. VALENZUELA, A.; GUERRA, R. y VIDELA, L.A. (1986) Antioxidant properties of the flavonoids silybin and (+)-cyanidanol-3: Comparison with butylated hydroxyanisole and butylated hydroxytoluene. *Planta Médica, 6:* 438-440.
6. VALENZUELA, A. y GUERRA, R. (1986) Differential effect of silybin on the Fe^{2+}-ADP and t-butil hydroperoxide-induced microsomal lipid peroxidation. *Experientia 42:* 139-141.
7. WILLS, E. (1969) Lipid peroxide formation of microsomes. The role of non-haem iron. *Biochem. J. 113:* 325-332.
8. SIES, H. (1986) Biochemistry of oxidative stress. *Angew. Chem. 25:* 1058-1071.
9. VALENZUELA, A.; RIOS, H. y NEIMAN, G. (1977) Superoxide radicals and the hemolytic mechanism of phenylhydrazine. *Experientia 33:* 962-963.
10. VALENZUELA, A. y GUERRA, R. (1985) Protective effect of the flavonoid silybin dihemisuccinate on the toxicity of phenylhydrazine on rat liver. *FEBS. Lett. 181:* 291-294.
11. VIDELA, L.A. y VALENZUELA, A. (1982) Alcohol ingestion, liver glutathione and lipoperoxidation: Metabolic interactions and pathological implications. *Life Scien. 31:* 2395-2399.
12. VALENZUELA, A.; GUERRA, R. y GARRIDO, A. (1987) Silybin dihemisuccinate protects rat erythrocytes against phenylhydrazine-induced lipid peroxidation and hemolysis. *Planta Médica, 53:* 402-405.
13. VIDELA, L.A.; FERNANDEZ, V.; DE MARINIS, A.; FERNANDEZ, N.; VALENZUELA, A. (1982) Liver lipoperoxidative pressure and glutathione status following acetaldehyde and aliphatics alcohols pretreatment in the rat. *Biochem. Biophys. Res. Comm. 104:* 945-949.
14. VIDELA, L.A.; FERNANDEZ, V.; FERNANDEZ, N.; VALENZUELA, A. (1981) On the mechanism of the glutathione induced depletion in the liver by acute ethanol ingestion. Substance and alcohol actions/misuse. *2:* 153-160.
15. VALENZUELA, A.; FERNANDEZ, V.; FERNANDEZ, N.; UGARTE, G.; VIDELA, L.A. (1980) Effect of acute ethanol ingestion on lipoperoxidation and on the activity of the enzymes related to peroxide metabolism. *FEBS. Lett. 111:* 11-14.
16. VIDELA, L.A.; FERNANDEZ, V.; UGARTE, G.; VALENZUELA, A. (1980) Effect of acute ethanol intoxication on the content of reduced glutathione of the liver in relation to its lipoperoxidative capacity in the rat. *FEBS. Lett. 111:* 6-10.
17. VALENZUELA, A.; LAGOS, C.; SCHMIDT, K. y VIDELA, L.A. (1985) Silymarin protection against hepatic lipid peroxidation induced by acute ethanol intoxication in the rat. *Biochem. Pharmacol. 34:* 2209-2212.
18. REICHMAN, P.; ORLOWSKI, M. ; MEISTER, A. (1973) Inhibition of glutamylcysteine synthetase by L-methionine-S-sulfoximide. *J. Biol. Chem. 248:* 6684-6690.
19. GARRIDO, A.; GUERRA, R.; VALENZUELA, A. (1988) Silymarin increases permeability of bone marrow cells to (^{3}H)-Uridine. *Res. Comm. Chem. Pathol. Pharm.* En prensa.
20. VALENZUELA, A.; BARRIA, T.; GUERRA, R. y GARRIDO, A. (1985) Inhibitory effect of the flavonoid silymarin on the erythrocyte hemolysis induced by phenylhydrazine. *Biochem. Biophys. Res. Comm. 126:* 712-716.
21. GILLETTE, J.R.; MITCHELL, J.R. y BRODIE, B.B. (1974) Biochemical mechanism of drug toxicity. *Ann. Rev. Pharmacol. 14:* 271-282.
22. CAMPOS, R.; GARRIDO, A.; GUERRA, R. y VALENZUELA, A. (1988) Silybin dihemisuccinate protects against glutathione depletion and lipid peroxidation induced by acetaminophen on rat liver. *En* Plant Flavonoids in Biology and Medicine: Biochemical, Cellular and Medicinal Properties. Alan Liss, Inc., New York. En prensa.
23. VALENZUELA, A.; ASPILLAGA, M.; VIAL, S. y GUERRA, R. (1988) Selectivity of silymarin on the increase of the glutathione content of different tissues of the rat. *Planta Médica,* en prensa.

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Biochemical aspects of cellular oxidative stress*

Aspectos bioquímicos del estrés oxidativo celular

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Oxidative stress, as proposed by H. Sies, indicates a change in the prooxidant/antioxidant balance of a biologic system in favour of the former. It is related to oxidative reactions that occur in aerobic metabolism which can damage biomolecules through generation of reactive oxygen species. The oxidative deterioration of polyunsaturated fatty acids of membrane phospholipids (lipid peroxidation) is one of the consequences of oxidative stress, and has been observed in the liver cell under the influence of ethanol or lindane intoxication, associated with the calorigenic action of thyroid hormones, induced by the rupture of t-butyl hydroperoxide by cellular hemoproteins, or in the autoxidation of a disrupted tissue. Appart from noxious challenges, oxidative free-radical processes are important in numerous physiological reactions, such as NADPH oxidase in the function of macrophages, ribonucleotide reduction in DNA metabolism, or in eicosanoids production.

General aspects of oxidative stress and antioxidant defense mechanisms in the cell

Due to the aerobic nature of cellular metabolism, oxygen reduction is a major event in biological systems. The electronic structure of dioxygen in the ground state has been described by the one-electron molecular orbital diagram (Fig. 1A), with two unpaired electrons as a reflection of the Pauli Principle. Thus, the chemistry of dioxygen is that of one-electron transfer reactions (Fig. 1D), leading to the generation of reactive intermediates (Fig. 1, Table 1) which can interact with biomolecules and induce their oxidative deterioration. Free-radical reactions, in turn, can generate electronically excited states, such as singlet oxygen and triplet carbonyls (Fif. 1C, Table 1). Since biological systems have antioxidant mechanisms, the term oxidative stress was introduced by Sies to denote a "shift in the prooxidant/antioxidant balance in favour of the former" (1, 2).

The detoxication of reactive oxygen is one of the prerequisites of aerobic life (2, 3). For this purpose, several enzymatic (Table 2) and nonenzymatic (Table 3) mechanisms of defense against

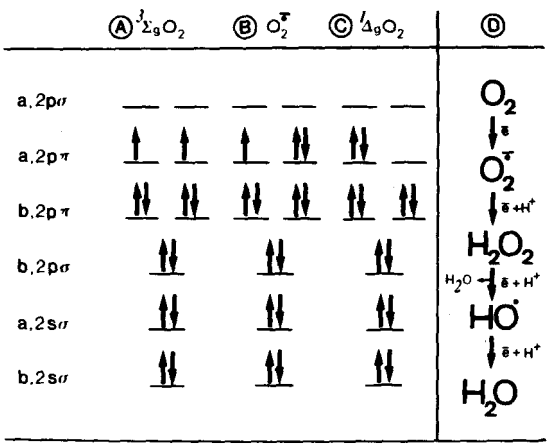


Fig. 1: Electronic structure of (A) dioxygen (triplet state), (B) superoxide radical, and (C) singlet oxygen (first excited state). (D), one-electron reduction of dioxygen.

oxidative damage are available in the different cell compartments (2-4). These include the enzymatic catabolism of some species (Table 2A), scavenging of free radicals, and quenching of excited states (Table 3) as primary mechanisms, plus the operation of secondary enzymatic systems to restore used cofactors and repair altered biomolecules (Table 2B) (2-4). Thus, antioxidant mechanisms must be coupled to the intermediary metabolism

* This paper was written as a tribute to Prof. Osvaldo Cori for his mastery in Biochemistry, research achievements, and, most important, for his friendship and support.

TABLE 1
Reactive oxygen species related to oxidative stress in cells*

Superoxide radical($O_2^{\cdot-}$)	One-electron reduction state of O_2 formed a) *enzymatically (microsomal oxygenation, mitochondrial respiratory chain, photosynthetic* O_2 reduction, flavin-dependent oxidations); b) by autoxidation of *flavins, hemoglobin, thiols, catecholamines, iron chelates;* c) by redox cycling of xenobiotics (adriamycin, paraquat, alloxan, bleomycin-Fe^{2+}); d) by physical factors (ultraviolet light, X-rays).
Hydrogen peroxide (H_2O_2)	Two-electron reduction state of O_2, formed by dismutation of $O_2^{\cdot-}$ or from O_2 reduction.
Hydroxyl radical ($HO^{\cdot}$)	Three-electron reduction state of O_2 formed by Fenton reaction ($Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + HO^{\cdot}$) or the iron-catalyzed Haber-Weiss reaction.
Alkoxy radical ($RO^{\cdot}$)	Oxygen-centered organic radical (e.g., fatty acid-derived radical formed in lipid peroxidation).
Peroxy radical ($ROO^{\cdot}$)	Radical species formed by O_2 incorporation into a fatty acyl radical ($R^{\cdot}$).
Hydroperoxide (ROOH)	Organic compound derived from fatty acids, thymine, cholesterol, etc.
Singlet oxygen ($^1\Delta g\ O_2$) (O_2^*)	First excited state of O_2, 22 Kcal/mol above ground state, with either red (dimol) or infrared (monomol) photoemission. Chemical excitation of O_2 can proceed either by peroxy radicals ($ROO^{\cdot}$) interaction (Russell's mechanism) or via oxene transfer using heme-Fe^{3+}.
Triplet carbonyls (R'R"CO*)	Excited carbonyl compound (formed via dioxetane rupture or $ROO^{\cdot}$ disproportionation) with blue-green photoemission.

* Adapted from Sies (2).

TABLE 2
Enzymatic antioxidants in biological systems*

A. Primary systems	
Superoxide dismutase (SOD)	Disproportionation of $O_2^{\cdot-}$ into O_2 and H_2O_2 by the CuZn-cytosolic enzyme or by the Mn-enzyme of the mitochondrial matrix in eukaryotic cells.
Catalase	Disproportionation of H_2O_2 into O_2 and H_2O by the heme-enzyme of the peroxisomal matrix.
Glutathione peroxidases	Reduction of H_2O_2 or hydroperoxides (ROOH) with GSH (GSSG Production) by the seleno-enzyme, non-Se-enzyme and some GSH transferases in the cytosol and mitochondrial matrix.
B. Secondary systems	
Phospholipase A_2	Hydrolysis of peroxidized fatty acid esters in phospholipid membranes to allow detoxication of the fatty acid hydroperoxides (ROOH) by glutathione peroxidases and repair by reacylation.
Glutathione reductase	Reduction of GSSG into GSH with NADPH, by the flavin-enzyme in cytosol and mitochondria.
NADPH-regenerating systems	Supply of NADPH in the cytosol (pentose phosphate pathway, isocitrate dehydrogenase and malate enzyme) or mitochondria (isocitrate dehydrogenase and energy-linked transhydrogenase) for GSSG or quinone reduction.
Glutathione synthesizing system	Part of the γ-glutamyl cycle formed by γ-glutamylcysteine synthetase and GSH synthetase, which produces GSH *de novo*.
NADPH-quinone oxidoreductase	Two-electron reduction of quinones without generation of reactive species.
Conjugation systems	Conjugation of xenobiotics or their metabolites, which can induce oxidative stress, by UDP-glucuronyl transferase, sulfotransferase and GSH transferases, plus GSSG and conjugate export from cells by transport systems.
Thioltransferase	Restoration of the thiol/disulfide status of cellular proteins altered by oxidative stress.
DNA repair systems	Oxidized DNA can be repaired enzymatically by the action of nucleases (nucleotide excision, base excision, recombinational, mismatch and error-prone repair) and glycosylases, followed by DNA polimerase.

* Adapted from Sies (2), Forman and Fisher (3), and Valenzuela and Videla (4).

TABLE 3

Nonenzymatic antioxidants in biological systems*

Reduced glutathione (GSH)	Enzymatic cofactor (e.g., GSH peroxidase, GSH-transferases, GSH-dehydroascorbate reductase and thioltransferase) and direct quencher of O_2^-, $HO^\bullet$ and O_2^*.
α-Tocopherol (vitamin E)	Membrane $ROO^\bullet$ scavenger and O_2^* quencher.
Ascorbic acid (vitamin C)	Water soluble scavenger of O_2^- and possible reduced vitamin E regenerator.
β-Catorene (vitamin A)	O_2^* quencher and chain-breaking action at low partial pressures of oxygen.
Flavonoids	Plant antioxidants (cyanidanol, silymarin, quercetin, etc.).
Nonspecific quenchers	Water, amino acids (histidine, trytophan, cysteine, tyrosine), formate, mannitol, etc.

* Adapted from Sies (2), Forman and Fisher (3), and Valenzuela and Videla (4).

for ATP, NADPH and precursors supply, and depend on the dietary replenishment of essential components (3, 4).

The main consequence of oxidative stress is the structural damage inflicted by reactive oxygen species to cellular compounds: DNA, proteins, carbohydrates and lipids. In fact, under the influence of ionizing radiation, photooxidation or oxidizing agents, DNA undergoes a radical-mediated (possibly initiated by $HO^\bullet$) strand scission via peroxyl radicals of bases (thymine and guanine) and of deoxyribose (2). Oxidative damage to amino acids side-chains in proteins can be a reversible phenomenon (e.g., the oxidation-reduction of thiol groups and methionine) or an irreversible process. In the latter case, histidine and tryptophan can undergo oxidative ring cleavage concomitantly with enzyme inactivation. This is also observed following ionizing radiation in which protein fragmentation seems to occur at proline residues via $HO^\bullet$ (2). In addition to these findings, polysaccharides (hyaluronic acid) and monosaccharides (deoxyribose) are also subjected to oxidative degradation. However, a major area of interest in oxidative stress has been the oxidation reaction pathways of polyunsaturated fatty acids present in membrane phospholipids (1-4). This can be accomplished by: a) a specific enzymatic process by which important biological signals are generated (prostaglandins, thromboxane A, prostacyclin and leukotrienes) and b) an unspecific oxidation pathway mediated by free radicals in which several degradation products are produced (2). The latter process is the damaging event known as lipid peroxidation (1-4), which will be illustrated in the following sections by the studies of our group in different experimental models. Discussion is centered on molecular mechanisms involved in the initiation of oxidative stress, metabolic consequences, contributory factors and exogenous protection.

Ethanol ingestion and liver oxidative stress

The relations between membrane damage, hepatic lipid peroxidation and ethanol have been extensively studied as a possible mechanism of cell necrosis after prolonged intake (5). Acute ethanol ingestion was found to induce an enhancement of hepatic lipid peroxidation, as evidenced by tissue accumulation of thiobarbituric acid reactive substance (TBAR) (6) and conjugated dienes (7), both intermediates of the process. This effect is also observed with non-invasive techniques such as the enhanced low-level chemiluminescense of the *in situ* rat liver (assay related to the steady state level of oxidative free radicals involved in lipid peroxidation) (8), the biliary release of TBAR in the anesthetized rat (assay related to enhanced tissue TBAR production) (9), and by the antioxidant-sensitive respiration in the isolated perfused rat liver (assay related to O_2 required for one-electron transfer reactions involved in the generation of active oxygen

species and/or in the lipid peroxidative process itself) (10) (Fig. 2). The lipid peroxidative effect of ethanol in the liver seems to be mediated by its first metabolite acetaldehyde (11, 12), exacerbated by fasting (6, 12) or iron exposure (9, 13), and diminished during aging (14), probably due to a decrease in the overall oxidative capacity of the liver observed in this situation (15). Furthermore, ethanol-induced liver peroxidation is completely suppressed by *in vivo* pretreatment with cyanidanol-3 (8, 16) or silymarin (17), flavonoids known to be effective free-radical scavengers (18, 19).

Ethanol-induced liver oxidative stress is associated with enhanced activities of microsomal NADPH oxidase (a O_2^- generating system) and SOD, as a compensatory adaptive mechanism (7), together with a drastic diminution in the content of hepatic GSH (6, 9, 11, 13, 14), the main cellular antioxidant (3, 4) (Table 3). Liver GSH depletion by ethanol is elicited by both acute and chronic ingestion (20), influenced by the lenght of abstinence and the presence of hepatocellular necrosis in human alcoholics (21), partly due to GSH oxidation in the tissue (22) and translocation into blodd plasma (23), and markedly reduced by prior antioxidant pretreatment (16, 17). Moreover, a significant inversed correlation is observed between liver lipid peroxidative indexes and GSH levels (6, 24), indicating an increased GSH demand of the liver to cope with the pro-oxidative condition imposed by ethanol. However, the exact nature of the chemical initiator of ethanol-induced oxidative stress remains to be elucidated.

Lindane hepatotoxicity via oxidative stress

Hexachlorocyclohexane is a powerful insecticide and ectoparasiticide constituted by various structural isomers, being the γ-isomer (lindane) the most active component capable of inducing liver necrosis and steatosis when administered to mammals. The administration of single does of lindane (20-80 mg/kg) to rats elicited a progressive increase in the hepatic content of microsomal cytochrome P-450 and in the rate of O_2^- generation, with tissue accumulation of TBAR (25). Also, microsomal SOD-and cyanidanol-sensitive respiration NADPH-dependent were drastically increased by lindane treatment (26). These changes can be interpreted in terms of the production of an oxidative stress via an increased O_2^- production, secondary to cytochrome P-450 induction with a concomitant enhancement in lipid peroxidation (25). The lindane-induced pro-oxidative condition seem to be favoured by the parallel decrease in superoxide dismutase and catalase activities (25), two of the main antioxidant components of the hepatocyte (Table 2), with an alteration in its glutathione status represented by the drastic decrease in the GSH/GSSG ratio (26). Apart from these findings, the reductive microsomal biotransformation of lindane can lead to the formation of a pentachlorocyclohexanyl radical (25),

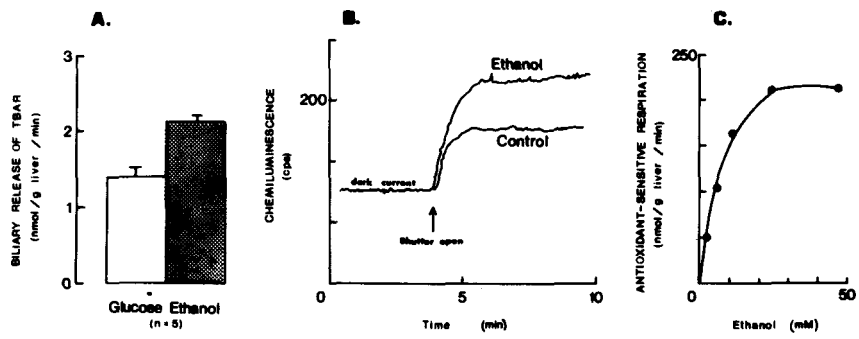


Fig. 2: Non-invasive lipid peroxidative indexes under the influence of ethanol: (A), biliary release of TBAR in the anesthesized rat after 6 h of treatment with 5 g of ethanol/kg (9); (B), chemiluminescense of the *in situ* rat liver (treatment as in (A)) (8); (C), ethanol-induced antioxidant-sensitive respiration in the perfused rat liver (10).

which could represent an additional source of oxidative stress.

Thyroid hormone, active oxygen and lipid peroxidation

Normal thyroid gland activity seems to be mainly concerned with the energy metabolism of most tissues of the body, which, in turn, is essential to support other specialyzed processes dependent upon thyroid gland secretion (27). Thyroid calorigenesis is characterized by an enhancement in the total oxygen consumption of the animal, which results from individual increases in the respiratory rate of target tissues such as the liver (27, 28). Although this effect is primarily due to an increased sodium pump activity, which determines higher rates of mitochondrial oxidative phosphorylation, a fraction of the augmented cellular oxygen uptake is not linked to active cation transport and could possible represent the production of active oxygen species by univalent reduction (28). In fact, as can be seen in Fig. 3, L -3,5,3' -triidothyronine (T_3)-induced thermogenesis in the rat is accompanied by an enhancement in the rat of oxygen uptake of the liver and in the activity of microsomal NADPH oxidase. This latter enzymatic activity reflects the maximal capacity of liver microsomes to oxidize NADPH aerobically in the absence of a drug substrate, generating O_2^- (27, 28). In agreement with these findings, T_3 markedly increases the rate of SOD-inhibitable oxidation of epinephrine to adrenochrome (Fig. 3), which reflects O_2^- generation, when epinephrine is added to liver microsomes supplemented with NADPH (28). This cellular response is accompanied by the stimulation of the lipid peroxidative process (Fig. 3), GSH depletion, cytochrome P-450 loss and a decreased unsaturation of membrane fatty acids, as secondary events of thyroid hormone action (27, 28). These combined changes could play a role in the production of hepatic metabolic and structural alterations observed in the hyperthroid state and in the increased susceptibility of the tissue to a toxic exogenous stress (27).

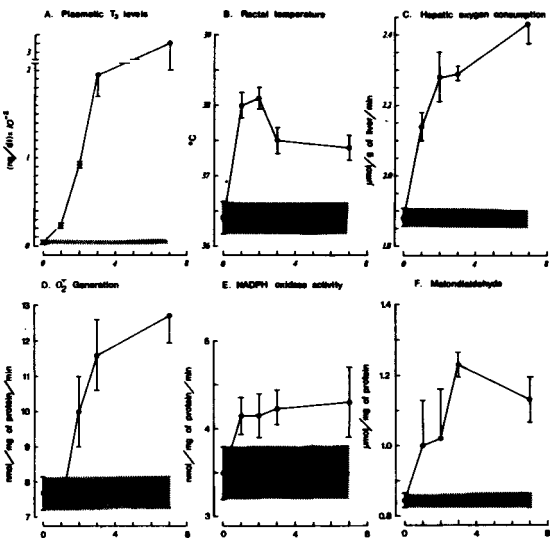


Fig. 3: Oxidative stress in an experimental hyperthyroid state: (A), concentration of T_3 in plasma; (B), rectal temperature of the animals; (C), rate of oxygen consumption by perfused rat livers; liver microsomal generation of O_2^- (D), NADPH oxidase activity (E), and TBAR formation (F), in fed rats given 0.1 mg of T_3/kg for 1 to 7 consecutive days (27).

t-Butyl hydroperoxide (t-BHP)-induced oxidative stress in rat erythrocytes and perfused liver

Red blood cells exposed to oxidative stress suffer a variety of changes, ranging from subtle alterations in their physicochemical properties to complete hemolysis. This oxidative action can be promoted by t-BHP (29) with extensive hemoglobin oxidation (methemoglobin formation), t-BHP cleavage (t-butanol formation) and lipid peroxidation (measured by TBAR production (29), oxygen consumption (Fig. 4A) and visible chemiluminescence (Fig. 4B)). These results are in agreement with a lipid peroxidation mechanism initiated by t-butoxy radicals produced by the interaction of hemoglobin and t-BHP (29). Of particular interest are the studies on oxygen uptake by erythrocytes exposed to t-BHP (Fig. 4A). In this system, the induction period (T_0) is related to the intracellular antioxidant mechanisms while the rate of oxygen uptake is associated with lipid peroxidation, owing to the anaerobic nature of the red blood cell

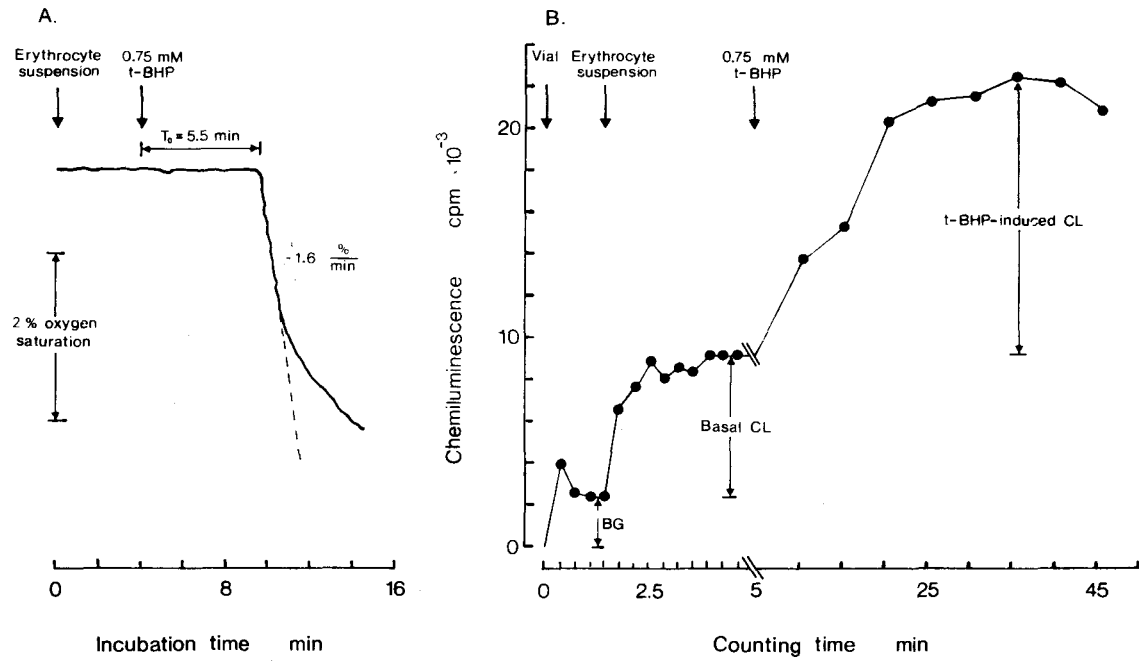


Fig. 4: t-Butyl hydroperoxide (t-BHP)-induced oxidative stress in human erythrocyte suspensions: (A) Oxygen uptake. T_0 corresponds to the induction period and the number adjacent to the trace indicates the initial decrease in oxygen tension per min; (B) Basal and t-BHP-induced chemiluminescence; BG, background.

metabolism (30). The determination of these parameters under different experimental conditions revealed that the protecting mechanism of the red blood cell against t-BHP-induced oxidative stress is constituted by inhibitors (e.g., blood plasma and vitamin E that elicit lower oxygen consumption rates and longer T_0) and retarders (e.g., chemical antioxidants and GSH which decrease O_2 uptake without affecting T_0) of the process (30).

The addition of t-BHP to perfused rat liver elicited a biphasic effect on hepatic respiration (Fig. 5). A rapid fall in liver oxygen consumption was initially observed, followed by a recovery phase leading to respiratory rates higher than the initial steady-state values of oxygen uptake (Fig. 5) (31). This overshoot in oxygen uptake is abolished by free-radical scavengers such as cyanidanol or butylated hydroxyanisole (BHA) (Fig. 5) at concentrations that did not alter mitochondrial respira-

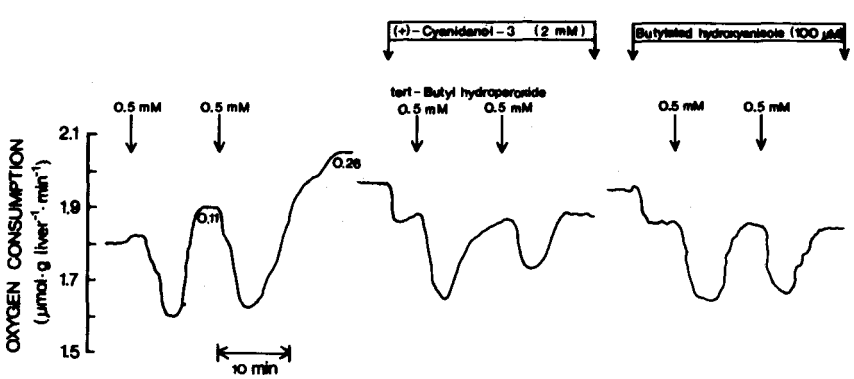


Fig. 5: Changes in oxygen consumption elicited by pulses of t-butyl hydroperoxide in recirculated perfused rat livers in the absence and presence of (+)-cyanidanol-3 and butylated hydroxyanisole (31).

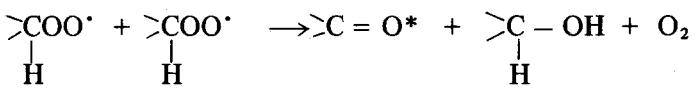
tion (31). Cyanidanol is also able to facilitate the recovery of respiration, the diminution in the calculated rate of hydroperoxide utilization and the decrease in liver GSH content elicited by two consecutive pulses of t-BHP (31). These findings indicate that t-BHP induced respiratory overshoot is related to an enhanced oxygen demand for the lipid peroxidation process that might be set in by the free radicals arising from the interaction of the hydroperoxide with cellular hemoproteins (31).

Visible chemiluminescence from rat brain homogenates undergoing autoxidation

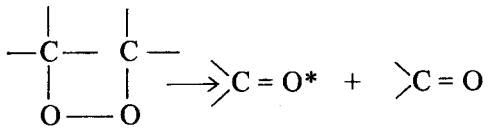
Autoxidation of brain homogenates is a model system characterized by a high and reproducible oxidation rate, with significant TBAR accumulation, oxygen uptake and visible chemiluminescence (32). Nevertheless, the time profile of the light emission markedly differs from that of other lipid peroxidation indicators, that is, light intensities are still emitted when the accumulation rate of TBAR has decreased to very low values (32), in agreement with studies in erythrocytes supplementes with t-BHP (29). The luminescence intensity would thus appear to be more closey related to the extent of lipid peroxidation than to the rate of peroxidation (32).

Light emission in the visible region is related to the production of excited carbonyls ($>C = O^*$), which can be generated by either:

a) Disproportionation of peroxy radicals (Russell's mechanism):



b) Breakdown of dioxetane-like intermediates:



or c) in electron transfer reactions (1). The luminescence emitted after the addition to preincubated samples of an excess of antioxidants is only partially quenched (33). From the kinetic analysis of the luminescent decay after antioxidant addition it was estimated that nearly 50% of the light arises from an intermediate that decays with a first order kinetics and with a lifetime of c.a. 40 sec at 32°C. The remaining light probably arises from the decomposition of several intermediates (lifetime of several hours) and exhibits a kinetics that is independent of the incubation time (33). These findings indicate that bimolecular free-radical processes such as mechanism (a) do not significantly contribute to the observed luminescence associated with this autoxidation system (32, 33).

Concluding remarks

The concept of oxidative stress has been discussed in terms of oxidative damage to biomolecules, including the process of lipid peroxidation in conditions associated with the action of toxic xenobiotics and hormonal disturbances. It must be pointed out that prooxidant states do have a definite role in other pathophysiological states such as inflammatory states, tumor promotion, spontaneous mutagenesis, radiation damage, postischemic tissue injury and shock (1, 2). Moreover, numerous physiologically important biochemical reactions occur with the participation of reactive oxygen species: formate-pyruvate lysase, ribonucleotide reductase, microsomal peroxidase reactions, prostaglandin-endoperoxide synthetase and NADPH

oxidase (2). The latter system is a crucial enzymatic activity for the normal function of macrophages which, under conditions of continous overstimulation, could lead to oxidative stress. This is the case of human subjects under the influence of excessive particulate air pollution, whom were recently reported to exhibit an enhanced peripheral blood macrophage activity with impairment of the antioxidant capacity of plasma (34).

ACKNOWLEDGMENTS

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REFERENCES

1. SIES, H. (Ed.) (1985) Oxidative Stress, London, Academic Press.
2. SIES, H. (1986) *Angew. Chem. Int. Ed. Engl. 25:* 1058-1071.
3. FORMAN, H.J.; FISCHER, A.B. (1981) In *Oxygen and Living Processess* (Gilbert, D.L., Ed.). New York, Springer-Verlag, pp. 235-249.
4. VALENZUELA, A.; VIDELA, L.A. (1983) *Rev. Méd. Chile. 111:* 829-836.
5. VIDELA, L.A.; VALENZUELA, A. (1982) *Life Sci. 31:* 2395-2407.
6. VIDELA, L.A.; FERNANDEZ, V.; UGARTE, G.; VALENZUELA, A. (1980) *FEBS. Lett. 111:* 6-10.
7. VALENZUELA, A.; FERNANDEZ, N.; FERNANDEZ, V.; UGARTE, G.; VIDELA, L.A. (1980) *FEBS. Lett. 111:* 11-13.
8. VIDELA, L.A.; FRAGA, C.G.; KOCH, O.R.; BOVERIS, A. (1983) *Biochem. Pharmacol. 32:* 2822-2825.
9. VALENZUELA, A.; FERNANDEZ, V.; VIDELA, L.A. (1983) *Toxicol. Appl. Pharmacol. 70:* 87-95.
10. VIDELA, L.A. (1984) *Biochem. J. 223:* 885-891.
11. VIDELA, L.A.; FERNANDEZ, V.; DE MARINIS, A.; FERNANDEZ, N.; VALENZUELA, A. (1982) *Biochem. Biophys. Res. Commun. 104:* 965-970.
12. VIDELA, L.A.; VILLENA, M.I. (1986) *Alcohol 3:* 163-167.
13. VIDELA, L.A.; FERNANDEZ, V.; VALENZUELA, A. (1985) *Alcohol 2:* 457-462.
14. VIDELA, L.A.; FERNANDEZ, V.; VALENZUELA, A. (1987) *Cell Biochem. Funct., 5:* 273-280.
15. FERNANDEZ, V.; KRIZ, A.; VIDELA, L.A. (1988) *Cell Biochem. Funct., 6:* 7-12.
16. VIDELA, L.A.; FERNANDEZ, V.; VALENZUELA, A.; UGARTE, G. (1981) *Pharmacology 22:* 343-348.
17. VALENZUELA, A.; LAGOS, C.; SCHMIDT, K.; VIDELA, L.A. (1985) *Biochem. Pharmacol. 34:* 2209-2212.
18. VIDELA, L.A. (1983) *Experientia 39:* 500-502.
19. VALENZUELA, A.; GUERRA, R.; VIDELA, L. A. (1986) *Planta Medica Nº 6:* 438-440.
20. FERNANDEZ, V.; VIDELA, L.A. (1981) *Experientia 37:* 392-394.
21. VIDELA, L.A.; ITURRIAGA, H.; PINO, M.E.; BUNOUT, D.; VALENZUELA, A.; UGARTE, G. (1984) *Clin. Sci. 66:* 283-290.
22. VIDELA, L.A.; FERNANDEZ, V.; FERNANDEZ, N.; VALENZUELA, A. (1981) *Substance & Alcohol Actions/Misuse 2:* 153-160.
23. FERNANDEZ, V.; FERNANDEZ, N.; VALENZUELA, A.; VIDELA, L.A. (1983) *Experientia 39:* 880-882.
24. VIDELA, L.A. (1984) *FEBS. Lett. 178:* 119-122.
25. JUNQUEIRA, V.B.C.; SIMIZU, K.; VIDELA, L. A.; BARROS, S.B.M. (1986) *Toxicology 41:* 193-204.
26. VIDELA, L.A.; BARROS, S.B.M.; SIMIZU, K.; JUNQUEIRA, V.B.C. (1988) *Cell Biochem. Funct., 6:* 47-52.
27. FERNANDEZ, V.; VIDELA, L.A. (1988) In *Handbook of Biomedicine of Free Radicals and Antioxidants* (Miquel, J., ed.). Boca Ratón, *CRC Press, Inc.,* in press.
28. FERNANDEZ, V.; BARRIENTOS, X.; KIPREOS, K.; VALENZUELA, A.; VIDELA, L.A. (1985) *Endocrinology 117:* 496-501.
29. VIDELA, L.A.; VILLENA, M.I.; DONOSO, G.; DE LA FUENTE, J.; LISSI, E. (1984) *Biochem. Intern. 8:* 821-830.
30. LISSI, E.; FRANZ, R.; CABEZAS, J.; FERNANDEZ, V.; VIDELA, L.A. (1986) *Cell Biochem. Funct. 4:* 61-68.
31. VIDELA, L.A.; VILLENA, M.I.; DONOSO, G.; GIULIVI, C.; BOVERIS, A. (1984) *Biochem. J. 223:* 879-883.
32. LISSI, E.A.; CACERES, T.; VIDELA, L.A. (1986) *J. Free Rad. Biol. Med. 2:* 63-69.
33. LISSI, E.A.; CACERES, T.; VIDELA, L.A. (1988) *Free Rad. Biol. Med. 4:* 93-97.
34. RUIZ, F.; VIDELA, L.A.; VARGAS, N.; PARRA, M.A.; TRIER, A.; SILVA, C. (1988) *Arch. Environ. Health.,* in press.

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Bases bioquímicas de la acción de drogas antichagásicas*

The biochemistry of the mode of action of drugs for the treatment of Chagas' disease

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El avance en el conocimiento de la bioquímica de parásitos, que ha ocurrido en los años recientes, ha conducido al desarrollo de nuevas drogas y ha permitido entender el modo de acción de muchas de ellas.

La acción de algunas drogas tripanosomicidas se debería a la generación de metabolitos que son radicales libres, incluyendo productos de reducción parcial del oxígeno El *T. cruzi* es muy susceptible al daño celular producido por estos metabolitos, ya que las enzimas que destruyen las especies activadas de oxígeno en mamíferos tienen muy baja actividad o no existen en el parásito. Drogas en uso, como son el nifurtimox, el benznidazol y el cristal violeta actuarían generando radicales libres.

Otro posible sitio de ataque quimioterapéutico en estudio es la biosíntesis de glutatión, el cual participa en la eliminación de radicales libres y en la conjugación y detoxicación de numerosas drogas. También se estudia la manera de interferir con la reducción del glutatión oxidado que en el *T. cruzi,* a diferencia del huésped, requiere del cofactor tripanotión.

Drogas experimentales como el alopurinol y análogos de purinas basan su modo de acción en la incapacidad del parásito de sintetizar purinas *de novo* y en una relativa baja especificidad de la enzima succino-AMP sintetasa.

La cadena respiratoria del parásito también presenta importantes diferencias con la del huésped.

El *Trypanosoma cruzi* es el parásito causal de la Trypanosomiasis americana. Esta enfermedad es un problema de salud pública que afecta a toda América Latina, desde el sur de los Estados Unidos hasta el paralelo 35 Sur en Chile y Argentina. Se estima actualmente en 24 millones las personas infectadas con *Trypanosoma cruzi* en toda la región. De ellos, 350 mil personas corresponden a nuestro país, donde la zona de endemia chagásica se extiende desde el límite norte hasta la Sexta Región (1). La Trypanosomiasis americana es más conocida como la Enfermedad de Chagas en homenaje al médico brasileño Carlos J. Chagas, quien, en 1909, describió esta patología, la asoció a un nuevo tipo de trypanosoma descubierto por él y lo llamó *Trypanosoma cruzi,* en homenaje a su maestro Oswaldo Cruz (2).

El nifurtimox (Bayer 2502, Lampit) y el benznidazol (RO-1051, Rodamil, Rochagan) son los fármacos actualmente en uso para el tratamiento de la Enfermedad de Chagas. Sin embargo, ambas drogas presentan una alta toxicidad, graves efectos laterales y existen dudas acerca de su efectividad. El cristal violeta es el fármaco en uso para tratar la sangre infectada con *T. cruzi* en los bancos de sangre; desgraciadamente este compuesto tiñe la sangre y en ocasiones los tejidos de los pacientes, razón por la cual es rechazado (Fig. 1).

La necesidad de nuevos, eficientes y seguros fármacos para el tratamiento de la Enfermedad de Chagas es urgente. Muchas drogas han sido probadas en forma empírica, ya que el desarrollo racional de las mismas no ha dado todavía los frutos esperados. El desarrollo racional de drogas se basa en el conocimiento de la fisiología y bioquímica del parásito. Diferencias en vías metabólicas o enzimas específicas entre el huésped y el parásito pueden ser utilizadas

* Dedicado a la memoria del Prof. Osvaldo Cori M., primer gran maestro de la Bioquímica moderna chilena.

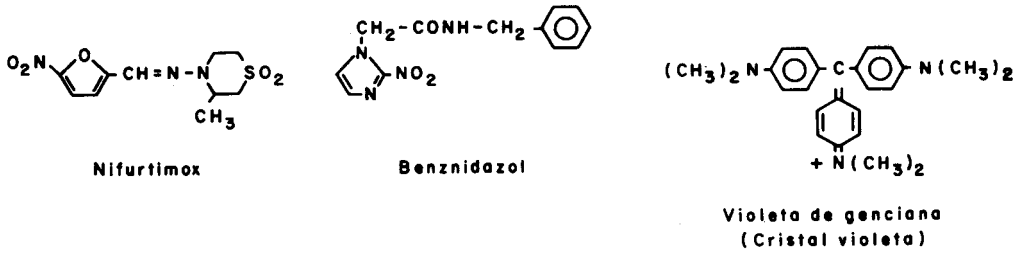

Fig. 1: Estructura de fármacos antichagásicos.

para desarrollar nuevos fármacos. A continuación se describen algunas de las diferencias encontradas y se discuten sus potencialidades terapéuticas.

Radicales libres y efecto tripanosomicida

Una droga puede generar un radical libre como intermediario y éste puede actuar directamente produciendo daño celular o desencadenar variados elementos como consecuencia de generar otros radicales a partir de aminoácidos, lípidos, nucleótidos y azúcares. El efecto final lo producen por mutagénesis o carcinogénesis al atacar ácidos nucleicos, por lipoperoxidación, por despolimerización de carbohidratos y por alteraciones en el metabolismo intermediario al dañar enzimas que participan en él.

Los radicales libres intermediarios tambien pueden actuar sobre oxígeno molecular, originando productos de reducción parcial del oxígeno como el anión superóxido ($O_2^{\cdot-}$), el cual espontáneamente o por acción de la enzima superóxido dismutasa genera peróxido de hidrógeno (H_2O_2). La interacción de $O_2^{\cdot-}$ y H_2O_2 puede llevar a la producción del radical hidróxilo ($OH^{\cdot}$), el cual se postula es el más dañino para la célula (Fig. 2).

Las células eliminan los radicales libres mediante un conjunto de enzimas (superóxido dismutasa, catalasa, glutatión peroxidasa) y de compuestos reductores (α-tocoferol, ácido ascórbico, β-caroteno, glutatión reducido). El *T. cruzi,* a diferencia del hospedero, es deficiente en los mecanismos defensivos contra radicales libres. No presenta actividad de las enzimas catalasa (3) y glutatión peroxidasa selenio dependiente (4) y presenta muy baja actividad de la enzima superóxido dismutasa (5). Su contenido de glutatión reducido es un décimo de la concentración en el hígado de rata (4) y no ha sido reportada la presencia de α-tocoferol, β-caroteno y ácido ascórbico (6).

Utilizando esta mayor sensibilidad a los radicales libres que presenta el *T. cruzi* muchas drogas tripanosomicidas actúan generando intermediarios que son radicales libres. A continuación se analizarán algunas de estas drogas.

Quinonas: La primera demostración en células eucarióticas intactas de la formación de un radical libre, a partir de una droga, fue usando β-lapachona. Al incubar epimastigotes de *T. cruzi* con esta droga, se genera

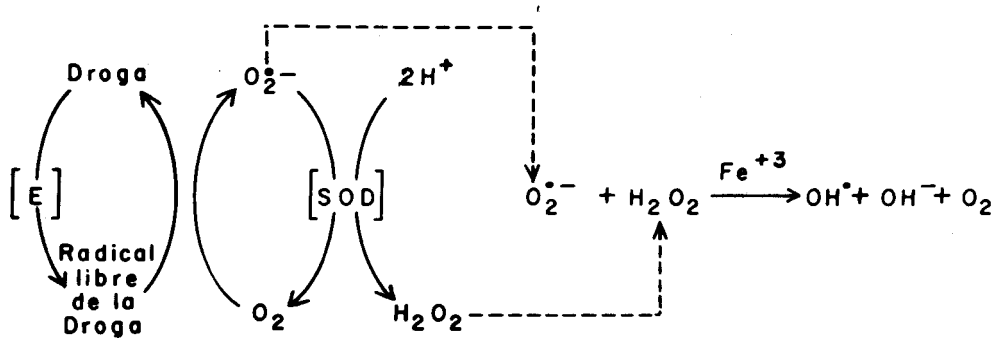

Fig. 2: Formación de radicales libres de drogas y del oxígeno en *Trypanosoma cruzi.* Reciclaje redox (E): Reacción catalizada por flavoproteínas tales como las reductasas del citocromo P-450 y del citocromo c; SOD: superóxido dismutasa (ecuación no balanceada).

un radical semiquinona, detectable por resonancia de spin electrónica (7). Este radical aumenta la lipoperoxidación y la liberación de productos de reducción parcial del oxígeno al medio de cultivo.

Similares resultados se han obtenido posteriormente con amastigotes y tripomastigotes aislados de la sangre, ya que ésta inactiva a la β-lapachona, no teniendo uso terapéutico en animales y en el hombre. Un derivado, la 3-alil-β-lapachona, no se inactiva en la sangre, siendo un eventual quimioprofiláctico de la transmisión de la Enfermedad de Chagas por transfusión sanguínea. Esta droga no es usada en la infección de mamíferos con *T. cruzi,* pues el sistema de detoxicación del hospedero la inactiva (8).

Compuestos nitroheterocíclicos: Las drogas actualmente en uso clínico contra el *T. cruzi* corresponden a este grupo. El nifurtimox es un derivado del nitrofurano y el benznidazol es un derivado nitroimidazólico (Fig. 1).

Ambos compuestos pueden originar radicales libres (9), pero se diferencian en que la concentración de nifurtimox (10-20 μM) a la cual se inhibe el crecimiento de *T. cruzi* en cultivo es similar a la requerida para la máxima producción de $O_2^{\cdot-}$ e iniciar la salida de H_2O_2 desde las células. En cambio, las concentraciones a las cuales el benznidazol inhibe el crecimiento de los cultivos son mucho más bajas que las concentraciones necesarias para producir $O_2^{\cdot-}$ y H_2O_2 y para detectar su reducción a un radical nitroanión (6, 10). De lo anterior, se concluye que el efecto tripanosomicida del nifurtimox se debe a los radicales libres derivados del oxígeno. No así el efecto del benznidazol cuyo mecanismo de acción estaría mediado por radicales libres de él mismo (Fig. 2).

Violeta de Genciana: En 1953 Víctor Nussenweig demostró efecto tripanosomicida del Violeta de Genciana contra tripomastigotes de *T. cruzi.* Tal actividad no se pierde en presencia de sangre y por ello ha sido hasta hoy la droga usada para eliminar la transmisión del parásito mediante transfusión sanguínea. El efecto contra tripomastigotes y también contra epimastigotes de *T. cruzi* se basa en la producción de un radical tri-(p-dimetilaminofenil)metilo, el cual explica el efecto antiparasitario. La producción de este radical es mayor en presencia de luz visible (11).

El gossypol, conocido anticonceptivo masculino, y la rosa de bengala deberían su acción tripanosomicida a la producción de radicales libres (12, 13).

Biosíntesis del glutatión y reducción del glutatión oxidado

Glutatión (L-γ-glutamil-L-cisteinilglicina) es probablemente el tiol de bajo peso molecular más abundante en los seres vivos. Se encuentra presente prácticamente en todas las células en rangos que fluctúan entre 0,5 y 10 mM. La mayoría de las funciones de este tripéptido se deben a su forma reducida (GSH), la cual es mantenida en niveles altos principalmente por acción de la glutatión reductasa, enzima que cataliza la reducción del disulfuro de glutatión (GSSG).

Glutatión atrapa radicales libres que se producen por radiaciones ionizantes o por reacciones catalizadas por las oxidasas de función mixta. Además, protege a la célula de procesos oxidativos tales como la peroxidación de lípidos. Este fenómeno de lipoperoxidación es uno de los principales mecanismos de toxicidad y se puede iniciar ya sea por oxígeno singlete como también por radicales libres producidos en la célula por productos metabólicos de xenobióticos (14, 15).

Glutatión se conjuga con una serie de compuestos tanto exógenos como endógenos en reacciones catalizadas por las glutatión S-transferasas (16 y Fig. 3).

Glutatión es sintetizado y degradado en el ciclo del γ-glutamilo donde participan seis enzimas (Fig. 3), una de las cuales está unida a membrana y las otras son solubles.

En el *T. cruzi* las únicas dos enzimas del ciclo del gama glutamilo estudiadas son la γ-glutamiltranspeptidasa y la γ-glutamilcisteína sintetasa. La γ-glutamiltranspeptidasa de *T. cruzi* ha demostrado ser diferente a la de mamíferos en relación a localización subcelular, aceptores del grupo glutamilo e inhibidores (17 y 18). En los mamíferos la enzima se encuentra unida fuertemente

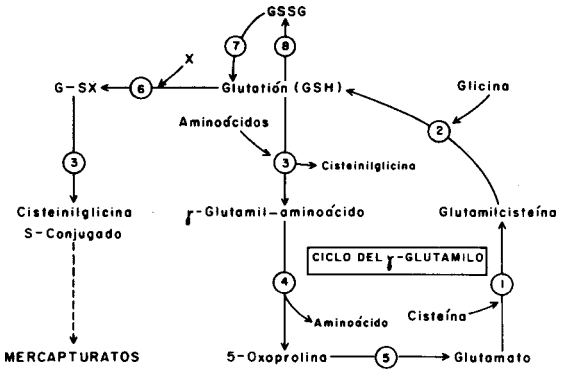

Fig. 3: Metabolismo y funciones del glutatión. Enzimas: (1) γ-glutamilcisteína sintetasa; (2) glutatión sintetasa; (3) γ-glutamiltranspeptidasa; (4) γ-glutamilciclotransferasa; (5) 5-oxoprolinasa; (6) glutatión-S-transferasa; (7) glutatión reductasa (tripanotión reductasa); (8) reacciones de protección enzimáticas y no enzimáticas.

a las membranas; en cambio, en el parásito se encuentra en parte libre en el citosol y en parte en microcuerpos parecidos a los glicosomas (17).

Moncada (17) determinó la concentración de glutatión en varias cepas de *T. cruzi*, encontrando valores en el rango de 0,60 a 1,0 mM. Cuando los cultivos de estas cepas fueron tratadas con butionina sulfoximina, que es un inhibidor de la glutamilcisteína sintetasa, la concentración de glutatión disminuyó al 50% (17). Estas diferencias observadas podrían eventualmente ser utilizadas en el desarrollo de agentes antichagásicos, como también contribuir a explicar las diferencias en resistencia a drogas encontradas entre diferentes cepas del parásito.

La función protectora del glutatión frente a los radicales libres da como producto glutatión oxidado (GSSG), el cual puede ser tóxico para las células (19). El glutatión oxidado no se acumula porque es reducido por una glutatión reductasa dependiente de $NADPH_2$ (20). Por lo tanto, esta enzima es un factor muy importante en la mantención del balance redox del glutatión.

Fairlamb y Cerani (21) han descrito que la glutatión reductasa de tripanosomatidas requiere en forma absoluta un cofactor para ser activa. Este cofactor fue identificado como un conjugado de glutatión con espermidina y se le denominó tripanotión (22, 23, 24). Ya que la reductasa del huésped no requiere este cofactor, la reductasa de los trypanosomas o la biosíntesis del tripanotión constituye un blanco quimioterapéutico capaz de alterar la relación GSH/GSSG y, por ende, producir efectos tóxicos en estos parásitos. Recientemente esta tripanotión reductasa ha sido purificada y caracterizada (25).

Efecto tripanosomicida del alopurinol y otros derivados de bases púricas

El alopurinol es la droga de elección en pacientes que presentan hiperuricemia. J.J. Mar y colaboradores (26, 27, 28) comprobaron que esta droga tenía efecto contra varias cepas de Leishmanias. Posteriormente, demostraron que el alopurinol también es activo contra epimastigotes de *T. cruzi*. El parásito lo metaboliza secuencialmente a HPPR-MP, Succino-APPR-APPR-MP, APPR-DP y APPR-TP (Fig. 4). De esta forma se puede incorporar al RNA, al actuar como un análogo tóxico de la adenina, y así tener efecto antiprotozoario. Este efecto se debería a la baja especificidad de la succino-AMP sintetasa del parásito (29). En el hombre la incorporación al RNA no ocurre, pues el 90% del alopurinol es metabolizado a oxipurinol, mediante una reacción catalizada por la enzima xantino oxidasa, en la cual es deficiente el *T. cruzi* y a la alta especificidad de la succino-AMP-sintetasa (Fig. 4). Por otra parte, se observó que epimastigotes de diferentes cepas de *T. cruzi* presentan diferente sensibilidad al

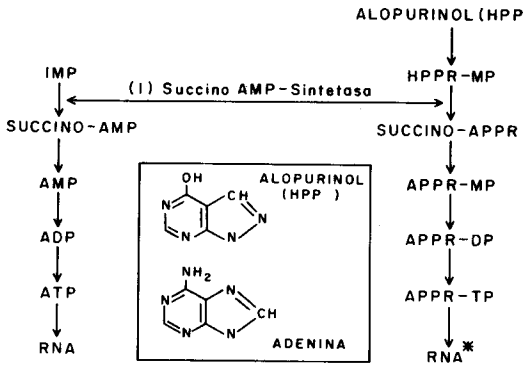

Fig. 4: Biotransformación del alopurinol en un análogo del ATP en *Trypanosoma cruzi*. A diferencia del huésped mamífero la enzima succino-AMP-sintetasa (1) del parásito metaboliza el derivado del alopurinol. HPPR-MP, 1 ribosil alopurinol 5'-fosfato; APPR-MP, 1 ribosil-4-aminopirazolo(3,4-d)pirimidina 5'-fosfato.

alopurinol (30). Estudios con tripomastigotes y amastigotes revelan que la metabolización en ambos estados es similar al epimastigote (9) y que también existen cepas con menos sensibilidad a la droga (31). Esta sensibilidad diferente entre cepa y cepa de *T. cruzi* también se obtiene al tratar los parásito no sólo con análogos de adenina, sino con un análogo de inosina, como es la Formicina B (32).

La cadena respiratoria como blanco de drogas antichagásicas

La cadena respiratoria del *T. cruzi* ha sido estudiada por los grupos de Agosin y de Stoppani (33, 34, 35) y parecerían existir tres oxidasas terminales (35).

Conociendo las peculiaridades que el proceso de obtención de energía presenta en el *Trypanosoma cruzi,* su eventual uso como blanco de acción de drogas antichagásicas es evidente. Nuestra aproximación a la cadena respiratoria del parásito fue, en gran parte, mediada por el azar. Al estudiar el sistema de las carboxilesterasas (36, 37), intentamos inducir estas enzimas usando 2(3)-tert-butil-4-hidroxianisol (BHA), el cual es conocido inductor de algunas enzimas detoxicantes de xenobióticos en mamíferos (38, 39). Grande fue nuestra sorpresa al comprobar que en los cultivos tratados con esta droga la cantidad de parásitos disminuía.

Pensando que ese efecto se debía a la generación de un radical libre y de productos de reducción parcial del oxígeno, estudiamos la acción del BHA sobre el consumo de oxígeno del *T. cruzi.* Mayor fue nuestra sorpresa al observar que no había aumento, situación esperable al producirse radicales del oxígeno, sino por el contrario, el BHA inhibía el consumo de oxígeno de los parásitos. Los estudios para dilucidar sus mecanismos de acción tripanosomicida y el de otros antioxidantes fenólicos indican que BHA inhibe *in vivo* la cadena respiratoria del parásito en un punto entre el NAD y el citocromo b (40 y Fig. 5).

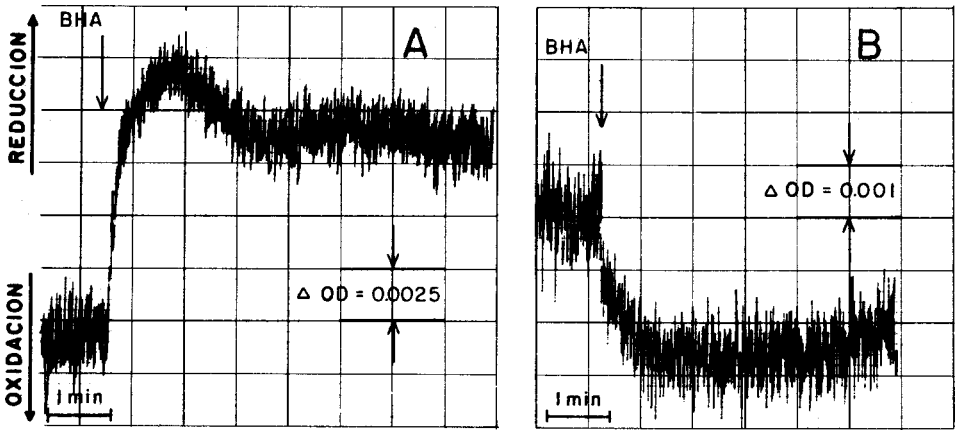

Fig. 5: Efecto del BHA sobre el estado redox del NAD (A) y el citocromo b (B) de epimastigotes intactos de *T. cruzi.* BHA se agregó a una concentración de 1 mM y los cambios de absorción se midieron en un espectrofotómetro Aminco DW2a de doble longitud de onda. NAD y citocromo b se siguieron a los pares de longitud de onda de 340-390 y 561-575 nanómetros, respectivamente.

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REFERENCIAS

1. MOLINEUX, D.H.; ASHFORD, R.W. (1983) The biology of Trypanosoma and Leishmania. Parasites of Man and Domestic Animals. Taylor and Francis Ltd., London, pp. 161-181.

2. SCHMIDT, G.D.; ROBERTS, L.S. (1977). Foundation of Parasitology. The C.V. Mosby Company, Saint Louis, Missouri, pp. 62-68.
3. DOCAMPO, R.; DE BOISO, J.F.; BOVERIS, A. and STOPPANI, A.O.M. (1976) Localization of peroxide activity in *Trypanosoma cruzi* microbodies. *Experientia, 32:* 972-975.
4. BOVERIS, A.; SIES, H.; MARTINO, E.E.; DOCAMPO, R.; TURRENS, J.F. and STOPPANI, A.O.M. (1980) Deficient Metabolic Utilization of Hydrogen Peroxide in *Trypanosoma cruzi. Biochem. J. 188:* 643-648.
5. BOVERIS, A. and STOPPANI, A.O.M. (1977) Hydrogen peroxide generation in *Trypanosoma cruzi. Experientia, 33:* 1306-1308.
6. DOCAMPO, R. and MORENO, S.N.J. (1984) Free Radical Metabolites, in Mode of Action of Chemotherapeutic agents and Phagocytic cells on *Trypasosoma cruzi. Rev. Infectious Diseases 6 (2):* 223-238.
7. DOCAMPO, R.; CRUZ, F.S.; BOVERIS, A.; MUNIZ, R.P.A. and ESQUIVEL, D.M.S. (1978) Lipid peroxidation and the generation of free radicals, superoxide anion, and hydrogen peroxide in β-lapachone-treated *Trypanosoma cruzi* epimastigotes. *Arch. Biochem. Biophys. 186:* 292-297.
8. GONÇALVES, A.M.; VASCONCELLOS, R.M.; DOCAMPO, R.; CRUZ, F.S.; DE SOUZA, W. and LEAR, W. (1980) Evaluation of the toxicity of 3-allyl-β-lapachone against *Trypanosoma cruzi* blood stream forms. *Mol. Biochem. Parasitol. 1:* 163-176.
9. DOCAMPO, R. and MORENO, S.N.J. (1984) Free radical intermediates in the trypanocidal action of drugs and phagocytic cells. In: *Oxygen radicals in Chemistry and Biology.* Walter de Gruyter, Berlin, pp. 749-751.
10. MORENO, S.N.J.; DOCAMPO, R.; MASSON, R.P.; LEON, W. and STOPPANI, A.O.M. (1982) Different behaviours of benznidazol as free radical generator with mammalian and *Trypanosoma cruzi* microsomal preparations. *Arch. Biochem. Biophys. 218:* 585-591.
11. DOCAMPO, R.; MORENO, S.N.J.; MUNIZ, R.P.A.; CRUZ, F.S. and MASON, R.P. (1983) Light-enhanced free radical formation and trypanocidal action of gentian violet (crystal violet). *Science 220:* 1291-1295.
12. MONTANANT, E.E.; BURGOS, C.; DE BURGOS, N.M.G.; ROVAI, L.A.; BLANCO, A. and SEGURA, E.L. (1982). Inhibitory action of gossypol on enzymes and growth of *Trypanosoma cruzi. Science 221:* 288-289.
13. CRUZ, F.S.; LOPES, L.A.V.; DE SOUZA, W.; MORENO, S.N.J.; MASON, R.P. and DOCAMPO, R. (1984) The photodynamic action of rose bengal on *Trypanosoma cruzi. Acta Tropica 41:* 99-108.
14. MEISTER, A. (1981) Metabolism and functions of glutathione. *Trends Biochem. Sci. 6:* 231-234.
15. MEISTER, A. (1983) Metabolism and transport of glutathione and other γ-glutamyl compounds. In: *Functions of Glutathione* (Edited by Larsson, A.; Orrenius, S.; Holmgren, A. and Mannervik, B.). Raven Press, New York, pp. 1-22.
16. JAKOBY, W.B. and HABIG, W.H. (1980) *Glutathione Transferases in Enzymatic Bases of Detoxication,* Vol. II (W.B. Jakoby ed.) Academic Press, N.Y., pp. 63-94.
17. MONCADA, C. (1987) γ-glutamiltranspeptidasa, glutatión S-transferasa y contenido de glutatión en diversas cepas de *Trypanosoma cruzi.* Tesis. Facultad. de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile.
18. REPETTO, Y.; LETELIER, M.E.; ALDUNATE, J. and MORELLO, A. (1987) The γ-glutamyltranspeptidase of *Trypanosoma cruzi. Comp. Biochem. Physiol. 87B:* 73-78.
19. MEISTER, A. and ANDERSON, M.E. (1983) Glutathione. *Annu. Rev. Biochem. 52:* 711-760.
20. WILLIAMS, C.H. Jr. (1976) Flavin-containing dehydrogenases. In: *The Enzymes* (editor P.D. Boyer). Vol. 13, Academic Press, New York, pp. 90-176.
21. FAIRLAMB, A.H. and CERAMI, A. (1985) Identification of a novel thiol-containing co-factor essential for glutathione reductase enzyme activity in trypanosomatids. *Mol. Biochem. Parasitol. 14:* 187-198.
22. FAIRLAMB, A.H.; BLACKBURN, P.; URICH, P.; CHAIT, B.T. and CERAMI, A. (1985) Trypanothione: a nivel bis(glutathionyl)spermidine cofactor for glutathione reductase in trypanosomatids. *Science 227:* 1485-1487.
23. FAIRLAMB, A.H.; HENDERSON, G.V. and CERAMI, A. (1986) The biosynthesis of trypanothione and N-glutathionyl spermidine in *Crithidia fasciculata. Mol. Biochem. Parasitol. 21:* 247-257.
24. SHAMES, S.L.; FAIRLAMB, A.H.; CERAMI, A. and WALSH, C.T. (1986) Purification and characterization of trypanothione reductase from *Crithidia fasciculata:* a new member of the family of disulfide-containing flavoprotein reductase. *Biochemistry 25:* 3519-3526.
25. KRAUTH-SIEGEL, R.L.; ENDERS, B.; HENDERSON, G.B.; FAIRLAMB, A.H. and SCHIRMER, R. H. (1987) Trypanothione reductase from *Trypanosoma cruzi.* Purification and characterization of the crystalline enzyme. *Eur. J. Biochem. 164:* 123-128.
26. MARR, J.J. and BERENS, R.L. (1983) Pyrazolopyrimidine metabolism in Leishmania and Trypanosomes: Significant differences between host and parasite. *J. Cell. Biochem. 22:* 187-196.
27. MARR, J.J. and BERENS, R.L. (1983) Pyrazolopyrimidine metabolism in the pathogenic Trypanosomatidae. *Mol. Biochem. Parasitol. 7:* 339-356.
28. MARR, J.J.; BERENS, R.L. and NELSON, D.J. (1978) Antitrypanosomal effects of allopurinol: conversion *in vitro* to aminopyrazolopyrimidine nucleotides by *Trypanosoma cruzi. Science 201:* 1018-1020.
29. SPECTOR, T.; BERENS, R.L. and MARR, J.J. (1982) Adenylsuccinate synthetase and adenylsuccinate lyase from *Trypanosoma cruzy.* Specificity studies with potential chemotherapeutic agents. *Biochem. Pharmacol. 31:* 225-229.
30. AVILA, J.L.; AVILA, A. and MUÑOZ, E. (1981) Effect of allopurinol in different strains of *Trypanosoma cruzi. Am. J. Trop. Med. Hyg. 30:* 169-774.
31. BERENS, R.L.; MARR, J.J.; LOOKER, D.L.; NELSON, D.L. and LA FON, S.W. (1984) Efficacy of pyrazolopyrimidine ribonucleosides against *Trypanosoma cruzi.* Studies *in vitro* and *in vivo* with sensitive and resistant strains. *J. Infec. Dis. 150:* 602-608.
32. McCABE, R.E.; REMINGTON, J.S. and ARAUJO, F.G. (1985) *In vitro* and *in vivo* activities of Formycin B against *Trypanosoma cruzi. Antimicrobial Agents and Chemotherapy 27:* 491-494.
33. AGOSIN, M.; NAQUIRA, C.; CAPDEVILA, J. and PAULIN, J. (1976) Hemoproteins in *Trypanosoma cruzi* with emphasis on microsomal pigments. *Int. J. Biochem. 7:* 585-593.

34. STOPPANI, A.O.M.; DOCAMPO, R.; DE BOISO, J.F. and FRASCH, A.C.C. (1980) Effect of inhibitors of electron transport and oxidative phosphorylation on *Trypanosoma cruzi* respiration and growth. *Mol. Biochem. Parasitol. 2:* 3-21.

35. AFRANCHINO, J.L.; TARLOVSKY, M.N.S. and STOPPANI, A.O.M. (1986) Terminal oxidases in the trypanosomatid *Trypanosoma cruzi. Comp. Biochem. Physiol. 85B:* 381-388.

36. REPETTO, Y.; ALDUNATE, J. and MORELLO, A. (1983) *Trypanosoma cruzi:* Carboxylesterase activity in intact epimastigotes. *Comp. Biochem. Physiol. 76B:* 61-64.

37. ALDUNATE, J.; REPETTO, Y.; LETELIER, M.E. and MORELLO, A. (1987) The carboxylesterases on *Trypanosoma cruzi* epimastigotes. *Comp. Biochem. Physiol. 65B:* 67-71.

38. BENSON, A.M.; BATZINGER, R.P.; AU, S.Y.L.; BUEDING, E.; CHA, Y.N. and TALALAY, P. (1978) Elevation of hepatic glutathione-S-transferase activities and protection against mutagenic metabolites of benzo(a) pirene by dietary antioxidants. *Cancer Res. 38:* 4486-4495.

39. BENSON, A.M.; CHA, Y.N.; BUEDING, E.; HEINE, H.S. and TALALAY, P. (1979) Elevation of extrahepatic glutathione-S-transferase and epoxide hydratase activities by 2(3)-t-butyl-4-hydroxyanisole. *Cancer Res. 39:* 2971-2977.

40. ALDUNATE, J.; FERREIRA, J.; LETELIER, M.E.; REPETTO, Y. and MORELLO, A. (1986) t-Butyl-4-hydroxyanisole, a novel respiratory chain inhibitor. Effects on *Trypanosoma cruzi* epimastigotes. *FEBS. Lett. 195:* 295-297.

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The relationship between the structures of four β-lactamases obtained from *Bacillus cereus**

Las relaciones entre las estructuras de cuatro β-lactamasas obtenidas de *Bacillus cereus.*

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Bacillus cereus has proved to be one of the most interesting microorganisms in the study of β-lactamases. It secrets these enzymes very efficiently and, frequently, in multiple forms. Three different forms are produced by strain 569/H; mutant 5/B of the same microorganism is constitutive for the secretion of β-lactamases I and II. The present study, based on secondary structure prediction by two independent methods, states the relationship among the structures of β-lactamases I, II and III produced by *B. cereus* 569/H and β-lactamase I from the strain 5/B of this microorganism. A strong similarity is also established for the enzyme type III of *B. cereus* and the enzyme type I produced by *B. licheniformis* which could have an evolutionary explanation.

A structural analysis of the leader peptide regions of these enzymes by the method of Mohana and Argos is also reported.

INTRODUCTION

β-lactamases have become one of the best studied groups of bacterial enzymes, due to their clinical importance and their ecological and evolutionary interest. These enzymes are, to a great extent, responsible for the bacterial resistance to β-lactam antibiotics, thus, much effort has been spent trying to solve their 3-dimensional structures (1-3): this knowledge would facilitate the design of, either specific inhibitors for the enzymes' activity, or, antibiotics resistent to their action. However, no details of the tertiary structure of any of the β-lactamases, perhaps with the only exception of the *S. aureus* enzyme recently published structure (4), are yet known.

This family of enzymes presents a great variety in size, amino acid composition and activity to the different β-lactam antibiotics (5). At least three different classes of enzymes have been defined: class A enzymes have molecular weights of about 30000 and do not require a cofactor for their function; class B enzymes require a Zn^{+2} to perform their catalytic action and have molecular weights of about 23000 (6). A third class of β-lactamases is represented by a 40000 Dalton serine cephalosporinase, obtained from *Escherichia coli.* Also small β-lactamases of molecular weights of only 12000 have been reported (6).

In the absence of a tertiary structure, a secondary structure prediction by two different prediction methods have been done for four class A β-lactamases, obtained from *Escherichia coli* R-TEM, *Bacillus cereus* 569/H, *Bacillus licheniformis* 749/C and *Staphylococcus aureus* PC1 (7). This study yielded a common structure for all these enzymes, consisting in two domains joined by a more or less flexible strand of the polypeptide chain.

Bacillus cereus 569/H produces three different β-lactamases, that have been named types I, II and III. β-lactamase I is a typical class A enzyme, and β-lactamase II is a class B penicillinase (6). β-lactamase III is a lipoprotein of molecular weight 32500,

* En homenaje a la memoria del Dr. Osvaldo Cori. Como reconocimiento al destacado rol que le cupo en la formación de los Bioquímicos de nuestra Universidad de Concepción.

and it has been shown to be highly homologous in primary structure to the class A β-lactamases (8). Unlike 569/H, strain 5 of *B. cereus* could not be induced to produce β-lactamases, but mutant 5/B is constitutive for the production of β-lactamases types I and II (9). The primary structures of the three enzymes obtained from strain 569/H and of the enzyme type I from the strain 5/B have been published (6, 8, 9, 10, 11). It seemed interesting, then, to compare the secondary structures predicted for the four enzymes obtained from *B. cereus* whose sequences are known, among them and with other β-lactamases of class A.

Since it is accepted that it is the spatial folding of the polypeptide chain, more than the primary structure, what confers the special properties of an enzyme, a secondary structure comparison could provide a better understanding of the specificity, origin and function of these enzymes. The fact that the primary structures of these four β-lactamases are known from DNA sequencing, have provide the sequences of the "leader or signal peptides" for all of them. Even though little sequence conservation exists in this part of the polypeptide chain, it seems interesting to analyze them with the Mohana and Argos method (12) to detect the existence of buried helical regions. It could occur, as in the case of the 4 class A β-lactamases mentioned above (7), that the conservation in the secondary structure in the signal peptide is more preserved than the sequence itself.

METHODS

Two methods to predict secondary structure of proteins from their primary structure which have been reported to give 80% reliability have been used, separately or successively; these are the Chou and Fasman's method and the method by Cid *et al.* (13, 14).

The Chou and Fasman's method:

It is based on empirical probabilities: it defines conformational parameters P_α, P_β and P_t, for the 20 natural amino acids, which represent the normalized frequence of occurrence of each amino acid residue in a particular type of secondary structure, as obtained from a data base of 29 fully determined protein structures. An average P_j greater than 1.0 obtained for a group of amino acids taken in sequence (6 for a helix, 5 for a β-strand and 4 for a β-turn) is an indication that the structure type j is likely to occur in that region of the sequence. The average of the conformational parameters has been replaced by their product, in order to improve the sensibility of the method in the vicinity of the limit value 1.0 (15).

The method by Cid et al.:

It combines physicochemical measurements of solubilities of amino-acids in polar and non polar solvents, with information obtained from a data base of 21 known protein structures (13). It gives the relative position of the polypeptide chain with respect to the protein surface, since it has been shown that exists a linear correlation between this distance and a hydrophobic coefficient, defined by Ponnuswamy and colaborators (16). The "hydrophobicity profile" of a protein is simply a plot of this coefficient H_f, versus the amino acid number in the sequence. Four basic profiles have been defined for 4 types of secondary structure: helix, β-turn, buried and exposed β-strands. The identification of these basic patterns in the hydrophobicity profile of the protein yields the predicted secondary structure.

The method of Mohana and Argos:

Both prediction methods described above, make use of parameters obtained from a data base of soluble proteins, whose tertiary structures are known. Therefore no use can be made of them to detect transmembrane helices in integral membrane proteins. Mohana and Argos (12) have designed an algorithm to predict the presence or absence of hydrophobic stretches in the protein sequence. They make use of five physical parameters that characterize the folding properties of the amino acids: hydration potential, membrane-buried free energy of transfer, polarity, residue turn propensity and residue bulk conformational preference. These five parameters were combined with appropriate weighing in a smoothed curve versus the bacteriorhodopsin amino acid sequence number. The smoothing process was performed by there cycles of least-squares fitting of the data to a quadratic polynomial, using a seven point moving window (17). The weighting factors assigned to the five parameters were chosen to give the best fit to a theoretical curve that displayed the helical and exposed regions of the bacteriorhodopsin molecule, the only membrane-bound protein known to a reasonable high resolution. The curve obtained after weighting, smoothing and summing the curves obtained for each one of the five parameters, was normalized to an average value of 0.0. After applying this algorithm to several families of integral membrane proteins, the authors defined a "conformational

preference parameter for membrane-buried helices". This parameter represents –as in the Chou and Fasman's method– the normalized frequence of occurrence of that amino acid residue in a membrane-buried helix (12). The prediction of the buried helical zones is made as follows: a curve of the buried helix parameter versus the sequence number is submitted to three cycles smoothing, as described above. All zones over a base line of 1.05, with a minimum length of 16 residues with no more than three charges, or more than five charged and strongly polar residues, are likely to correspond to buried helices.

Computer programs that perform the three types of secondary structure prediction on a given aminoacid sequence are in operation at the University of Concepción, and are available on request.

Model building

Three dimensional models of the predicted secondary structure can be easily built using rigid arrows, cylinders and "hair pins" to represent the β-strands, helical zones and β-turns, respectively. These elements are joined by mobile connections and by flexible wire that represents the random coiled zones. The lengths of the building elements are scaled to the number of amino acids involved and to the distance between α-carbons in that particular type of structure. The following complementary information is considered: distinction between exposed and buried β-strands (14), preferences for β-strands to participate in a parallel or antiparallel β-sheet (18), stabilization of helices and β-strands in a super secondary structure (19) and, proximity of some amino acid residues according to chemical evidence.

RESULTS AND DISCUSSION

The predicted secondary structures of β-lactamases I from *B. cereus* 5/B, II and III from *B. cereus* 569/H, compared to those previously obtained for β-lactamases I from *B. licheniformis* 749/C and *B. cereus* 569/H (7), are aligned to best fit on Table 1. The alignement was made attending the shape of the hydrophobicity profiles for the three β-lactamases type I and that of type III. No similarity, either in the primary or secondary structures, could be detected between these enzymes and the only β-lactamase type II. Fig. 1, illustrates the shape of the hydrophobicity profiles in the region near Ser 70, for four of these enzymes. It is remarkable the conservation of the profiles shape in a region with only a 52% invariability of the primary structure.

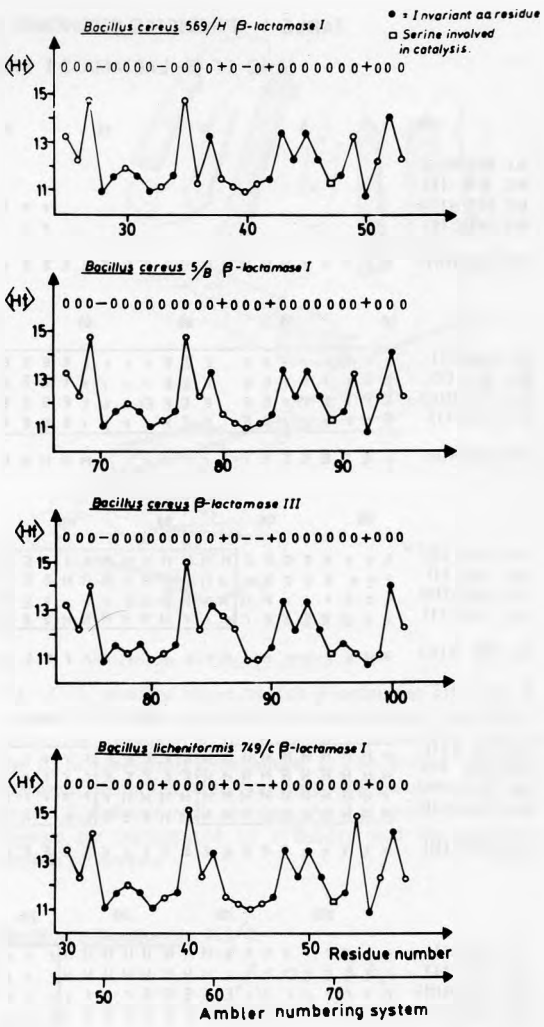
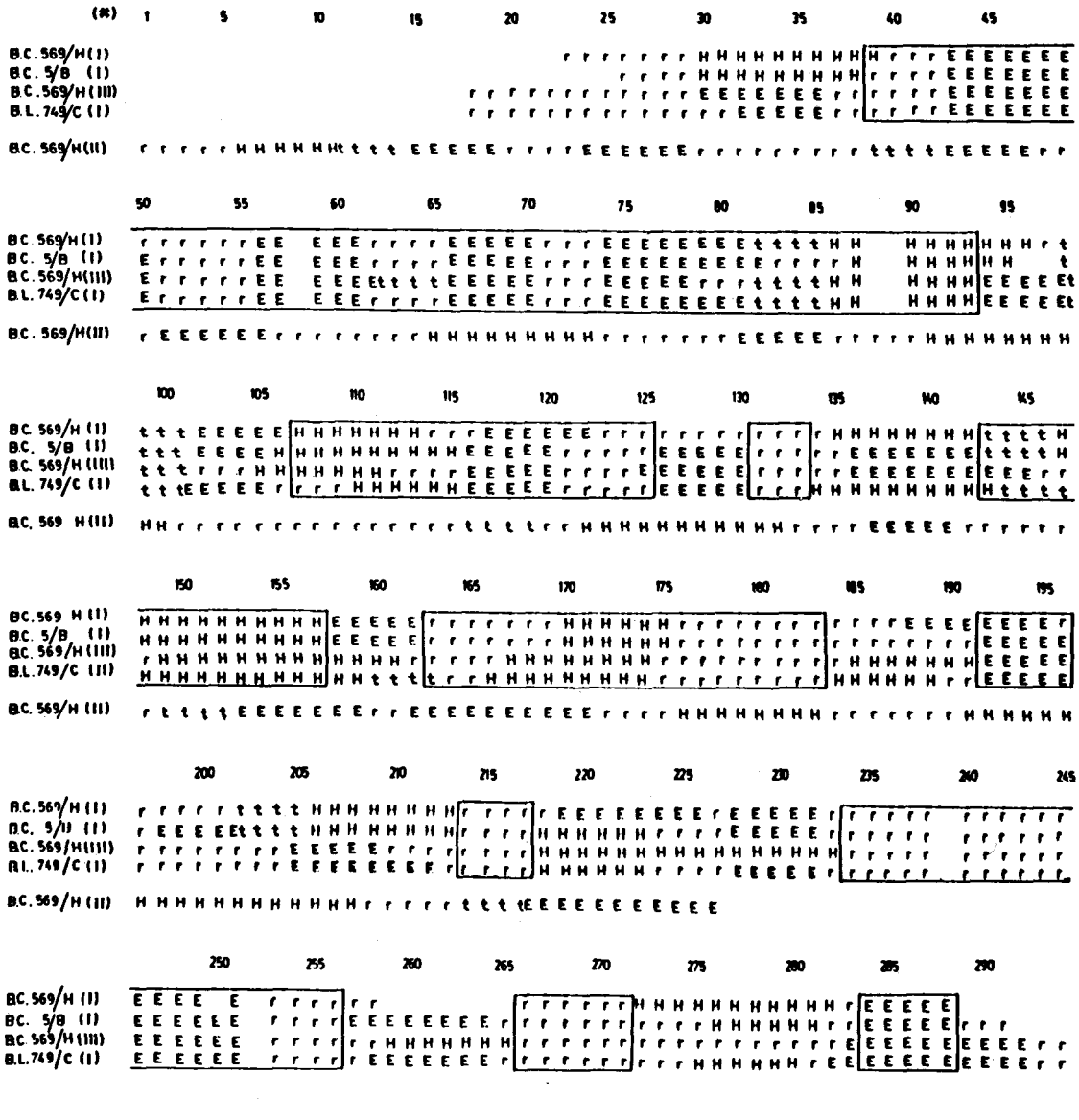


Fig. 1: Hidrophobicity profiles in the vicinity of Ser 70, amino acid residue involved in the catalytic process, of four class A β-lactamases. The sequences were aligned on the basis of the profiles' shape. Note the similarity of the profiles for only a 51% conservation of the primary structure in this region.

The close relation between the three β-lactamases type I and the enzyme called type III, obtained from *B. cereus,* is summarized on Table 2. There, a comparison between the complete primary and secondary structures of these enzymes is made: more than 54% of the amino acid sequence and more than 61% of the secondary structure are preserved when these enzymes are considered pairwise. 43% of the primary and 57% of the secondary structures are common to the four enzymes.

86% of both the primary and the predicted secondary structures of the β-lactamase I produced by *B. cereus* 569/H are conserv-

TABLE 1 PREDICTED SECONDARY STRUCTURES FOR 4 β-LACTAMASES FROM Bacillus cereus, AND OF β-LACTAMASE I FROM Bacillus licheniformis 749/C.



B.C.569/H(I) = Bacillus cereus 569/H β-lactamase I
B.C. 5/B (I) = Bacillus cereus 5/B β-lactamase I
B.C.569/H(III) = Bacillus cereus 569/H β-lactamase III
B.L.749/C(I) = Bacillus licheniformis 749/C β-lact. I
B.C.569/H(II) = Bacillus cereus 569/H β-lactamase II

*) The Ambler numbering system is used except for β-lactamase II from B cereus, where a sequence or secondary structure alignement was unsuccessful.

ed in the enzyme secreted by the strain 5/B of this microorganism. However it is interesting to point out that, in the terminal region, starting on residue 240 (Ambler notation) there is a stronger similarity of this last enzyme with the β-lactamase I produced by *B. licheniformis* than with that produced by strain 569/H of its parent microorganism (Fig. 2).

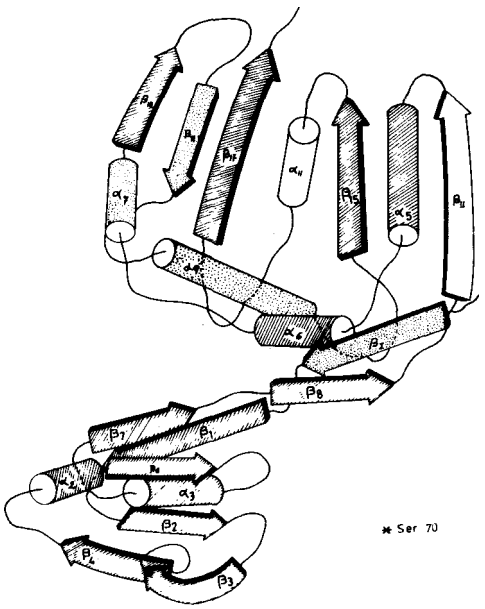
It is evident, from the data shown on Tables 1 and 2, that β-lactamases I, and III from *B. cereus* 569/H, β-lactamase I from *B. cereus* 5/B as well as β-lactamase I from *B. licheniformis* 749/C, can be described by

TABLE 2

Percent similarity matrix for 3 B. cereus β-lactamases and a β-lactamase I from B. licheniformis.

	% of secondary structure similarity			
B. C. 569/H (I)	100	86	61	78
B. C. 5/B (I)	86	100	76	83
B. C. 569/H (III)	57	59	100	85
B. L. 749/C (I)	54	54	68	100
	B.C. 569/H (I)	B.C. 5/B (I)	B.C. 569/H (III)	B.L. 749/C (I)

% of primary structure similarity



β lactamase III Bacillus cereus 569/H

Fig. 3: A possible structure for β-lactamase III from *B. cereus* 569/H. The stippled elements represent secondary structures conserved in the *B. cereus* β-lactamases I and in the *B. licheniformis* β-lactamase I. The dotted elements represent structures common only to β-lactamase III and to the *B. licheniformis* enzyme. As it is usual, the helical regions are represented by cyllinders and the extended strands by arrows.

very similar secondary structures. Fig. 3 shows a possible folding for the polypeptide chain of β-lactamase III which strongly resembles the two-domain structure proposed in (7) for the class A enzymes obtained from *B. cereus* 569/H and *B. licheniformis* 749/C. This result confirms, at the sec-

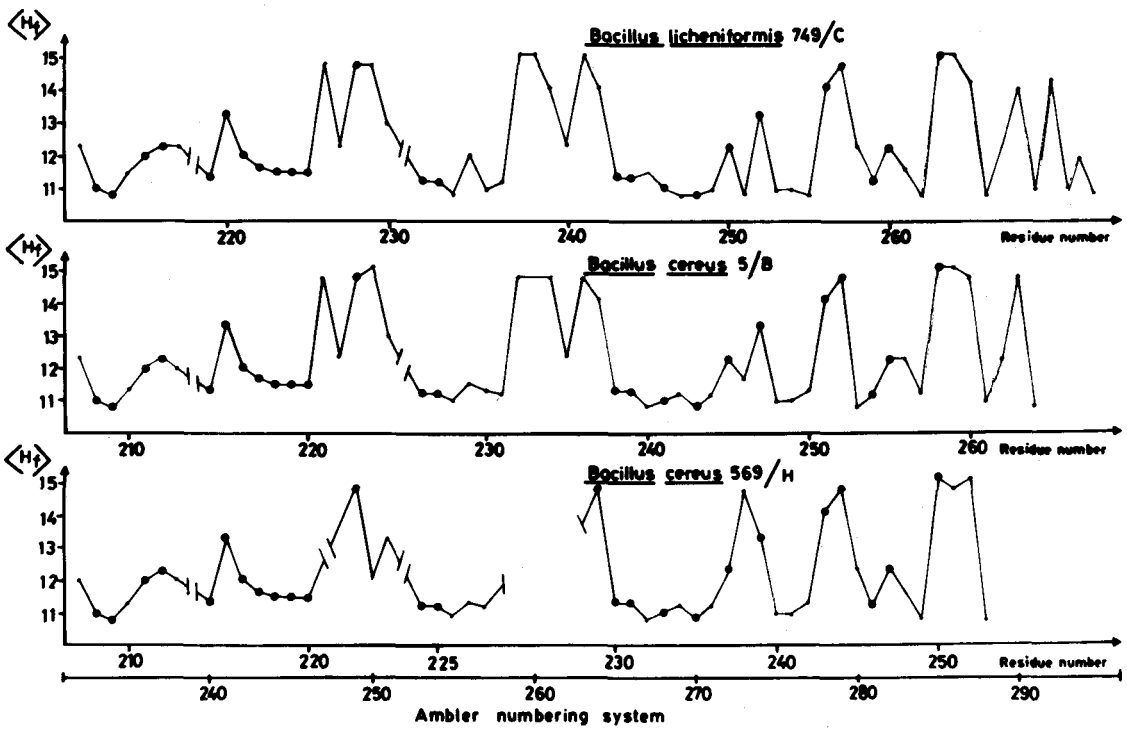


Fig. 2: Hydrophobicity profiles of the carboxy terminal regions of three class A β-lactamases. The invariant amino acid residues are represented by filled circles. Note that the *B. Cereus* 5/B profiles resembles more that of the *B. licheniformis* enzyme.

ondary structure level, what has been proposed on the basis of the primary structure (8): the lipoprotein β-lactamase III from *B. cereus* 569/H is a class A enzyme, a similar, but a different protein from the type I enzyme produced by *B. cereus* 569/H.

A two-domain structure, with a close contact between both domains, has been found for the 2.5 Å tertiary structure of the *S. aureus* PCl recently determined (4). The limits of the domains determined by X-ray methods are different from those proposed by secondary structure prediction, even though there are several coincidences between the predicted and determined secondary structure elements.

The second result, expected from the lack of coincidence of the primary structures, is that there are no similarities in the secondary structures of the class B β-lactamase II from *B. cereus,* and the other four class A enzymes listed on Table 1. The characteristic "active site region" of these last enzymes shown on Fig. 1 could not be found for β-lactamase II, thus suggesting a completely different catalytic mechanism of action.

The secondary structure predicted for the β-lactamase II from *B. cereus* is so different from that proposed for the class A enzymes that it would be sufficient to classify it as an enzyme with a completely different catalytic mechanism, without knowing that it is a Zn^{+2} requiring protein. The structure proposed for it (Fig. 4) is a one-domain structure, where the most

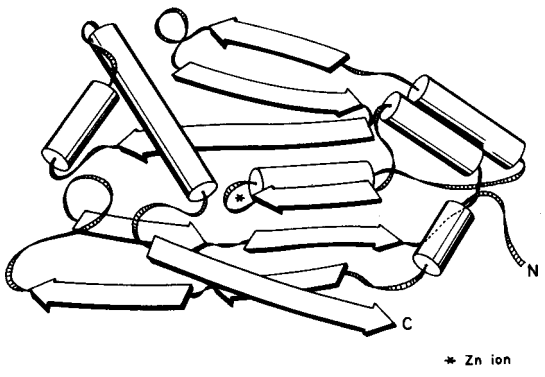


Fig. 4: A possible structure for β-lactamase II from *B. cereus* 569/H. It is a one domain structure type α + β, where the most prominent feature is an antiparallel β-pleated sheet. The location of the Zn ion required for catalysis is marked in the structure.

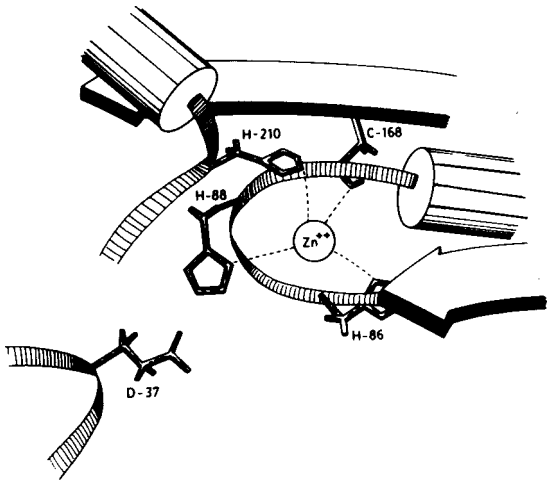


Fig. 5: The location of the Zn ion in the proposed structure for β-lactamase II from *B. cereus* 569/H. The lateral chains of four amino acid residues known to be ligands to the Zn are marked. The position of Asp 37, which is assumed to participate in the orientation of a water molecule (fifth ligand to Zn ion) is also shown.

remarkable feature is an antiparallel β-sheet which resembles the structure of other Zn-dependent proteins, like carbonic anhydrase (20) and carboxypeptidases A and B (21). Fig. 5 shows the probably location of the Zn ion in this structure; three out of the four residues that coordinate to the Zn^{+2} according to chemical evidence (10), are located on a loop or a random coiled zone. This coordination resembles more the carboxypeptidase structure than that of the carbonic anhydrases. Therefore, a first hypothesis to test would be that the catalytic mechanism of the class B β-lactamases should be similar to that of the carboxypeptidases, in agreement with a proposition made by Bicknell *et al.* (22) based on a cryostudy of the cobalt-substituted enzyme.

The comparison among the four enzymes obtained from *B. cereus* and that obtained from *B. licheniformis* was extended to their leader peptides. On Fig. 6 are shown the Mohana and Argos profiles for these five sequences: Both the profiles' shape, and the lenght of the hydrophobic region that could represent a buried helical structure, are almost identical for the *B. licheniformis* β-lactamase I and the *B. cereus* β-lactamase III. If these enzymes present a buried helix in this region, it should have only 13 to 14 amino acid residues. By contrast, the β-lac-

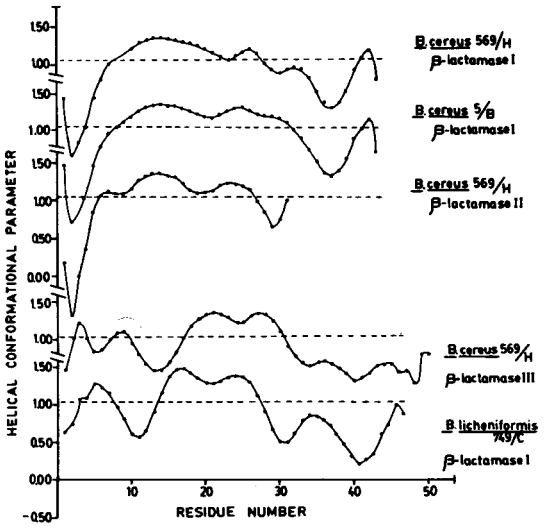


Fig. 6: Mohana and Argos profiles for five β-lactamases. The regions over the dotted line are likely to be buried helices. Note the similarity of the profiles of both β-lactamases I from *B. cereus*, and between β-lactamase III and β-lactamase I from *B. licheniformis*.

tamases I from both strains of *B. cereus* present a continuous hydrophobic region 20 to 23 residues long, which resembles more the 21 residue long hydrophobic strand of the β-lactamase II leader peptide.

It has been suggested (6) that some class A β-lactamases could have evolved from a common ancestor: this seems certainly true for the four enzymes listed on Table 2. Moreover, the conservation of the primary, and of the predicted secondary structures, between any of these enzymes with respect to the *B. licheniformis* β-lactamase I, is certainly higher than what could be expected for non related isoenzymes.

The results reported indicate also that the lipoprotein β-lactamase III from *B. cereus* resembles more β-lactamase I from *B. licheniformis* than its homologue in *B. cereus*. The conservation in the primary structures are 68% and 57%, and this difference raises to 85% and 61% respectively for the secondary structures. The relationship of the lipoprotein to the *B. licheniformis* enzyme also includes the leader peptides, as shown by the Mohana and Argos profiles of Fig. 6. All these results would support the hypothesis suggested in (8) that the β-lactamase III possibly did not evolve within *B. cereus*, but it could had been transferred, relatively recently from *B. licheniformis* to *B. cereus*.

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REFERENCES

1. KNOX, J.R.; ZORSKY, P.E. and SANJEEVA MURTHY, N. (1973) J. Mol. Biol. *79:* 597-598.
2. ASCHAFFENBURG, R.; PHILLIPS, D.C.; SUTTON, B.J.; BALDWIN, G.; KEINER, P.A. and WALEY, S.G. (1978) J. Mol. Biol. *120:* 447-449.
3. DIDEBERG, O.; LIBERT, M.; FRERE, J.M.; CHARLIER, P.; ZHAO, H. and KNOX, J.R. (1985) J. Mol. Biol. *181:* 145-146.
4. HERZBERG, O. and MOULT, J. (1987) Science *236:* 694-701.
5. CITRI, N. and POLLOCK, M.R. (1966) Adv. Enzimology *28:* 237-323.
6. AMBLER, R.P. (1980) Phil. Trans. R. Soc. Lond. B. *289:* 321-331.
7. BUNSTER, M. and CID, H. (1984) FEBS Lett. *175:* 267-274.
8. HUSSAIN, M.; PASTOR, F.I.J. and LAMPEN, J.O. (1987) Preprint from J. of Bacteriology. Vol. 169(2).
9. WANG, W.; MEZES, P.S.F.; YANG, Y.Q.; BLACHER, R.W. and LAMPEN, J.O. (1985) J. of Bacteriology. *163(2):* 487-492.
10. AMBLER, R.P.; DANIEL, M.; FLEMING, J.; HERMOSO, J.M.; PANG, C. and WALEY, S.G. (1985) FEBS Lett. *175:* 267-274.
11. HUSSAIN, M.; CARLINO, A.; MADONNA, M.J. and LAMPEN, J.O. (1985) J. of Bacteriology. *164(1):* 223-229.
12. MOHANA RAO, J.K. and ARGOS, P. (1986) Biochim. Biophys. Acta, *869:* 197-214.
13. CHOU, P.Y. and FASMAN, G.D. (1974) Biochemistry *13:* 211-245.
14. CID, H.; BUNSTER, M.; ARRIAGADA, E. and CAMPOS, M. (1982) FEBS Lett. *150:* 247-254.
15. DUFTON, P.Y. and HIDER, R.C. (1977) J. Mol. Biol. *115:* 117-193.
16. PONNUSWAMY, P.K.; PHRABHAKARAN, M. and MANAVALAN, P. (1980) Biochim. Biophys. Acta *623:* 301-316.
17. MINEUR, H. (1966) Techniques de calcul numérique, Dunod, Paris, pp. 445-471.
18. LIFSON, S. and SANDER, C. (1979) Nature *282:* 109-111.
19. LEVITT, M. and CHOTHIA, C. (1976) Nature. *261:* 552-558.
20. KANNAN, K.K.; NOSTRAND, B.; FRIDBORG, K.; LÖVGREN, S.; OHLSSON, A. and PETEF, M. (1975) Proc. Nat. Acad. Sci. (USA) *72(1):* 51-55.
21. SCHMIDT, M.F. and HERRIOT, J.R. (1976) J. Mol. Biol. *103:* 175-190.
22. BICKNELL, R.; SCHÄFFERT, A.; WALEY, S.G. and AULD, D.S. (1986) Biochemistry. *25:* 7208-7215.

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An approach to the three-dimensional structure of bovine growth hormone based on chemical modification and secondary structure prediction

Una aproximación a la estructura tridimensional de la hormona de crecimiento de bovino basada en modificación química y predicción de estructura secundaria

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Three different methods have been applied to the prediction of secondary structure. The prediction that better fitted the chemical data was chosen.

Two regions of the bovine growth hormone molecule (111-117 and 166-174) appear to be exposed to the solvent, according to hydropathic analysis but have several charged residues not reactive towards their specific reagents.

Two molecular domains are postulated, each one bearing a region with charged residues on its surface and interacting with the other in the molecule by means of saline bridges. The hydrophobic core of the molecule is formed by the ensemble of the hydrophobic region predicted between residues 81 and 108, and the hydrophobic faces of the amphiphilic helices 109-127 and 9-33.

INTRODUCTION

Crystal X-ray diffraction is the most powerful method for obtaining the three-dimensional structure of a protein molecule. Nevertheless, it can only provide a static image that does not take into account the molecular fluctuations. It does not reflect all of the possible multiple energy minima that are found in proteins, as has been shown by molecular dynamic analysis of myoglobin (1) and magnetization transfer NMR spectroscopy of staphylococcal nuclease (2). In spite of the valuable contribution of X-ray crystallography to the knowledge of the tertiary structure of proteins, some doubts have arisen about the analogy of the protein structure in solution to that in its crystallized state. For example, bGH has been crystallized as dimer of hormone dimers (3), while in solution this protein is in a monomer-dimer equilibrium.

Despite the highly developed techniques in the X-ray crystallography field, the difficulties inherent to this method led to many attempts to predict the three-dimensional structure of proteins. Prediction is possible by taking into account that the secondary and tertiary structure are governed by the amino acid sequence (4). There are different predictive methods for secondary structure, based on statistical analysis of the tendency of amino acid types to adopt different structures. Each of these methods has an accuracy of about 60%. When several methods are used simultaneously, the accuracy never exceeds 80%. Needless to say, prediction of tertiary structure based on secondary structure has also limitations of its own. The most accurate methods require a good sequence homology with a protein whose structure is already known by X-ray diffraction. When this is not available, the general approach is to minimize an energy function

Abbreviation: bGH, bovine growth hormone.

that represents the chemical specification of the protein. This is a difficult approach which requires additional information.

A better knowledge of the three-dimensional structure of a protein in solution can be obtained by the integration of the information yielded b y X-ray diffraction and chemical modification.

Chemical modification has played an important role in establishing the structure of enzyme active sites. A model of pancreatic ribonuclease A, based on chemical information, was proposed as early as 1966 by Hammes and Scheraga (5). That model proved to be remarkably similar to another determined, some years later, on the basis of crystallographic data (6). Chemical modifications are used to explore reactivity of the different amino acid residues, which depends on their accessibility and microenvironment. It also depends on the characteristics of the reagents used, which determine whether they can reach the residues involved. For instance, tetranitromethane can react with tyrosine residues located in the interior of the molecule by entering hydrophobic channels (7).

Plenty of information about the reactivity of different amino acid residues in bGH has accumulated especially in the last 20 years: (8-32). All the information obtained –bearing in mind the nature of the reagent used (size) and hydrophillicity or hydrophobicity– has led to several hypotheses about the relative positioning of such residues on the surface or in the interior of the molecule (21, 24). The use of bifunctional reagents has recently enabled us to establish that some residues are close to one another (29, 32). Although the prediction of the secondary structure of bGH was performed by Chen and Sonenberg (33), recent advances in this field have allowed us to apply three different predictive methods to bGH.

In this paper we present predictions on the three-dimensional structure of bGH on the basis of the application of predictive methods for secondary structure and the data obtained by chemical modifications.

METHODS

The predictive methods of Chou and Fasman (34), Dufton and Hider (35) with Levitt's parameters (36) and Garnier *et al.* (37) were used to evaluate the secondary structure.

Chou and Fasman's algorythm was slightly modified as follows: according to the original method, predicted α-helix or β-sheets may vary in position as well as in length depending on the nucleation segment used. In order to eliminate this ambiguity, a computer program that registered the predictions based on all possible α and β nucleation sites was developed. The boundary potential of these segments was calculated as indicated by the authors. All possible β-turns were also identified and registered. The final prediction was based on examination of the different regions, choosing the combinations that maximized the conformational parameters avoiding overlapping. In the few cases overlapping could not be avoided, the choice was made not to interrupt an α-helix with a β-sheet unless in resulted in a substantial increase in the prediction parameters, or it allowed to predict two antiparallel β-sheets separated by a turn.

Hydropathic analysis was performed according to Kyte and Doolittle (38). Amphipathic analysis was carried out as described by Eisenberg *et al.* (39).

RESULTS AND DISCUSSION

Figure 1 shows the amino acid sequence of bGH (40-43) and the reactivity of the different residues towards their specific reagents. The amino acid residues whose reactivity has been studied are divided into three categories: those which readily react with hydrophilic reagents, those which react slowly or do not react at all and the ones which possess an intermediate reactivity.

Although the reactivity of a functional group does not only depend on its accessibility to the solvent but also on its microenvironment, there are segments which –on the basis of their reactivity– may be positioned either on the surface or in the interior of the molecule. For example, between residues 166 and 178 there are tyrosine, lysine, aspartic acid, glutamic acid, histidine and methionine residues which react very slowly or not at all with their specific reagents (19, 21, 23-25). The inaccessibility of this segment to hydrophilic reagents suggests that it may be located in the interior of

the molecule, in spite of the hydrophilic character of its residues. Hydrophilicity, hydrophobicity, polarity and hydropathy of the polypeptide chain were analyzed. A close agreement with the chemical data was obtained by means of the hydropathic index.

Figure 1 shows the results obtained using the hydropathic index (38) in which each residue is assigned a relative value according to its statistical chance of being oriented towards the inside or the outside of the molecule.

This index (38) combines the data obtained by X-ray crystallography and the water vapor transfer free energy. Segments tending to be inside or outside the molecule are indicated in the same figure. Comparison of the residues reactivity with the corresponding values obtained from hydrophatic analysis shows a reasonable agreement in most cases: residues with the higher reactivity are generally located in predicted accessible regions while those with low or no reactivity correspond to the regions predicted to be in the interior of the molecule.

The only segments which show a discrepancy from one method to another lie between residues 109 and 117 and between 166 and 174; these segments mainly contain charged amino acids. In effect, the hydropathic index predicts that they are located on the surface of the molecule. On the other hand, experimental evidence obtained by chemical modifications of different amino acid residues (Tyr (23) Glu, Asp (24, 31) and Lys (19) for the segment 109-117 and Lys (19), His (25), Asp and Glu (24, 31) for the segment 166-174) suggests that they are located in the interior of the molecule.

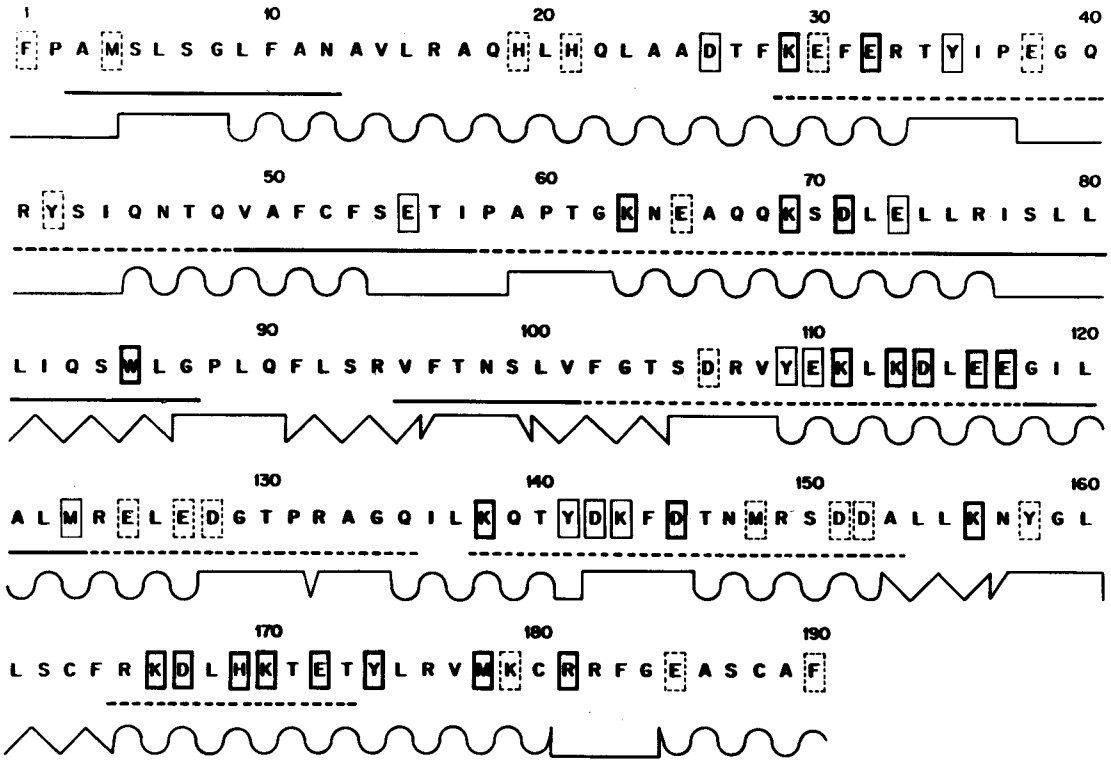

Fig. 1: Amino acid sequence of bovine growth hormone (40-43). Residues in dotted boxes are those which readily react with hydrophilic reagents, those in thinly lined boxes are of intermediate reactivity and those in heavily lined boxes react very slowly or not at all. The reagents used to determine the reactivity of tyrosine, methionine lysine, histidine, aspartic and glutamic acids are: N-acetylimidazole (23), hydrogen peroxide (16), trinitrobenzenesulfonic acid (19), ethoxyformic anhydride (25), and glycine methyl ester in the presence of a water soluble carbodiimide (24, 31), respectively. Full-line underscoring in the amino acid sequence indicates that the residues are in the interior of the molecule and dotted-line underscoring indicate that they are predicted to be on the surface –both by hydropathic analysis (38). Predicted secondary structure is represented below. α-helix ($\sim\sim$), β-sheet ($\wedge\wedge$), random coil (——) and β-turn ($\backslash/$).

Several predictive methods for protein secondary structure are available. Figure 2 shows results obtained by applying the method of Chou and Fasman (34), Dufton and Hider (35) and Garnier *et al.* (37) to the bGH polypeptide chain. Such results are highly coincident. None of the three methods was adopted in particular. For each residue we selected the prediction of secondary structure that better fitted the chemical data previously obtained. For example, the spatial proximity between residues 29 and 35, detected by using a bifunctional reagent (32), is incompatible with the α-structure predicted or with a β-sheet structure (Fig. 2). Therefore, we predict an α-helix from residues 9 through 33 followed by a β-turn. Figure 1 shows our secondary structure prediction.

Several predictions on the three dimensional structure of bGH may be postulated on the basis of the above data and those obtained by chemical modifications.

Reports reviewed by Paladini *et al.* (44) on the action of different proteolytic enzymes on hGH showed a preferential proteolytic cleavage between residues 134 and 135. This allowed the obtention –after disulfide bridge reduction– of fragments 1-134 and 141-191 (45, 46), both of which had some biological activity. When the fragments were put together, the product had the same biological potency as that of the native hormone (45).

From these results, it could be inferred that the two fragments retain their original conformation and are folded independently from each other, thus constituting two different molecular domains. According to the above, we can predict that there are two different molecular domains in bGH, constituted as follows: One by residues 1-134 –including the α-helix 109-127– and another by residues 135-190 including α-helix 165-180. It is likely that either helix lies on the surface of its domain once this folds, just as could be inferred from hydropathy data. When both domains interact, electrostatic bonds are probably

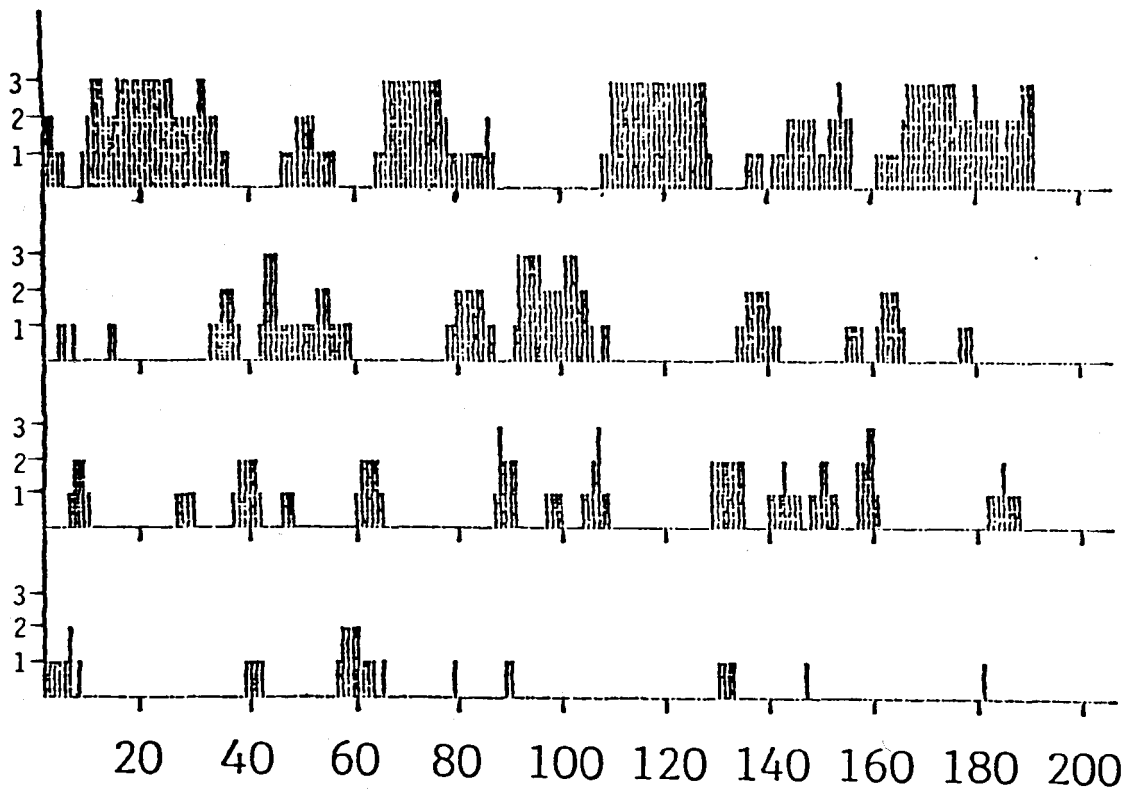

Fig. 2: Combined predictions for α-helix (top), β-sheet (second row), β-turn (third row) and random coil (bottom) for the molecule of bGH. The vertical axis represents the number of predictions for each structural element and the horizontal axis shows the residue number.

generated between them, removing charged residues from exposure to the solvent, thus rendering them inaccessible to chemical reagents, as was found by chemical reactivity (17, 22).

It has been proposed (47, 48) that protein local sites of maximal hydrophobicity in the amino acid sequence fold together establishing the molecular core. Figure 1 shows that the hydrophobic core of the region 1-134 might be formed by three antiparallel β-sheets predicted to be between residues 81-108. This feature has been called a β-meander (49) and found in a number of proteins: Y4-lysozyme (50), staphylococcus nuclease (51), Ser-proteases (52), etc. The hydropathy values indicate that this β-meander tends to be buried in the interior of the bGh molecule.

The amphiphilicity of the α-helical segment 109-127 (Fig. 3) suggests that it has a hydrophobic face which may interact with the hydrophobic segment 81-108;

As discussed above, the hydrophilic face may establish electrostatic bonds with segment 165-180, thus rendering this region of the molecule hydrophobic, as per the hypothesis proposed by Chotia (53). Therefore, the ensemble of the segment 81-108 and the hydrophobic face of α-helices 109-127 and 165-180 may integrate the hydrophobic core of the molecule.

The hydrophobic face of the remaining amphiphilic α-helix 9-33 (Fig. 3) is the most likely to interact with the still exposed side of the predicted β meander.

Ermácora *et al.* (32) and Nowicki *et al.* (29), using a hydrophobic bifunctional reagent, established covalent bridges between tyrosine 174 and lysines 29 and 111. These three residues react very slowly or do not react at all towards hydrophilic reagents such as N-acetylimidazole (23) and trinitrobenzenesulfonic acid (19). These results suggest that residues 29, 111 and 174 belong to a region only accessible to hydrophobic reagents.

Cascone *et al.* (21) suggest that the C-terminal region of bGH, at least from tyrosine 174, is buried in a hydrophobic

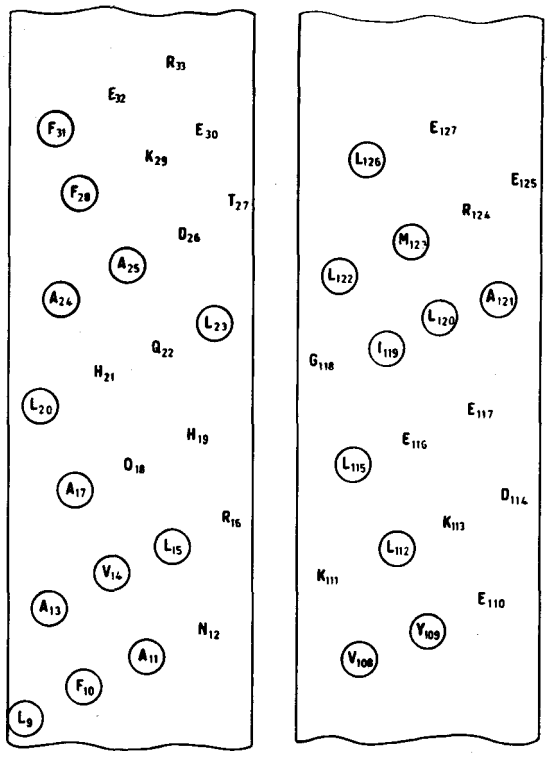

Fig. 3: Cylindrical plotting of amphiphilic α-helices 9-33 and 109-127. Hydrophobic residues are encircled.

region probably located near the surface of the molecule. These authors take into account the lack of reactivity of methionine 178 towards a hydrophilic reagent hydrogen peroxide (16), its susceptibility to chloramine-T and the fact that tyrosine 174 is modified by tetranitromethane (18) but has a low reactivity towards N-acetylimidazole (23). Delfino *et al.* (24), on the basis of the chemical reactivity of different residues suggest that the buried regions is located at least from residue 166 through 178 and that the polypeptidic chain probably becomes accessible to the solvent from lysine 179 up to the C-terminus. These authors add that carboxyl groups of residues 185 and 190 have a high reactivity towards carbodiimide although this region evidences a certain degree of steric hindrance since alanine 189 is removed by carboxypeptidase only after addition of a denaturing agent (54).

Carboxyl groups of residues 114, 116 and 117 react slowly with carbodiimide (24) in a fashion similar to that shown

by lysines 111 and 113 towards trinitrobenzenesulfonic acid (19), suggesting that region 111-117 is not easily accessible to the solvent (24). These facts and predictions support the proposed interaction between helices 9-33 and 165-180.

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REFERENCES

1. ELBERT, R. & KARPLUS, M. (1987) *Science 235:* 318-321.
2. FOX, R.O.; EVANS, P.A. & DOBSON, C.M. (1986) *Nature 320:* 192-193.
3. BELL, J.A.; MOFFAT, K.; VONDERHAAK, B.K. & GOLDE, D.W. (1985) *J. Biol. Chem. 260:* 8520-8525.
4. ANFINSEN, C.B. (1973) *Science 181:* 223-230.
5. HAMMES, G.G. & SHERAGA, H.A. (1966) *Biochemistry 5:* 3690-3693.
6. MEANS, G.E. & FEENEY, R.E. (1971) in "Chemical modification of proteins", p. 11, Holden-Day, Inc., San Francisco, Cambridge, London, Amsterdam.
7. DICKERSON, R.E.; TAKANO, T.; EISENBERG, D.; KALLAI, O.B.; SAMSON, L.; COOPER, A. & MARGOLIASH, E. (1971) *J. Biol. Chem. 246.* 1511-1535.
8. LI, C.H.; SIMPSON, M.E. & EVANS, H.M. (1948) *J. Biol. Chem. 176:* 843-846.
9. MILLS, J.B. & WILHELMI, A.E. (1965) *Endocrinology 76:* 522-525.
10. OIKAWA, A.; DELLACHA, J.M. & SONENBERG, M. (1967) *Biochem. J. 104.* 947-952.
11. MILLS, J.B. & WILHELMI, A.E. (1968) *Ann. N.Y. Acad. Sci. 148:* 343-351.
12. LIBERTI, J.P.; ALFANO, J. & SONENBERG, M. (1969) *Biochim. Biophys. Acta 181:* 176-183.
13. BISCOGLIO, M.J.: DELLACHA, J.M.; PALADINI, A.C. & SANTOME, J.A. (1971) *Ciencia y Cultura 23:* 191-198.
14. WALLIS, M. (1972) *FEBS Lett. 21:* 118-122.
15. GLASER, C.B.; BEWLEY, T.A. & LI, C.H. (1973) *Biochemistry 12:* 3379-3387.
16. GLASER, C.B. & LI, C.H. (1974) *Biochemistry 13:* 1044-1047.
17. GRAF, L.; LI, C.H. & BEWLEY, T.A. (1975) *Int. J. Peptide Protein Res. 7:* 467-473.
18. DAURAT-LARROQUE, S.T.; MOYA-PORTUGUEZ, M.E. & SANTOME, J.A. (1977) *Int. J. Peptide Protein Res. 9:* 119-128.
19. BISCOGLIO DE JIMENEZ BONINO, M.J.; CASCONE, O. & SANTOME, J.A. (1979) *Int. J. Peptide Protein Res. 14:* 107-112.
20. NOWICKI, C. & SANTOME, J.A. (1979) *Int. J. Peptide Protein Res. 13:* 379-384.
21. CASCONE, O.; BISCOGLIO DE JIMENEZ BONINO, M.J. & SANTOME, J.A. (1980) *Int. J. Peptide Protein Res. 16:* 299-305.
22. NOWICKI, C. & SANTOME, J.A. (1981) *Int. J. Peptide Protein Res. 18:* 52-60.
23. BLUMGRUND DE SATZ, V. & SANTOME, J.A. (1981) *Int. J. Peptide Protein Res. 18:* 492-499.
24. DELFINO, J.M.; FERNANDEZ, H.N. & SANTOME, J.A. (1983) *Int. J. Peptide Protein Res. 21:* 440-450.
25. FUKUSHIMA, J.G.; BISCOGLIO DE JIMENEZ BONINO, M.J.; CASCONE, O. & SANTOME, J.A. (1983) *Int. J. Peptide Protein Res. 21:* 451-457.
26. WOLFENSTEIN-TODEL, C. & SANTOME, J.A. (1983) *Int. J. Peptide Protein Res. 22:* 611-616.
27. OPPEZZO, O.J.; BISCOGLIO DE JIMENEZ BONINO, M.J.; CASCONE, O.; NOWICKI, C.; BLUMGRUND, V.; SANTOME, J.A. & FERNANDEZ, H.N. (1984) *Acta Physiol. Pharmacol. Latinoam. 34:* 175-184.
28. FUKUSHIMA, J.; DELFINO, J.M.; WOLFENSTEIN-TODEL, C.; BISCOGLIO DE JIMENEZ BONINO, M.; CASCONE, O.; FERNANDEZ, H.N. & SANTOME, J.A. (1984) *Acta Physiol. Pharmacol. Latinoam. 34:* 151-156.
29. NOWICKI, C.; WOLFENSTEIN-TODEL, C. & SANTOME, J.A. (1985) *Int. J. Peptide Protein Res. 26:* 568-574.
30. MARTAL, J.; CHENE, N. & DE LA LLOSA, P. (1985) *FEBS Lett., 180:* 295-299.
31. DELFINO, J.M.; FERNANDEZ, H.N. & SANTOME, J.A. (1986) *Int. J. Peptide Protein Res. 28.* 307-314.
32. ERMACORA, M.; NOWICKI, C.; WOLFENSTEIN-TODEL, C. & SANTOME, J.A. (1987) *Int. J. Peptide Protein Res.* (in press).
33. CHEN, C.H. & SONENBERG, M. (1977) *Biochemistry 16:* 2110-2117.
34. CHOU, P.Y. & FASMAN, G.D. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol. 47:* 45-148.
35. DUFTON, M.J. & HIDER, R.C. (1977) *J. Mol. Biol. 115:* 177-191.
36. LEVITT, M. (1978) *Biochemistry 17.* 4277-4285.
37. GARNIER, J.; OSGUTHORPE, D.J. & ROBSON, B. (1978) *J. Mol. Biol. 120:* 97-120.
38. KYTE, J. & DOOLITTLE, R.F. (1982) *J. Mol. Biol. 157:* 105-132.
39. EISENBERG, D.; WEISS, R.M. & TERWILLIGER, T.C. (1984) *Proc. Natl. Acad. Sci. USA 81:* 140-144.
40. SANTOME, J.A.; DELLACHA, J.M.; PALADINI, A.C.; WOLFENSTEIN, C.E.M.; PEÑA, C.; POSKUS, E.; DAURAT, S.T.; BISCOGLIO, M.J.; SESE, Z.M. & SANGUESA, A.V.F. (1971) *FEBS Lett. 16:* 198-200.
41. SANTOME, J.A.; DELLACHA, J.M.; PALADINI, A.C.; PEÑA, C.; BISCOGLIO, M.J.; DAURAT, S.T.; POSKUS, E. & WOLFENSTEIN, C.E.M. (1973) *European J. Biochem. 37:* 164-170.
42. WALLIS, M. (1973) *FEBS Lett. 35:* 11-14.
43. SANTOME, J.A.; DELLACHA, J.M. & PALADINI, A.C. (1976) *Pharmac. Ther. B, 2:* 571-590.
44. PALADINI, A.C.; PEÑA, C. & POSKUS, E. (1983) CRC Critical Reviews in *Biochemistry 15:* 25-56.
45. LI, C.H. & BEWLEY, T.A. (1976) *Proc. Natl. Acad. Sci. USA 73:* 1476-1479.
46. LI, C.H.; HAYASHIDA, T.; DONEEN, B.A. & RAO, J. (1976) *Proc. Natl. Acad. Sci. USA 73:* 3463-3465.
47. KAUZMANN, W. (1959) *Adv. Protein Chem. 14:* 1-63.
48. ROSE, G.D. & ROY, S. (1980) *Proc. Natl. Acad. Sci. USA 77:* 4643-4647.

49. SCHULZ, G.E. & SCHIRMER, R.H. (1979) in "Principles of Protein Structure", Cantor, C.R., ed., p. 83, Springer-Verlag, New York, Heidelberg, Berlín.
50. MATTHEWS, B.W. & REMINGTON, S.J. (1974) *Proc. Natl. Acad. Sci. USA 71:* 4178-4182.
51. ARNONE, A.; BIER, C.J.; COTTON, F.A.; DAY, V.W.; HAZEN, Jr., E.E.; RICHARDSON, D.C.; RICHARDSON, J.S. & JONATH, A. (1971) *J. Biol. Chem. 246:* 2302-2316.
52. BIRKTOFT, J.J. & BLOW, D.M. (1972) *J. Mol. Biol. 68:* 187-240.
53. CHOTHIA, C. (1974) *Nature 248:* 338-339.
54. SANTOME, J.A.; WOLFENSTEIN, C. & PALADINI, A.C. (1965) *Biochem. Biophys. Res. Commun. 20:* 482-485.

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Amino-terminal sequence of spinach chloroplast fructose-1,6-bisphosphatase*

Secuencia amino terminal de fructosa-1,6-bisfosfatasa de cloroplastos de espinaca

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The sequence of the NH_2-terminal 25-amino acid residues of purified spinach chloroplast fructose-1,6-bisphosphatase was determined by automated Edman degradation. The amino acid sequence is as follows: Ala-Ala-Val-Gly-Glu-Ala-Ala-Thr-Gln-Thr-Lys-Ala-Arg-Thr-Arg-Ser-Lys-Tyr-Glu-Ile-Glu-Thr-Leu-Thr-Gly. A comparison of this sequence with the corresponding region of pig kidney and yeast (*Saccharomyces cerevisiae*) fructose-1,6-bisphosphatases shows that the sequence of residues 1-19 of the chloroplast enzyme has no homology with the other fructose-1,6-bisphosphatases, but homology is evident after residue 20. The dissimilar sequence contains a region (residues (8-17) rich in basic and hydroxylated amino acids, a structure which is typical of presequences of mitochondrial and chloroplast proteins. Since chloroplast fructose-1,6-bisphosphatase is nuclear in origin, these results suggest that the chloroplast targeting region may have been retained within the amino acid sequence of the mature protein.

Fructose-1,6-bisphosphatase catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate. Because of its key role in gluconeogenesis, the enzyme isolated from gluconeogenic tissues of animals has been extensively studied (for reviews, see refs. 1 and 2). Two forms of the enzyme exist in plant leaves, one is cytosolic and the other chloroplastic, each having an important metabolic role. Cytosolic fructose-1,6-bisphosphatase of leaves is a key enzyme in the pathway of biosynthesis of sucrose, an important transported sugar in most plants (3). The cytosolic enzyme has characteristics which are typical of the so-called gluconeogenic fructose-1,6-bisphosphatases (4). By contrast, chloroplast fructose-1,6-bisphosphatase which is an essential enzyme in carbon dioxide fixation into sugars (5) has unique characteristics. These include insensitivity to AMP inhibition (6) and light dependent activation via a ferredoxin/thioredoxin system (7). However, we have demonstrated from structural studies that a high degree of sequence homology exists among all fructose-1,6-bisphosphatases in spite of their different functions and modes of regulation (8, 9).

The amino acid sequence information so far available for chloroplast fructose-1,6-bisphosphatase accounts for more than 3/4 of the primary structure of the enzyme, but does not include the amino-terminal region (9). This paper gives this information which was obtained by direct sequencing of purified chloroplast fructose-1,6-bisphosphatase. It was somehow expected that data could be obtained by direct sequencing of the purified protein, since chloroplast fructose-1,6-bisphosphatase (10) is as most chloroplast proteins encoded by the nuclear genome (11). These

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* One of us (F.M.) dedicates this paper to the memory of his mentor, Professor Osvaldo Cori.

Keywords: Fructose-1,6-bisphosphatase/amino terminal sequence/chloroplast/transit peptide/sequence homology.

nuclear-encoded chloroplast proteins contain a "transitpeptide" which is usually removed proteolytically after import into chloroplasts (12), exposing a new unblocked N-terminal residue.

EXPERIMENTAL

Fructose-1,6-bisphosphatase from spinach chloroplasts was purified to homogeneity by a procedure which consisted of the following steps: extraction, ammonium sulfate fractionation, Sephadex G-100 gel filtration, DEAE-cellulose chromatography, hydroxylapatite chromatography, and fast protein liquid chromatography on a Pharmacia Mono Q HR 5/5 column (9).

Direct sequencing of purified spinach chloroplast fructose-1,6-bisphosphatase was performed by a technique similar to that recently reported for the sequencing of pea chloroplast NADP-malate dehydrogenase (13). In this procedure, 20 nmol of the native protein were precipitated by the addition of 4 volumes of cold acetone. The pellet was dried and resuspended in 120 μl of 0.3% sodium dodecyl sulfate. The sample was applied to the protein sequencer filter disk in its cartridge, dried, and then 30 μl of trifluoroacetic acid were added. After drying the filter disk, it was assembled into the sequencer. Sodium dodecyl sulfate was then removed by washing the disk for 2 min with butylchloride prior to sequencing.

Automated microsequencing was performed in in Applied Biosystems 470A gasphase protein sequencer using the standard sequencing program and the reagents provided by the manufacturer. The phenylthiohydantion derivatives of amino acids liberated after each degradation cycle were identified and quantitated as such by high performance liquid chromatography using a modification of the procedure described by Hunkapiller (14). The analyses were performed with a Waters Model 840 system equipped with an Altex Ultrasphere ODS 5 μm column (4.6 x 250 mm).

RESULTS AND DISCUSSION

Fructose-1,6-bisphosphatase from spinach chloroplasts was purified to homogeneity and then subjected to N-terminal protein sequence analysis as described under "Experimental Procedures". Automated Edman degradation carried out for 25 cycles yielded the amino acid sequence: Ala-Ala-Val-Gly-Glu-Ala-Ala-Thr-Gln-Thr-Lys-Ala-Arg-Thr-Arg-Ser-Lys-Tyr-Glu-Ile-Glu-Thr-Leu-Thr-Gly (Table I). The sequence of residues 16-25 of spinach chloroplast fructose-1,6-bisphosphatase corresponded to the first 10 residues of the previously sequenced tryptic peptide T-22, a peptide that aligned with residues 6-19 of pig kidney fructose-1,6-bisphosphatase (15). The sequence of residues 1-11 of spinach chloroplast fructose-1,6-bisphosphatase corresponded to the previously sequenced peptide T-3. This peptide had been erroneously aligned in the middle of the enzyme's structure (15) because it showed 27% homology with the sequence of residues 160-170 of pig kidney fructose-1,6-bisphosphatase (16). Although we were cautious about the tentative alignment of peptide T-3, we expressed confidence about the alignment of the rest of the partial structure of 284 residues (15). Table I also shows (on the right hand side the sequence of residues 1-19 of pig kidney fructose-1,6-bisphosphatase (16) aligned with NH_2-terminal sequence of spinach chloroplast fructose-1,6-bisphosphatase, an alignment based on the existence of the common sequence -I-X-T-L-T-(residues 10-14 in the pig kidney enzyme). Homology between the two fructose-1,6-bisphosphatases in this region, however, is rather low. A comparison of the NH_2-terminal sequence of spinach chloroplast fructose-1,6-bisphosphatase with those of the corresponding region of pig kidney and *Saccharomyces cerevisiae* enzymes (17) conforms the dissimilar NH_2-terminal region of the chloroplast enzyme (Fig. 1). Indeed, no homology whatsoever is found in the first 19 residues, but homology begins al residue 20. The currently known sequence of spinach chloroplast fructose-1,6-bisphosphatase comprises 299 residues.

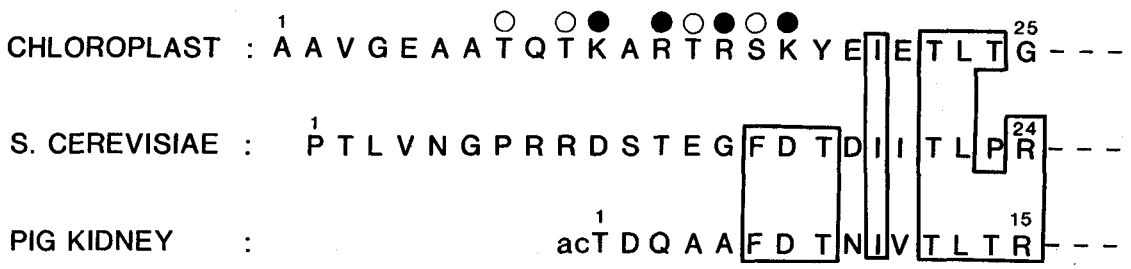

Fig. 1: Alignment of the NH_2-terminal sequences of spinach chloroplast, yeast (*S. cerevisiae*), and pig kidney fructose-1,6-bisphosphatases. The chloroplast sequence was obtained from the data reported herein. Symbols above residues in the chloroplast fructose-1,6-bisphosphatase sequence indicate the presence of hydroxylated (○) and basic (●) residues. The sequences of the NH_2-terminal region of pig kidney and yeast fructose-1,6-bisphosphatase are from Rittenhouse *et al.* (17). Amino acid residues common to at least two sequences are enclosed in boxes. Amino acids are indicated by the single-letter code, and the abbreviation acT in position 1 of the pig kidney enzyme indicates acetyl threonine.

This includes the 284 amino acid residue structure shown in Fig. 1 of Marcus and Harrsch (15), plus residues 1-15 of Table I above. The overall homology of this structure with the corresponding amino acid sequence of pig kidney (16) and *S. cerevisiae* yeast (D.T. Rogers, personal communication) fructose-1,6-bisphospha-

TABLE I

Sequences analysis of spinach chloroplast fructose-1,6-bisphosphatase[a]

Cyclye of Edman degratation	Residue identified (nmol)	Corresponding residue in: previously sequenced tryptic peptides[b]		pig kidney FbPase (residues 1-19)[c]
1	A (3611)	A		
2	A (3042)	A		
3	V (1727)	V		
4	G (2061)	G		
5	E (1851)	E		
6	A (2196)	A	T3	
7	A (2127)	A		
8	T (1550)	T		
9	Q (1339)	Q		
10	T (1662)	T		
11	K (1504)	K		T
12	A (1628)			D
13	R (472)			Q
14	T (1317)			A
15	R (452)			A
16	S (1005)	S		F
17	K (1206)	K		D
18	Y (1092)	Y		T
19	E (1078)	E		N
20	I (1133)	I		I
21	E (669)	E		V
22	T (687)	T	T22	T
23	L (1221)	L		L
24	T (726)	T		T
25	G (1233)	G		R
		W		F
		L		I
		L		I
		K		E

[a] Automated microsequencing was performed as described under "Experimental". Recoveries in picomoles are given in parentheses.

[b] Data from Marcus and Harrsch (15).

[c] Data from Marcus *et al.* (16).

tase is of 43 and 37%, respectively. This indicates that the chloroplast enzyme has a structure closely related to other fructose-1,6-bisphosphatases but a dissimilar NH_2-terminal region. We have also suggested (15) that a dissimilar region may exist in the middle of the chloroplast enzyme molecule to accomodate a unique pair of cysteine residues involved in the light-dependent activation of chloroplast fructose-1,6-bisphosphatase.

The determined NH_2-terminal sequence of spinach chloroplast fructose-1,6-bisphosphatase is shown in Fig. 1 aligned with the corresponding region of *S. cerevisiae* yeast and pig kidney fructose-1,6-bisphosphatases (17). This figure serves to illustrate at least two structural characteristics: (a) the presence of a region where the sequence homology among the three enzymes begins (shown by the boxed areas). This high degree of homology continues to be a distinctive feature of the not shown rest of the structures; (b) the unique nature of the NH_2-terminal sequences of the three enzymes: (i) the pig kidney fructose-1,6-bisphosphatase is the shortest and residue 1 is acetylated; (ii) the first 14 residues of the yeast *S. cerevisiae* NH_2-terminal enzyme sequence have no equal in the other sequences. It has been established that Ser-11 of this sequence is the site of *in vitro* (17) and *in vivo* (18) phosphorylation of yeast fructose-1,6-bisphosphatase; (iii) the distinct nature of the NH_2 terminal region of the chloroplast enzyme which is rich in basic (•) and hydroxylated (○) amino acids. This characteristic of the chloroplast enzyme structure is discussed separately below in the final paragraph.

As mentioned in the introductory section, chloroplast fructose-1,6-bisphosphatase is nuclear encoded (10) and likely to have an amino-terminal transit peptide. The transit peptides are usually removed proteolytically after import of nuclear-encoded proteins into chloroplasts (12). However, as noted above the sequenced NH_2-terminal region of spinach chloroplast fructose-1,6-bisphosphatase contains a region (residues 8-17) mostly composed of basic and hydroxylated amino acids. Thus, spinach chloroplast fructose-1,6-bisphosphatase has an NH_2-terminal sequence with features which are typical of presequences of imported mitochondrial and chloroplast proteins (19, 20). It is suggested that chloroplast fructose-1,6-bisphosphatase belongs to a yet rare class of proteins that do not carry transient pre-sequences and that the chloroplast targeting region is retained within the amino acid sequence of the mature protein. Examples of this class have already been reported for nuclear-encoded mitochondrial proteins (21, 22), but not yet for chloroplast proteins. Definitive proof of the non-removal of a pre-sequence present in chloroplast fructose-1,6-bisphosphatase should be obtained upon completion of our current work on the sequencing of chloroplast fructose-1,6-bisphosphatase cDNA.

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We thank Dr. David T. Rogers, Genetics Institute, Cambridge, Massachusetts, USA, for communicating to us the amino acid sequence of yeast *Saccharomyces cerevisiae* fructose-1,6-bisphosphatase prior to publication. This work was supported by grants from the U.S. Department of Agriculture (83-CRCR-1-1299), and the National Institutes of Health DK 26564).

REFERENCES

1. BENKOVIC, S.J. and DeMAINE, M.M. (1981) *Adv. Enzymol. 53:* 45-82.
2. TEJWANI, G.A. (1982) *Adv. Enzymol. 54:* 121-194.
3. HARBRON, S.; FOYER, C. and WALKER, D. (1981) *Arch. Biochem. Biophys. 212:* 237-246.
4. ZIMMERMAN, G.; KELLY, G.J. and LATZKO, E. (1978) *J. Biol. Chem. 253:* 5952-5956.
5. HALLIWELL, B. (1981) in *Chloroplast Metabolism,* pp. 66-88, Oxford University Press, New York.
6. PREISS, J.; BIGGS, M.L. and GREENBERG, E. (1967) *J. Biol. Chem. 242:* 2292-2294.
7. BUCHANAN, B.B.; WOLOSIUK, R.A. and SCHURMANN, P. (1979) *Trends Biochem. Sci. 4:* 93-96.
8. MARCUS, F.; GONTERO, B.; HARRSCH, P.B. and RITTENHOUSE, J. (1986) *Biochem. Biophys. Res. Commun. 135:* 374-381.
9. MARCUS, F.; HARRSCH, P.B.; MOBERLY, L.; EDELSTEIN, I. and LATSHAW, S.P. (1987) *Biochemistry,* in press.
10. CHUECA, A.; LAZARO, J.J. and LOPEZ-GORGE, J. (1984) *Plant Physiol. 75:* 539-541.
11. CHUA, N.-H. and SCHMIDT, G.W. (1979) *J. Cell Biol. 81:* 461-483.
12. SCHMIDT, G.W. and MISHKIND, M.L. (1986) *Ann. Rev. Biochem. 55:* 879-912.
13. FICKENSCHER, K.; SCHEIBE, R. and MARCUS, F. (1987) *Eur. J. Biochem.,* in press.

14. HUNKAPILLER, M.W. (1985) Protein Sequencer User Bulletin Nº 14, Applied Biosystems, Foster City, California.
15. MARCUS, F. and HARRSCH, P.B. (1987) *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. 3, pp. 471-474, Martinus Nijhoff, Dordrecht, The Netherlands.
16. MARCUS, F.; EDELSTEIN, I.; REARDON, I. and HEINRIKSON, R.L. (1982) *Proc. Natl. Acad. Sci. USA 79:* 7161-7165.
17. RITTENHOUSE, J.; HARRSCH, P.B.; KIM, J.N. and MARCUS, F. (1986) *J. Biol. Chem. 261:* 3939-3943.
18. RITTENHOUSE, J.; MOBERLY, L. and MARCUS, F. (1987) *J. Biol. Chem. 262:* 10114-10119.
19. ALLISON, D.S. and SCHATZ, G. (1986) *Proc. Natl. Acad. Sci. USA 83:* 9011-9015.
20. HURT, E.C.; SOLTANIFAR, N.; GOLDSCHMIDT-CLERMONT, M.; ROCHAIX, J.-D. and SCHATZ, G. (1986) *EMBO J.* 1343-1350.
21. RIDLEY, R.G.; PATEL, H.V.; GERBER, G.E.; MORTON, R.C. and FREEMAN, K.B. (1986) *Nucleic Acids Res. 14:* 4025-4035.
22. ARAKAWA, H.; TAKIGUDI, M.; AMAYA, Y.; NAGATA, S.; HAYASHI, H. and MORI, M. (1987) *EMBO J. 6:* 1361-1366.

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Photoaffinity labeling of pyruvate kinase from rabbit muscle*

Marcación por fotoafinidad de la piruvato quinasa de músculo de conejo*

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Several studies have been performed on the structure of muscle pyruvate kinase. X-ray diffraction has provided a three-dimensional picture of the active site, and chemical modification studies have reveales essential amino acid residues for substrate binding or catalysis. We have shown that 8-azido-ADP (N_3 ADP) behaves as a photoaffinity label for the enzyme. This reagent upon irradiation produces inactivation of the enzyme, and the activity loss is protected by nucleotides. The partially modified enzyme shows the same Km for ADP as the native one suggesting an "all or none" inactivation effect. The incorporation of 1 mole of ^{14}C-N_3 ADP per subunit correlates with complete inactivation. A radioactive peptide was isolated from the enzyme labeled with ^{14}C-N_3 ADP. The partial sequence of this peptide showed that it corresponds to the same peptide isolated from rabbit muscle pyruvate kinase labeled with dialdehyde-ADP and with trinitrobenzenesulfonate. This peptide is identical to a region in the cat and chicken muscle enzymes, and also a high degree of homology is found in a region of the rat liver and yeast enzymes. These studies show that N_3 ADP binds to the same site as dialdehyde-ADP in rabbit muscle pyruvate kinase, and this site seems to be the nucleotide binding site.

Many studies have been performed on the active site of enzymes in order to understand their catalytic mechanism. Chemical modification is one method for obtaining information about active or allosteric sites. This may be accomplished by using group-specific reagents directed to distinct chemical groups in the protein or by means of affinity labels. The objective is to produce a change in some property of the enzyme that can be correlated with the modification of specific amino acid residues. The specific chemical modification should result in the quantitative modification of a unique functional amino acid residue withouth affecting other functional groups or the conformation of the molecule.

Affinity labels have proven to be very effective tools for the study of the relationships between structure and fuction of proteins. These reagents, also called site-specific reagents, are structurally similar to known substrates, competitive inhibitors, allosteric effector or other kind of ligands that bind to proteins. They posses reactive groups capable of forming covalent bonds with amino acid side chains; the binding is helped by a local increase of the reagent concentration at the ligand binding site.

A very important class of affinity reagents are the photoaffinity labels, which have photolabile groups in their structure. These reagents show several advantages in comparison with affinity labels (1). First, they are activated *in situ* by irradiation forming very reactive functional groups (usually carbenes or nitrenes), which can react not only whith nucleophilic residues but also with hydrophobic amino acids involved in the binding of ligands. Second, photoaffinity labels do not react after addition to the enzyme unless they are irradiated. This property is very useful to study specific interactions of these analogs with enzymes. Another advantage of photoaffinity labels is that

* This article has been written in memory of Dr. Osvaldo Cori in recognition of his mastery and friendship.

the time and rate of labeling can be easily controlled by irradiation.

Azido-nucleotides have been employed to study the nucleotide binding site of various enzymes (2-5). These compounds produce upon irradiation highly reactive nitrenes that rapidly react with amino acid residues located at the site of binding.

In our laboratory we have employed 8-azido-ADP (N_3 ADP) to study the active site of rabbit muscle pyruvate kinase as a means to understand the mechanism of action of the enzyme.

Pyruvate kinase (E.C.2.7.1.40) catalyzes the following reaction:

Phosphoenol pyruvate (PEP) + ADP ⟶ Pyruvate + ATP

The enzyme is a tetramer composed of identical subunits of molecular weight ranging from 55,000 to 60,000. In higher animals four isoenzymes have been described (6). They are called M_1, M_2, L and R. The M_1 isoenzyme found in muscle has been the most studied.

Muscle pyruvate kinase requires for activity the presente of monovalent (K^+) and divalent (Mg^{+2}) metal cations. The enzyme presents hyperbolic kinetics for the two substrates, PEP and MgADP, and is competitively inhibited by ATP with respect to both substrates (7). A mechanism is postulated by which the substrates must be simultaneously present in order for the phosphate transfer to take place (7).

Several chemical modification studies have been made on muscle pyruvate kinase. These studies have shown the participation of different amino acid residues in the binding site of ADP and PEP. Thus, a lysyl residue has been involved in the binding of ADP (8, 9) and an arginyl residue in the binding of PEP (10, 11).

A tryptic peptide from bovine muscle pyruvate kinase has been isolated by Johnson *et al.* (12). This 34-residue peptide labeled with trinitrobenzenesulfonate (TNBS) was sequenced and the binding of the reagent to a lysyl residue was established. Rabbit muscle pyruvate kinase was modified with the affinity label 2',3-dialdehyde ADP and a tryptic peptide identical to the one found in the bovine enzyme was sequenced (9). An identical sequence is present in the primary structure of the chicken (13) and cat muscle (14) enzymes and also, with a high degree of homology, in the rat liver (15, 16) and yeast (17) pyruvate kinases. Therefore, this region is highly conserved, as would be expected for a catalytically active site.

N_3 ADP does not behave as a substrate for rabbit muscle pyruvate kinase (18). However, the photoaffinity reagent behaves as a competitive inhibitor with respect to ADP as shown in Figure 1. A Ki_{app} of 3mM was estimated for N_3 ADP. The fact that N_3 ADP does not behave as a substrate may be explained by the different conformation of N_3 ADP and ADP; the azido derivate present a *syn* conformation induced by the azido group in position 8 of the purine ring (19, 20). Studies performed by circular dicroism indicate that nucleotides bound to isolated subunits of enzymes present the *anti* conformation (21).

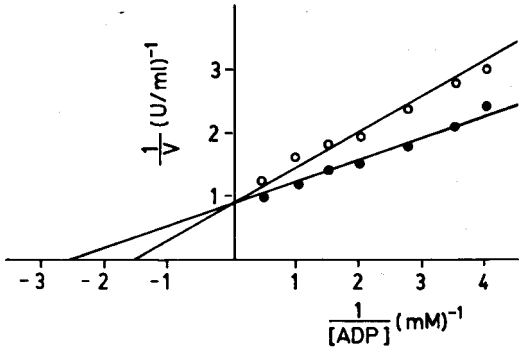

Fig. 1: N_3ADP as a competitive inhibitor with respect to ADP. The assay of activity was made in the absence (•) or in the presence (o) of 1.8 mM N_3ADP.

Figure 2 shows the activity loss of the enzyme when it is irradiated in the presence of N_3 ADP. A rapid inactivation is observed during the first few minutes, and the rate decreses progressively until a plateau is reached after 20-30 minutes. This effect is observed in the presence or in the absence of Mg^{+2}. Both the initial rate and the degree of inactivation reached are dependent on the concentration of N_3 ADP.

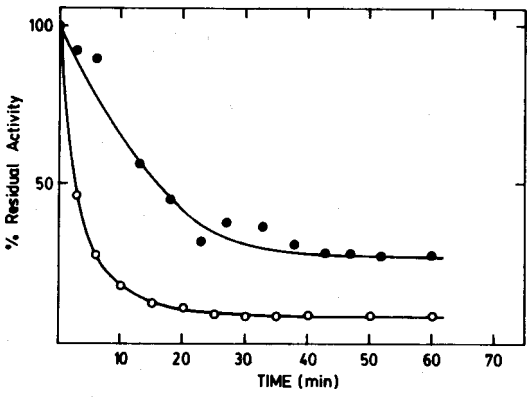

Fig. 2: Inactivation of rabbit muscle pyruvate kinase by N_3ADP. The enzyme was irradiated in the presence of 0.17 mM (•) and 0.36 mM (o) N_3ADP. The enzyme concentration was 0.30 mg/ml in 50 mM Tris-HCl, pH 7.5 containing 20 mM $MgSO_4$.

If the maximal inactivation is plotted as a function of the concentration of N_3 ADP, saturation kinetics is observed. This is in agreement with the formation of a noncovalent enzyme-N_3ADP complex before the covalent light-induced binding (22). Figure 3 shows the plot of the reciprocal of maximum inactivation as a function of the reciprocal of the N_3ADP concentration. The extrapolation of the straight line on the ordinate axis gives the maximum level of inactivation at infinite concentration of the reagent. This value is close to 100%, suggesting that an essential site for the catalytic activity of the enzyme is modified.

Magnesium has a small effect on the inactivation. It was found that inactivation increased slightly up to 1.5 mM Mg^{+2}. The inactivation observed at higher concentration (8 mM) of Mg^{+2} is similar to the one observed in the absence of Mg^{+2}. Hence, in some experiments, 1.5 mM Mg SO_4 was included.

N_3ADP presents a characteristic ultraviolet spectrum with an absorbtion maximum at 282 nm. Upon irradiation, this maximum shifts to lower wavelenghts (274 nm) with a reduction in peak height. The change of the spectrum as a function of the illumination time shows the degree of photolability of the reagent in aqueous solution. The new maximum at 274 nm corresponds to the appearance of an hydroxylamine derivative of ADP (23). The change of the absorbance maximum shows a kinetics which is very similar to that observed for the inactivation of the enzyme in the presence of N_3ADP. Therefore, the reagent decomposes along with the inactivation of pyruvate kinase, and hence a maximum value of inactivation is reached. If an additional amount of N_3ADP is added to a sample of enzyme previously irradiated in the presence of N_3ADP, an additional inactivation is observed with a similar kinetics.

The Km for ADP of the partially modified enzyme is the same as that for the native enzyme (Figure 4). This value was estimated

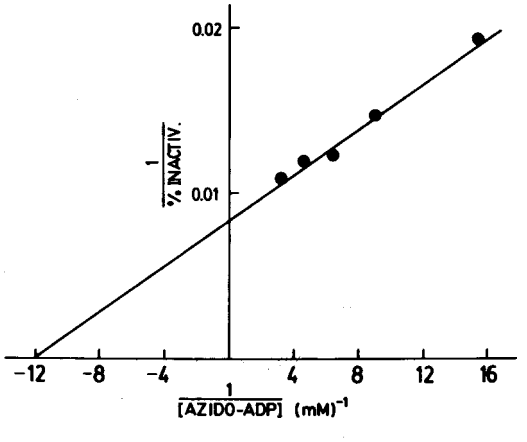

Fig. 3: Effect of the N_3ADP concentration on the degree of inactivation of pyruvate kinase. The enzyme at a concentration of 0.24 mg/ml in 50 mM Tris-HCl, pH 7.5 was inactivated by 0.13 mM, 0.22 mM, 0.31 mM, 0.43 mM and 0.64 mM N_3ADP.

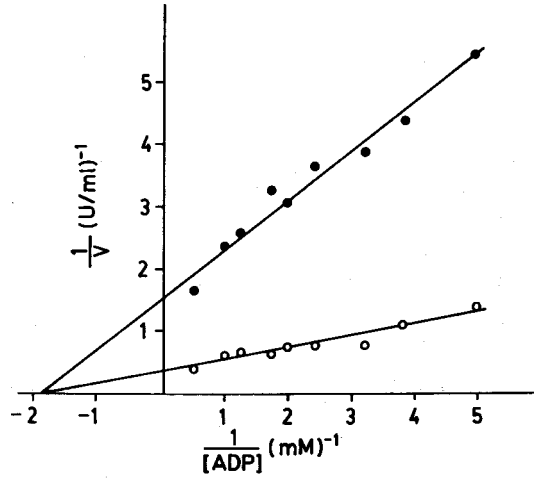

Fig. 4: Kinetic properties of the partially inactivated enzyme. Initial velocities as a function of ADP concentration were measured for the native (o) and modified (•) enzyme.

at 0.55 mM. This fact indicates that a partially inactivated enzyme is composed of two populations of molecules, one of completely inactivated and the other of native enzyme.

Experiments were performed to determine the protective effect of different compounds on the inactivation of the enzyme. Table I shows that the best protection is afforded by ADP and ATP. AMP protects slightly and adenosine shows no effect. PEP gives significant protection while protection by pyruvate is only slight. These results suggest that N_3 ADP modifies the mucleotide or the phosphate binding site of the enzyme.

TABLE I

Effect of substrates on the inactivation of pyruvate kinase by N_3-ADP

Additions to the reactions mixture	% Residual Activity
None	9
Mg^{+2} (2 mM)	7
Adenosine (8 mM)	9
AMP (8 mM)	24
ADP (8 mM)	63
ATP (8 mM)	68
Pyruvate (8 mM)	22
PEP (8 mM)	44
Adenosine (8 mM) + Mg^{+2} (10 mM)	18
AMP (8 mM) + Mg^{+2} (10 mM)	27
ADP (8 mM) + Mg^{+2} (10 mM)	51
ATP (8 mM) + Mg^{+2} (10 mM)	57

Pyruvate kinase (0.48 mg/ml) was irradiated in the presence of 1.06 mM N_3-ADP with the indicated additions. Values represent residual activity after reaching the plateau.

In order to obtain more precise information on the binding site of N_3 ADP to the enzyme, an experiment based on the ability of TNBS to inactivate pyruvate kinase was done. The incorporation of 1 mole of TNBS per subunit of pyruvate kinase produces total inactivation (8) and the reagent binds to a lysine residue located at the nucleotide binding site of the enzyme (12). A sample of the enzyme was inactivated with N_3 ADP to 23% residual activity, the enzyme was dialyzed, treated with TNBS and the incorporation of the reagent was measured (24). A control experiment was performed by measuring the incorporation of TNBS to the native enzyme. Native enzyme incorporated 1.2 and the treated enzyme 0.52 moles of TNBS per subunit. This result suggests that N_3 ADP and TNBS bind to the same locus on the enzyme, the nucleotide binding site.

The stoichiometry of binding was studied by measuring the extent of inactivation by ^{14}C-N_3 ADP as a function of the number of moles of reagent incorporated per subunit of the enzyme. Figure 5 shows that by extrapolation to 100% inactivation the binding of 1 mole of N_3 ADP per subunit of enzyme would result in complete inactivation. Experiments for the isolation of a labeled peptide were performed. For this purpose, fifty mg of enzyme were labeled with ^{14}C-N_3 ADP. The enzyme was inactivated by 20% and incorporated 0.15 moles of N_3 ADP per subunit. The modified enzyme was reduced, carboxymethylated (25) and, digested with trypsin (12). The tryptic digest was applied to a Sephadex G-25 columm (Figure 6). Three peaks absorbing at 230 nm were found and most of the radiactivity was associated to the ascending part of the first peak. Tubes containing the radioactive peak were pooled and rechromagraphed through Sephadex G50 where a peak containing most of the radioactivity

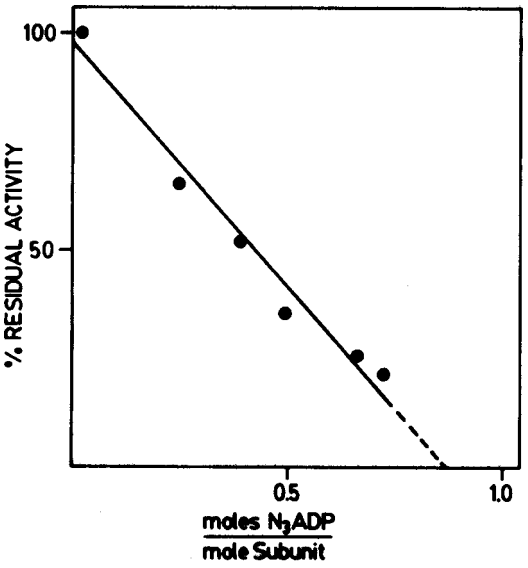

Fig. 5: Relationship between residual activity and moles of ^{14}C-N_3-ADP incorporated into the enzyme. Pyruvate kinase (1 mg/ml) was incubated in 50 mM Tris-HCl pH 7.5, 1.5 mM $MgSO_4$ and 0.5 mM ^{13}C-N_3 ADP. Aliquots were taken at different times for activity assay and for measuring radioactive incorporation.

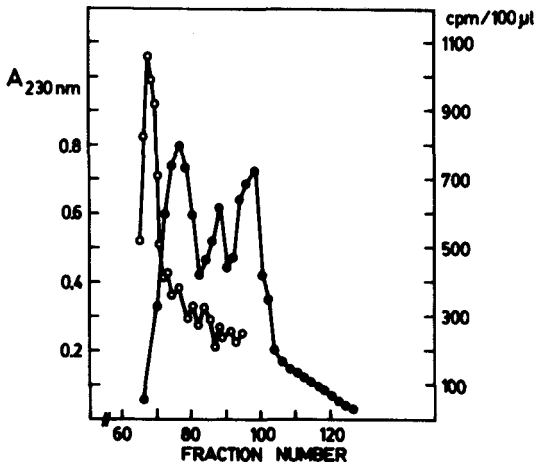

Fig. 6: Elution profile of tryptic digest of ^{14}C-N_3ADP-labeled pyruvate kinase. Elution was performed with 0.5% ammonium bicarbonate, pH 8.0.

was obtained again. This material was further purified by HPLC and the pure peptide was submitted to sequencing. A small amount of a pure peptide was obtained, corresponding to the first 25 residues of the 34 residue peptide isolated by Johnson *et al.* (12) and by Bezares *et al.* (9). The carboxy-terminal lysine would not appear to be the site of modification because trypsin cleaved at that site. This fact indicated that TNBS, oADP and N_3ADP bind to the same site on muscle pyruvate kinase being this site near or at the nucleotide binding site.

X-ray diffraction studies at a resolution of 0.26 nm have shown that cat muscle pyruvate kinase consists of three structural domains, called A, B and C (26). The active site of the enzyme is located in a pocket between domains A and B. Domain A is the biggest and has the eight stranded alfa/beta barrel structure also found in other enzymes. Betwen the third beta-sheet strand and the third helix of domain A, the chain forms domain B (27). The lysyl group modified by TNBS and probably by oADP correspond to Lys-366 in cat muscle pyruvate kinase and is located between the eight beta-sheet strand and the eight helix of domain A (14). In the three-dimensional model of the enzyme this corresponds to the entrance of the active site, which helps explain its high reactivity.

One of the purposes of employing N_3ADP was that in virtue of its high reactivity this reagent could label other amino acid residues within the active site. At the present time work is in progress to determine which other amino acids are labeled.

REFERENCES

1. SCHÄFER, H.-J. (1987) Photoaffinity labeling and Photoaffinity Crosslinking of Enzymes, in Chemical Modification of Enzymes (Eyzaguirre, J., Ed) pp. 45-62, Ellis Horwood Limited, Chichester, England.
2. SCHEURICH, P.; SCHÄFER, H.-J.; DOSE, K. (1978) *Eur. J. Biochemistry 88:* 253-257.
3. KERLAVAGE, A.R.; TAYLOR, S.S. (1980) *J. Biol. Chem. 255:* 8483-8488.
4. HOLLEMANS, M.; RUNSWICK, M.J.; FEARNLEY, I.M.; WALKER, J.E. (1983) *J. Biol. Chem. 258:* 9307-9313.
5. BUBIS, J.; TAYLOR, S.S. (1985) *Biochemistry 24:* 2163-2170.
6. HALL, E.R.; COTTAM, G.L. (1978) *Int. J. Biochem. 9:* 785-793.
7. KAYNE, F.J. (1973) *The Enzymes (3rd ed.) 8:* 353-382.
8. HOLLENBERG, P.F.; FLASHNER, M.; COON, M.J. (1972) *J. Biol. Chem. 246:* 946-953.
9. BEZARES, G.; EYZAGUIRRE, J.; HINRICHS, M.V.; HEINRIKSON, R.L.; REARDON, I.; KEMP, R.G.; LATSHAW, S.P.; BAZAES, S. (1987) *Arch. Biochem. Biophys. 253:* 133-137.
10. BERGHAUSER, J. (1977) Hoppe Seyler's Z. *Physiol. Chem. 358:* 1565-1572.
11. CARDEMIL, E.; EYZAGUIRRE, J. (1979) *Arch. Biochem. Biophys. 192:* 533-538.
12. JOHNSON, S.C.; BAYLEY, T.; BECKER, R.R.; CARDENAS, J.M. (1979) *Biochem. Biophys. Res. Comm. 90:* 525-530.
13. LONBERG, N.; GILBERT, W. (1983) *Proc. Nat. Acad. Sci. USA, 80:* 3661-3665.
14. MUIRHEAD, H.; CLAYDEN, D.A.; BARFORD, D.; LORIMER, C.G.; FOTHERGILL-GILMORE, L.A.; SCHILTZ, E.; SCHMITT, W. (1986) *EMBO J. 5:* 475-481.
15. INQUE, H.; NOGUCHI, T.; TANAKA, T. (1986) *Eur. J. Biochem. 154:* 465-469.
16. LONE, Y.-Ch.; SIMON, M.-P.; KAHN, A.; MARIE, J. (1986) *FEBS. Lett. 195:* 97-100.
17. BURKE, R.L.; TEKAMP-OLSON, P.; NAJARIAN, R. (1983) *J. Biol. Chem. 258:* 2193-2201.
18. KOBERSTEIN, R.; SUND, H. (1975) *Z. Physiol. Chem. 356:* 246.
19. IKEHARA, M.; VESUGI, S.; HOSHIDA, K. (1972) *Biochemistry 11:* 830-836.
20. SARMA, R.H.; LEE, C.H.; EVANS, F.E.; YATHRINDA, N.; SUNDARALINGAM, M. (1974) *J. Am. Chem. Soc. 96:* 7337-7348.
21. OHTA, S.; TSUBOI, M.; OSHIMA, T.; YOSHIDA, M.; KAGAWA, Y. (1980) *J. Biochem. 88:* 1609-1617.
22. MUNSON, K.B. (1981) *J. Biol. Chem. 256:* 3223-3230.
23. KOBERSTEIN, R.; COBIANCHI, L.; SUND, H. (1976) *FEBS. Letters 64:* 176-180.
24. OKUYAMA, T.; SATAKE, K. (1960) *J. Biochem. 47:* 454-466.
25. CRESTFIELD, A.M.; MOORE, S.; STEIN, W.H. (1963) *J. Biol. Chem. 238:* 622-627.
26. STUART, D.i.; LEVINE, M.; MUIRHEAD, H.; STAMERS, D.K. (1979) *J. Mol. Biol. 134:* 109-142.
27. MUIRHEAD, H. (1983) *Trends Biochem. Sci. 8:* 326-330.

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Starch, adenine nucleotides and apyrase changes during potato tuber development

Cambios en almidón, nucleótidos de adenina y apirasa durante el desarrollo del tubérculo de papa

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Concentration of ATP, ADP, orthophosphate, starch, total proteins and apyrase were determined during the growth of the potato tuber (*S. Tuberosum* var. Desirée). Electrofocusing was employed for the quantitative analysis of this enzyme.

Starch, apyrase and total proteins increased progressively between the 76th and 140th days of growth. ATP differed reaching its maximum concentration at the 91st day and then decreased. ADP had a lower level than ATP, and decreased continously. Orthophosphate had an initial high concentration and then decreased to an approximatly constant value. There was no direct relation between the changes in the ATP, ADP and orthosphosphate concentration and the increasing concentration of apyrase. A possible metabolic role of apyrase has been proposed related with the diphosphate nucleotide hydrolysis connected with the turnover of starch in the plant tissue.

Antibodies against apyrase have been developed as a tool for further studies of the physiological role of this enzyme.

Apyrase (ATP-diphosphohydrolase E.C. 3.6.1.5.) is an enzyme that catalyzes the hydrolysis of pyrophosphoric bonds in the presence of bivalent metals, with sequencial release of orthophosphate (Traverso-Cori *et al.*, 1965). The adenine nucleotides (ATP and ADP) are the substrates most commonly used to measure enzymatic activity.

Apyrase is widely distributed in animal (LeBel *et al.*, 1980; Knowles *et al.*, 1983; Ribeiro *et al.*, 1985) and plant tissues (Traverso-Cori *et al.*, 1965; Vara and Serrano, 1981).

In this laboratory it has been found several forms of apyrases extracted from different varieties of *S. tuberosum* tuber. Enzymes extracted from var. Pimpernel and var. Desirée potatoes have been purified to homogeneity and characterized from a kinetical and structural point of view (Kettlun *et al.*, 1982; Mancilla *et al.*, 1984).

The purification procedure is the same for both isoenzymes (Pimpernel and Desirée). They are very similar in their molecular mass (50 kDa), metal dependence, amino acid composition (only one polypeptide chain) but they differ in their isoelectric point, in the ratio of ATPase and ADPase hydrolytic activities and in the degree of involvement of the amino acid residues in the active site. This last idea has been suggested from the inactivation studies with chemical modifiers (Kettlun *et al.*, 1982).

The purpose of this paper is the approach to the understanding of the physiological role of apyrase in the cellular metabolism of potato tuber through the establishment of several metabolites in this plant tissue.

Adenine nucleotides (ATP and ADP), inorganic phosphate, total proteins, starch and apyrase mass levels were measured during the potato development. The conception involves in this work is that all of these metabolites might be interconnected with apyrase activity.

Este artículo ha sido escrito en memoria del profesor Osvaldo Cori (Q.E.P.D.), cuya imagen como maestro y científico admiraremos y respetaremos siempre.

MATERIALS AND METHODS

Apyrase activity was followed by Pi liberation from ATP or ADP (Traverso-Cori *et al.*, 1965). Two methods of Pi determination were used: Fiske and SubbaRow (1925) and Ernster *et al.* (1950). Proteins were determined by their UV absorption (Warburg and Christian, 1941). In the homogenized tissue proteins were measured by a turbidimetric method with TCA (Stadtman and Novelli, 1951). The Lowry method (Lowry *et al.*, 1951) was used to determine the concentration of purified enzyme.

Determination of ATP and ADP content in the S. tuberosum tuber. Adenine nucleotides were extracted according to the method described by Barua *et al.* (1981). Approximatly 5 g of thin slices of peeled tuber were received in 20 ml of 2.5 M $HClO_4$ previously cooled to -10^o and homogenized in an Omni-Mixer (4 times for 15 s at maximum speed). The homogenate was centrifuged at 27,000 x g during 30 min at 4°. The pellet was washed with 5 ml of 2.5 M $HClO_4$ and centrifuged. Both supernatants were mixed, neutralized with 8 M KOH, freezed dried, suspended in H_2O and stored at -30^o.

In order to ckeck the complete recovery of nucleotide concentration in the homogenate a parallel experiment was performed adding increasing amount of nucleotides to the homogenate. The recovery varied between 93 and 108%. During this treatment apyrase was completely inactivated so it did not nodify the initial nucleotide concentration.

Adenine nucleotides were determined by enzymatic methods, ATP was measured according to Lamprecht and Trautschold (1974); ADP was determined using the technique described by Jaworek *et al.* (1974).

Starch determination in potato tuber. 2 g of sliced potatoes were boiled for 10 min in 40 mL of water: cooled and homogenized in an Omni-Mixer (15 s x 2 times); filtrated through Whatman paper N° 4 and the filtrate was used to measure starch concentration by the Iodine-Iodide Method (Plumer, 1981).

Apyrase, protein and inorganic phosphate determination in the potato tuber. 25 g of tuber were homogenized (Waring Blendor at 4°) in 100 mL of 20 mM thioglycollic acid pH 4.0, filtrated through paper (Whatman N° 4), centrifuged at 27,000 x g during 20 min. In this supernatant proteins, inorganic phosphate (Pi) and apyrase activity were measured. For electrofocusing the fraction mentioned above was freeze dried and dialized against 0.1 M NaCl.

Electrofocusing in polyacrylaminde gels. The electrofocusing procedure was done according to Pearce *et al.* (1972). Protein staining was done as described by Winter *et al.* (1977). Apyrase activity in 2 mm slices of gel was measured by the method of Ernster *et al.* (1950). Protein stained bands were quantified by densitometry in a Quick Scan Model R4-077 (Helena Laboratories).

Obtention of antibodies against apyrase of S. tuberosum var. Desirée. Three rabbits were immunized with a semipurified apyrase (var. Desirée). This apyrase preparation was 80% pure determined by electrofocusing. Serum tittle was determined by ELISA test (Voller *et al.*, 1978).

Determination of the specificity of the antisera against apyrase. After apyrase electrofocusing, the gels were cut in 2 mm slices where enzymatic activity was detected (Ernster *et al.*, 1950) and antibody reaction was followed by ELISA test (Voller *et al.*, 1978).

Inhibition of apyrase activity by antibodies. The enzyme was preincubated with logaritmic dilutions in bovine serum albumin of immunized serum. The reaction was started by adding ATP or ADP. As a control, apyrase was preincubated with not immunized rabbit serum.

RESULTS

Metabolites and apyrase activity during development of potato tuber var. Desirée.

The age of the tuber was considered as the number of days from the sow till the crop.

Slices of potatoes from different parts of the tuber were taken in order to determine if the starch and metabolites were or not homogeneously distributed. Results (not shown) indicate that starch is distributed in a relatively homogeneous way.

ATP and ADP concentrations in the tuber during development. Two shrubs of potatoes var. Desirée were cropped forthnightly, from the tenth week of sowing on. From each plant a few tubers were chosen at random and processed. Fig. 1 shows that ATP and ADP concentration are different during development. ADP level is low and decrease continously, while ATP has a maximum around the 91st day.

Starch and Pi determination during the tuber development. Pi decreased after day 76th and then stayed relatively constant (Fig. 1). These results agree with those found in the literature for *S. tuberosum* var. Kennebec and Norland (Snyder *et al.*, 1977; Quick *et al.*, 1979) which showed a high Pi concentration during the initial states of development of the tuber. This initial level disminished to a constant value which was reached between the eighth and the tenth week after sowing. On the other hand, starch increased in the first stages and afterwards stabilized as shown in Fig. 1.

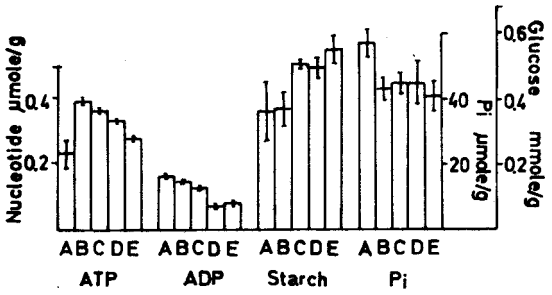

Fig. 1: Nucleotides, starch and Pi levels during potato tuber development. A) 76 days; B) 91 days; C) 106 days; D) 119 days; E) 140 days. All data are expressed per g of fresh tissue.

These results were the same as those found by the authors mentioned above, although these studies were done in different varieties of *S. tuberosum*. These same authors defined that tuber have reached maturity when the levels of starch and Pi have very small changes.

Total protein concentration during the development of the tuber. The protein content in *S. tuberosum* var. Desirée changed during the development of the tuber. Total protein concentration increased to high level until the day 120th of growth, then it stayed roughly constant (as shown in Fig. 2).

Apyrase activity determination during the tuber development. ATPase and ADPase activities were measured as a signal of apyrase presence during the tuber development. The ATPase/ADPase ratio in the extracts of Desirée tuber of different ages was roughly constant (around 1,0), this activity ratio is characteristic of apyrase obtained from potato tuber var. Desirée. Phosphomonoesterase activity was not detected in the potato extracts measured in the apyrase assay conditions.

Apyrase specific activity increased as the tuber developed (Fig. 2). This activity enhancement could be attributed to a larger mass of enzyme suggested by the experimental evidence presented below.

Apyrase quantification in potato extract by gel electrofocusing. A good linearity was found between total protein added to a electrofocusing gel and the corresponding integrated apyrase band. The shaded area in Fig. 3, represents the apyrase band detected by its enzymatic activity. The pI value of this band with apyrase activity agrees with the value obtained for a pure enzyme (var. Desirée). The relationship between the total area of the densitogram and the area corresponding to the enzyme gave the percentage of apyrase respect to the total proteins of the extract. The results of the integration of the protein bands of potato extracts in different states of development are shown in Fig. 2. These experiments support the hypothesis that the enhancement of enzymatic activity (Fig. 2) is due to an increase of the enzyme mass rather than to an increase in its activity.

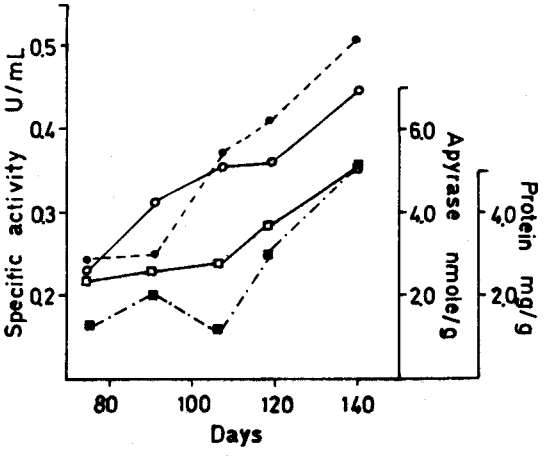

Fig. 2: Apyrase activity, protein and apyrase mass changes during potato tuber development. ATPase activity ○—○; ADPase activity ●- -●; total protein □—□; apyrase mass ■-·-■

Antibodies against apyrase

To confirm that apyrase has an increase in mass during the growth of potato tuber, antibodies against apyrase were produced in order to quantify this enzyme. The

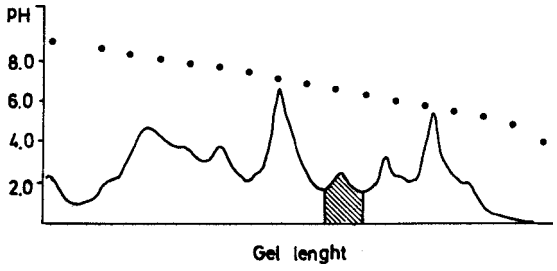

Fig. 3: Densitometric trace of a electrofocused gel of potato tuber extract. pH profile ● ●; shadowed area represents the apyrase peak.

antigenicity of the serum against apyrase was determined through ELISA test and this value was $1:2^{13}$.

The immune serum against apyrase had an inhibitory effect on both activities of the enzyme: ATPase and ADPase. Electrofocusings of apyrase of diverse stage of purification were performed. Enzymic activity and ELISA test were followed along the gels. A good correspondence between antigen-antibody reaction and enzymic activities was observed only in the purified fraction. In partially purified preparations of apyrase there was not a complete correspondence between ELISA test and the protein band with apyrase activity (Fig. 4). A possible explanation for these results is that apyrase preparation used to induce antibodies was not homogeneous. Although these antibodies developed were not completely specific against apyrase, as it is shown in Fig. 4, it was important to verify that apyrase has a good antigenic capacity in rabbit. The next step will be to employ a homogeneous preparation of apyrase to immunize rabbits.

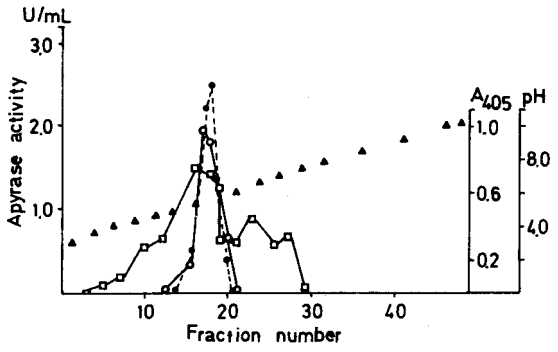

Fig. 4: Polyacrylamide gel electrofocusing of a partially purified apyrase fraction. pH profile ▲ ▲; ATPase activity ○—○; ADPase activity ●···●; A_{405} □ □, produced by the *p*-nitrophenol released in the ELISA test.

DISCUSSION

A possible role of apyrase in the tuber tissue during potato evolvement could be insert in the Leloir's pathway of starch biosynthesis. In the tuber during growth ATP increases before a rise in the starch content (Fig. 1). ATP is utilized to produce ADP-glucose reaction catalyzed by a specific pyrophosphorylase. This enzyme is allosterically regulated, Pi is an inhibitor while 3-phosphoglycerate is a positive effector (Bonner and Varner, 1976). The fact that this enzyme is allosterically inhibited by Pi means that Pi concentration must diminish in order to increase the starch synthesis; this also agrees with the results shown in Fig. 1.

Starch in the tuber is continuosly synthetized and degraded. Starch degradation is catalyzed by a phosphorylase in the presence of Pi (Bonner and Varner, 1976). This reaction must be utterly important for the utilization of the stored starch during bud formation. It is possible that apyrase could have a role at this stage because this enzyme would deliver the Pi needed for starch degradation. This proposal could be valid only for sprouting, because during tuberization apyrase enlarges four times while starch increases one and a half times.

Apyrase activity in salivary glands of blood-sucking arthropods, is correlated with the degree of inhibition of ADP-induce platelets aggregation *in vitro* (Ribeiro *et al.*, 1985). Because platelets aggregation in vertebrates largely determines haemostasis and ADP is one of the most important stimuli for this activity, ADP-degrading enzymes might serve as a universal salivary component of blood feeders.

It is possible to propose a similar role for plant apyrase related to the hydrolysis of the ADP produced along the different steps of starch synthesis. Several glycosyltransferases reactions are commonly inhibited by the resulting nucleoside diphosphate (Sadler *et al.*, 1982). Apyrase could enhance these reactions by removing inhibitory products. Both sucrose synthesis and starch synthesis in plants involve reversible reactions releasing UDP or ADP as products (Preiss, 1982) and apyrase may drive those reactions to completion. In addition, apyrase might have similar role in the biosynthesis of many sugar nucleotides. The biosynthesis of cell wall components containing various sugars are usually

catalyzed by transglucosylases that utilize as their substrates, sugar nucleotides with diphosphate nucleosides as a product (Bonner and Varner, 1976).

Recently activating and inhibitory proteins of apyrase activity have been described in our laboratory (Mancilla *et al.*, 1987). This is a further evidence for the importance of apyrase in nucleotide metabolism probably linked to carbohydrate synthesis and degradation.

REFERENCES

BARUA, K.; CHOUDHURI, M.M.; GHOSH, B. (1981) *Plant. Sci. Lett. 21:* 357-360.

BONNER, J.; VARNER, J. (1976) In *Plant Biochemistry.* Academic Press. New York.

ERNSTER, L.; ZETTERSTROM, R.; LINDBERG, O. (1950) *Acta Chem. Scand. 4:* 942-947.

FISKE, C.H.; SUBBAROW, Y. (1925) *J. Biol. Chem. 66:* 375-400.

JAWOREK, D.; GRUBER, W.; BERGMEYER, H.U. (1974) In *Methods of Enzymatic Analysis.* (Bergmeyer, H.U. Ed. 2nd Ed. Academic Press. New York. Vol. 4, pp. 2117-2131.

KETTLUM, A.M.; URIBE, L.; CALVO, V.; SILVA, S.; RIVERA, J.; MANCILLA, M.; VALENZUELA, M.A.; TRAVERSO-CORI, A. (1982) *Phytochemistry 21:* 551-558.

KNOWLES, A.F.; ISLER, R.E.; REECE, J.F. (1983) *Biochim. Biophys. Acta 731:* 88-96.

LAMPRECHT, W.; TRAUTSCHOLD, I. (1974) In *Methods of Enzymatic Analysis.* (Bergmeyer, H.U. Ed. 2nd Ed.). Academic Press. New York. Vol. 4 pp. 2101-2110.

LEBEL, D.; POIRIER, G.G.; PHANEUF, S.; ST-JEAN, P.; LALIBERTE, J.F.; BEAUDOIN, A.R. (1980) *J. Biol. Chem. 255:* 1227-1233.

LOWRY, O.H.; ROSEBROUGH, N.J.; FARR, A.L.; RANDALL, R.J. (1951) *J. Biol. Chem. 193:* 265-275.

MANCILLA, M.; KETTLUN, A.M.; VALENZUELA, M.A.; TRAVERSO-CORI, A. (1984) *Phytochemistry 23:* 1397-1400.

MANCILLA, M.; VALENZUELA, M.A.; ANICH, M.; KETTLUN, A.M.; JARA, O.; TRAVERSO-CORI, A. (1987) *Phytochemistry 26:* 2471-2475.

PEARCE, E.L.; BANKS, B.E.C.; BANTHORPE, D.V.; BERRY, A.R.; DAVIES, H.S.; VERNON, C.R. (1972) *Eur. J. Biochem. 29:* 417-425.

PLUMMER, D.T. (1981) In *Introducción a la Bioquímica Práctica.* Ed. McGraw-Hill. Latinoamericana, S.A. Bogotá, pp. 170-171.

PREISS, J. (1982) *Annu. Rev. Plant Physiol. 33:* 431-454.

QUICK, W.A.; SNYDER, J.C.; DESBOROUGH, S.L. (1979) *Potato Res. 22:* 1-17.

RIBEIRO, J.M.C.; ROSSINGNOL, P.A.; SPIELMAN, A. (1985) *J. Insect. Physiol. 31:* 689-692.

SADLER, E.; BEYER, T.A.; OPPENHEIMER, C.L.; PAULSON, J.C.; PRIEELS, J.P.; REARICK, J.I.; HILL, R.L. (1982) *Methods Enzymol. 83:* 458-514.

SNYDER, J.; DESBOROUGH, S.; HOLM, D. (1977) *Am. Potato, J. 54:* 545-555.

STADTMAN, E.; NOVELLI, G.D. (1951) *J. Biol. Chem. 191:* 365-376.

TRAVERSO-CORI, A.; CHAIMOVICH, H.; CORI, O. (1965) *Arch. Biochem. Biophys. 109:* 173-184.

VARA, F.; SERRANO, R. (1981) *Biochem. J. 197:* 637-643.

VOLLER, A.; BARTLETT, B.; BIDWELL, D. (1978) *J. Clin. Path. 31:* 507-520.

WARBURG, O.; CHRISTIAN, W. (1941) *Biochem. Z. 310:* 384-415.

WINTER, A.; EK, K.; ANDERSSON, U. (1977) LKB Application Note 250, December.

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Role of cytochrome P-450 monooxygenases in the activation of chemical carcinogens

El papel de las monooxigenasas dependientes de citocromo P-450 en la activación de cancerígenos químicos

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Several studies have pointed out that the environment is a significant determinant in the incidence of human cancer (Haenszel and Traueber, 1964), although many of the specific causal agents have yet to be identified.

Lung cancer is the most important cause of death from cancer in the world, with an estimated total deaths in excess of one million annually. It is generally accepted that the major cause of the disease is the smoking of different forms of tobacco (cigarrettes, cigars, pipe, etc.). The occurrence of malignant tumors of other parts of the respiratory tract, of the upper digestive tract, of the bladder, renal pelvis and pancreas, is also causally related to smoking (IARC Monographs, 1986). Epidemiological evidence though inconclusive, points to urban air pollution as a possible contributory agent in respiratory cancers specially in cities with high levels of smog. Polycyclic aromatic hydrocarbons (PAH) are ubiquitous pollutants of air, soil, and water and are major components of smog and cigarrette smoke. Major sources of PAH include emissions from transport systems, heat and power generation, refuse burning, and industrial process (National Academy of Science Reports, USA, 1972). One of the most abundant of this class of compounds is benzo(a)pyrene (BaP).

THE CYTOCHROME P-450 MONOOXYGENASES SYSTEM

Living organism are exposed to PAH and thousands of other hydrophobics, man-made chemicals which are not normal components of the body (xenobiotics). These compounds are metabolized to more hydrophilic products and rapidly eliminated by the excretory systems. If foreign chemicals were not metabolized and eliminated, many of them would accumulate and kill the organism.

Oxidative metabolism of xenobiotics is catalyzed by the cytochrome P-450 monooxygenase system. This system has been found in most species and organs (Lu and West, 1980), and it is particulary active in the liver. Within the cell it is located mainly in the endoplasmic reticulum, but it is also found in the nuclear membrane (Guengerich, 1979) and in the mitochondria (Takemori and Kominami, 1984). The monooxygenase system is also capable of metabolizing endogenous compounds such as steroids, fatty acids, and prostaglandins. In microsomes the system is composed by NADPH-cytochrome P-450 reductase, a flavoprotein which contains FAD and FMN as prosthetic groups. This flavoprotein reduces by a two electron transfer mechanism (White and Coon, 1980) the other component, the hemoprotein cytochrome P-450. In this system, the flavoprotein and the cytochrome P-450 are tightly attached to the membrane and their interaction requieres the presence of phosphatidylcholine.

The cytochrome-P-450 is the site of substrate binding, molecular oxygen activation and substrate hydroxylation. This terminal oxidase catalyzes the insertion of one oxygen atom in the substrate, while the other is incorporated to water. The

monooxygenase system exhibits unusually broad substrate specificity which is related to the presence of multiple forms (isozymes) of cytochrome P-450 (reviewed by Waxman, 1986). The relative amounts of the individual cytochrome P-450 isozymes are markedly dependent on the sex, species, age, hormonal and genetic constitution, nutritional status as well as prior exposure to inducers. It is generally accepted that the different forms of cytochrome P-450 represent separate gene products, rather than derivatives of a common precursor protein and, therefore, constitute a set of isozymes.

The oxidative xenobiotics pathway is usually a detoxification process since the products are generally pharmacologically inactive and rapidly excreted. However, sometimes, the system works as a double-edged sword, given rise to highly reactive electrophilic metabolites capable of covalent interaction with critical macromolecules such as proteins, DNA and RNA. The latter is an activation process and the biologically active metabolites exert profound cellular effects including, mutagenicity, cytotoxity, and malignant transformation (Fig. 1). The predominancy of either the detoxification or activation pathways might result in the fact that the carcinogenic activity of the PAH can be abolished, diminished or enhanced by metabolism (Gelboin, 1967; Gelboin, 1980). The alteration in specific P-450 activity can also result in changes in the relative predominance of activation versus detoxification pathways.

The oxidative products of xenobiotics metabolism by the monooxygenases system usually are substrates for other enzymes which catalyze conjugative or synthetic reactions between the newly-formed functional groups and small-molecular weight endogenous compounds derived from carbohydrates, aminoacids, or other polar groups (Caldwell, 1982). Most of these conjugates, ionized at physiological pH as glucuronides, sulphate esters, and glutathione derivatives, are readily excreted and usually they behave as pharmacologically inactive compounds.

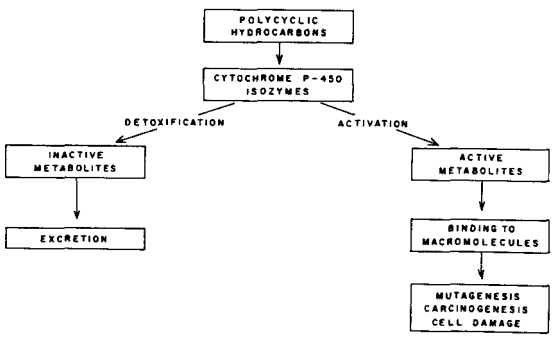

Fig. 1: Polycyclic aromatic hydrocarbons metabolism. Fate of the oxidation products.

METABOLISM OF POLYCYCLIC HYDROCARBONS

The PAH are metabolized by the cytochrome P-450 monoxygenase system to epoxides, phenols, dihydrodiols and quinones, many of these metabolites are also conjugated with UDPGA, glutathione and PAPS. These metabolic interrelationships are illustrated inf Fig. 2 for BaP. This hydrocarbon is initially oxidized by cytochrome P-450 to several arene oxides. These compounds may rearrange spontaneously to phenols or undergo hydration to trans-dihydrodiols in a reaction mediated by microsomal epoxide hydratase (E.C.4.2.1.63.). Cytochrome P-450 also catalyze the direct formation of phenols from the parent compound. One of these phenols the 6-OH BaP is oxidized spontaneously or metabolically to several quinones. Others however such as 9-OH BaP are oxidized to the 4,5-oxide, which on hydrated to the corresponding dihydrodiol. Dihydrodiols are also P-450

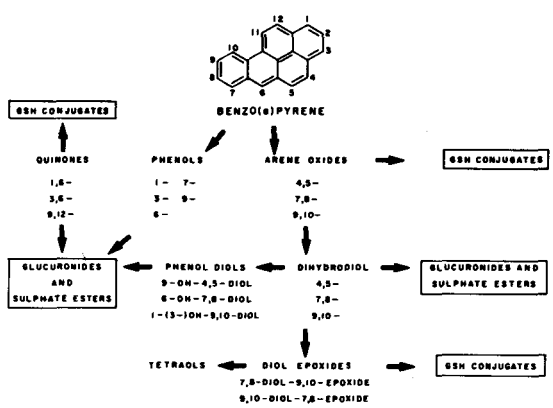

Fig. 2: Metabolic pathways of Benzo(a)Pyrene.

substrates to give the diol epoxides, which are further hydrolyzed spontaneously to tetrols. Phenols, quinones and dihydrodiols can be further conjugated to glucuronides and sulphate esters. Whereas arene oxides, diol epoxides and quinones can also be conjugated with glutathione by a reaction catalyzed by a cytosolic enzyme, the glutathione S-transferase (E.C.2.5.1.18). Thus, BaP and PAH metabolism is mediated by several sequential transformations which lead to activation or detoxification products.

THE IMPORTANCE OF THE STEREOSELECTIVE METABOLISM OF PAH IN THE EXPRESSION OF THEIR BIOLOGICAL ACTIVITY

Studies of several PAH have either shown or implicated bay-region diol epoxides as their ultimate carcinogenic metabolites (Levin *et al.*, 1982; Sims and Grover, 1981). A bay-region occurs in a PAH when and angulary-fused benzo-ring is present, for example, the sterically-hindered region between the 10- and 11- positions constitutes the bay-region of BaP (Fig. 2). When formed in mammals, these bay-region diol epoxides consist of enantiomeric pairs of diastereoisomers in which the benzylic hydroxyl group is either cis (diol epoxide 1) or trans (diol epoxide-2). Thus, since each diastereoisomer can be resolved in two enantiomers a total of four stereoisomers are possible (Fig. 3). All these diol epoxides stereoisomers have been synthesized for BaP, benz(a)anthracene, benzo(c)phenanthrene and chrysene and evaluated as mutagenic and tumorigenic agents. Tumor studies in newborn and adult mice have established that the isomer with RS- diol SR- epoxide absolute configuration has the highest tumorigenic activity among the four stereoisomers, for all four hydrocarbons. Furthermore, this is the predominant isomer formed from the parent hydrocarbon by the stereoselective action of one P-450 isozyme (P-450 c) and epoxide hydratase in rat liver (Thakker *et al.*, 1977; Vyas *et al.*, 1982), and it is the predominant isomer found covalently bound to DNA in a variety of

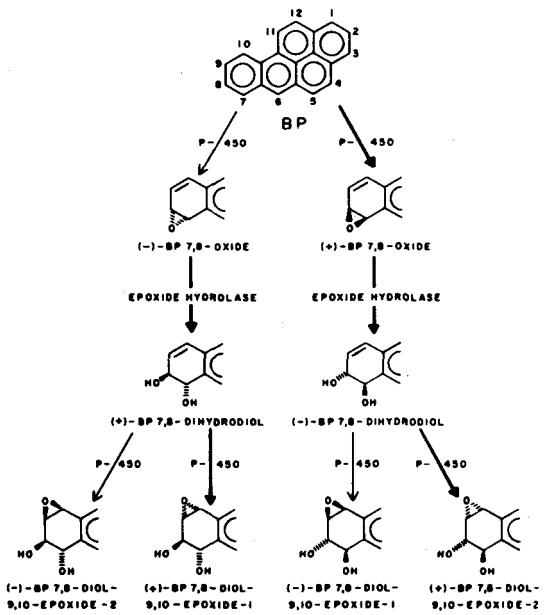

Fig. 3: Stereoselectivity of the 7,8-diol-9,10-epoxides derivatives from Benzo(a)Pyrene.

mammalian cells and organs exposed to these hydrocarbons. Recent studies have indicated that bay-region diol epoxides are ultimate carcinogenic metabolites for about a dozen of PAH.

On the other hand, metabolic studies with PAH as benzo(e)pyrene which is attacked by the monooxygenase system mainly at the K- region instead of the bay-region (Fig. 4), have shown that this parent compound is almost completely inactive as carcinogen (Slaga *et al.*, 1980). However, it must be clear that the fact that the P-450 catalyzed formation of bay-region diol-epoxides leads to the most biologically

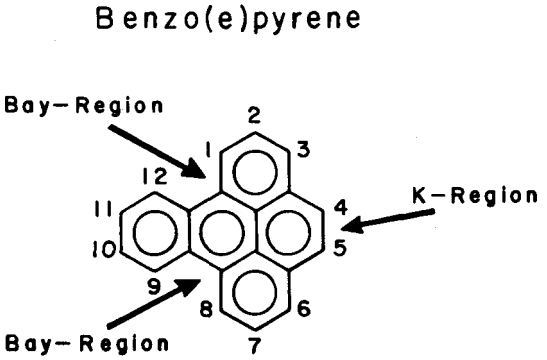

Fig. 4: Structure of Benzo(e)Pyrene showing the bay region and K-region.

active compounds, does not preclude the existence of other ultimately carcinogenic metabolites of PAH. Furthermore, not all carcinogenic hydrocarbons have bay-regions. Another interesting finding reported by Thakker *et al.*, 1981; shows that the addition of 7,8- benzoflavone to human and rabbit microsomal incubations enhanced the formation of bay-region diol epoxides with high mutagenic activity from B(e)P. This is a clear example of a cosubstrate altering the extent to which a PAH is metabolized *in vitro* along a specific pathway. This type of metabolic alterations may contribute to the cocarcinogenic effects induced by certain chemicals. An even more attractive possibility is that compounds might be found which could divert metabolism of carcinogens along pathways other than those which lead to ultimate carcinogenic metabolites. Along this line, Safirman *et al.*, 1987, have tested the effect of several antioxidants as carcinogenic inhibitors.

METABOLIC FATE OF POLYCYCLIC AROMATIC HYDROCARBONS IN THE CELL

The most studied site of metabolic activation of these xenobiotics is the endoplasmic reticulum, but metabolism of PAH has been found in isolated nuclei. Active metabolites can also reach other cellular organelles as mitochondria. Since the target (DNA, RNA and nuclear proteins) is closer to nuclear membranes than to endoplasmic reticulum enzymes, the production of reactive metabolites of PAH by the nuclei membrane might be a key step in their availability to target sites. Thus, problems like stability of reactive metabolites produced by the endoplasmic reticulum and transport by carrier proteins can be overcome by the close proximity between the nuclear membrane and the genetic material.

Results obtained by Salazar *et al.*, 1985, and presented in Table 1, show that nuclei purified from livers of untreated rats have activity to oxidate BaP *in vitro*. But, if rats are injected with BaP, the liver nuclei show a dramatic enhancement in the BaP oxidation activity. The increase after hydrocarbon injection is much higher in the nuclei than in the microsomal fraction. This difference is not due to a higher activity of cytochrome P-450 reductase in the nuclear membranes of rats injected with BaP, since it was determined that the specific activity of this enzyme in nuclear membrane of treated animals is 80% lower than that of microsomes from the same animal. From these results it is suggested that the nuclear membranes preferentially produce active metabolites and/or these metabolites are detoxified at a lower rate. This idea is supported by studies of Lesko *et al.*, 1978, which clearly demonstrated that nuclear membranes produce a ratio of B(a)P-quinones/B(a)P-phenols equal to 3.9 whereas this ratio is only 0.5 in microsomes.

It might be pointed out that these results are in agreement with the idea that

TABLE 1

Benzo(a)pyrene oxidation activity in liver nuclei and microsomes from normal and B(a)P-treated rats*

In vivo treatment	[ηmoles B(a)P metabolized x hr^{-1} x (mg protein)$^{-1}$] MICROSOMES	NUCLEI
Control (6)	6.80 ± 0.37	0.34 ± 0.02
B(a)P (5)	26.49 ± 4.21	3.25 ± 0.12

* The hydrocarbon was injected in one single dose i.p. (40 mg/kg body wt) 48 hr before the animals were killed. Values are the mean ± S.D. in different subcellular preparations. The number of animal groups is indicated in brackets (four animals in each group).

BaP oxidation activity present in purified nuclei might be involved in the inhibition of DNA synthesis by BaP and others PAH. This is supported by the more pronounced inhibition of DNA synthesis observed in nuclei from BaP treated rats, when the incubation was performed in the presence of NADPH, as is shown in Fig. 5. Furthermore, the same figure indicates that this cofactor of monooxygenase activity has no effect on nuclei from control animals. On the other hand, the incubation of nuclei from untreated rats with BaP alone, show small inhibition of DNA synthesis as compared with incubation in the presence of the hydrocarbon plus NADPH (Fig. 6). It has been suggested that the microsomal activation system is involved mainly in the process of xenobiotic detoxification whereas the nuclear oxidation activity might be related to the effect of PAH on the expression of the nuclear genome (Gelboin, 1980). These results strongly support this idea.

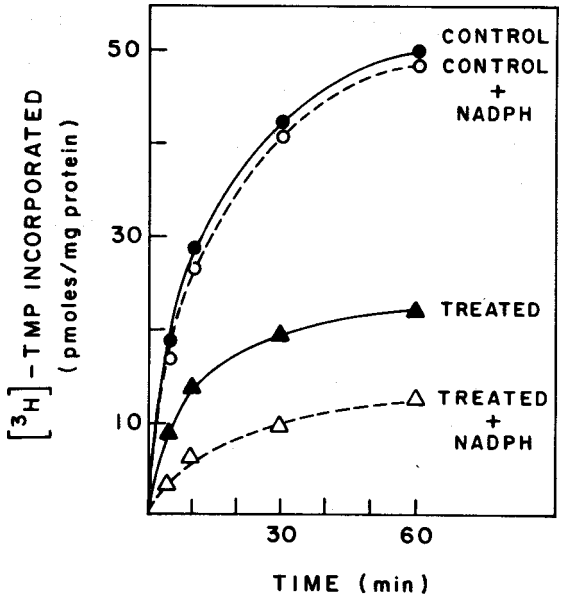

Fig. 5: Kinetics of DNA synthesis in intact nuclei purified from BaP-treated and control rats, in the absence and presence of NADPH. NADPH concentration was 1.2 mg/ml. DNA values were: 0.48 mg DNA/mg protein (control) and 0.51 mg DNA/mg protein (BaP-treated). The data represent the mean of four experiments in four differents nuclear preparations with duplicate determination in each experiment. Standard deviation of the mean was always least than 10% of the values.

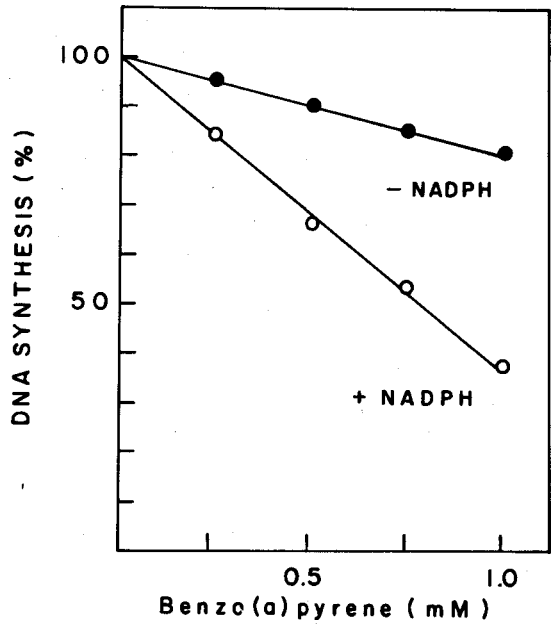

Fig. 6: In vitro effect of BaP on DNA synthesis in nuclei purified from control rats in the presence and absence of NADPH (1.2 mg/ml). The [3H]TMP incorporation in the absence of BaP (100% activity) was 51 pmoles x mg protein $^{-1}$ x h^{-1}.

An interesting finding can be observed, when animal cells are incubated with radiactively labeled BaP, hydrocarbon metabolites are covalently linked to both nuclear and mitochondrial DNA, although the amount of the adduct with DNA is higher in the mitochondrial genome (Backer and Weinstein, 1980; Allen and Coombs, 1980). In agreement with these results, it was found in our laboratory, that DNA synthesis in liver mitochondria purified from BaP treated rats is 65% lower than in those purified from untreated animals (Salazar *et al.*, 1982), and in constrast to the nuclear system, this inhibition of mitochondrial DNA synthesis was not dependent of NADPH.

In this same line of research, Oesch *et al.*, 1985, using HPLC have studied the BaP metabolites formed by rough and smooth endoplasmic reticulum, nuclei, plasma membrane, as well as mitochondrial fractions of rat liver. They have reported that the metabolic profiles produced by the smooth and rough endoplasmic reticulum were similar to each other, but different from those produced by the other three subcellular fractions. The metabolite pattern produced by nuclear

fractions differed slightly from that produced by the endoplasmic reticulum, but plasma membrane and mitochondria produced markedly different patterns. These results might indicate differences in the patterns of citochrome P-450 isozymes and/or differential control of the various metabolic pathways in the individual cellular membranes.

MODULATION OF POLYCYCLIC HYDROCARBON METABOLISM BY THE NUTRITIONAL STATUS

The relationship between nutritional status and microsomal PAH oxidation is poorly understood in molecular terms, although the effects of short fasting periods (Wiebel and Gelboin, 1975) or low- and high-protein diet have been reported (Clinton *et al.*, 1979; Hietanen, 1980). In our laboratory, we have studied the nutritional influence in the cytochrome P-450 monooxygenase activity towards chemical carcinogens as BaP (Salazar *et al.*, 1983). Table 2 shows that protein-energy malnutrition decreases BaP oxidation activity to almost undetectable levels and shift maximal wavelength absoption of the CO-reduced cytochrome P-450 spectra to 452 nm. Refeeding with a protein diet, drastically enhanced BaP microsomal oxidative activity to levels close to those of rats fed a normal diet and shift the CO reduced cytochrome P-450 spectra to 451 nm after 4 days of refeeding and to 450 nm after 6 days. It is also interesting to note in the same Table that BaP administration rapidly induce BaP microsomal oxidation in malnourished rats and shift the reduced cytochrome P-450 spectra to 448 nm. These data and other evidence suggest that a deficit of protein for a long period of time provoke drastic changes in cytochrome P-450 isozymes patterns as well as in BaP microsomal oxidation activity. The murine Ah locus controls the induction of many drugs and carcinogens metabolizing enzymes. It is possible that under this extreme nutritional condition, some forms of cytochrome P-450 species related to BaP oxidation activity are either not synthesized or synthesized at a lower rate. Another possibility is that the rate of protein degradation is enhanced. Thus, at least some structural gene products of the Ah murine complex are sensitive to the protein deficiency. However, the regulatory gene product, the cytosolic receptor, is still responsive since an important induction of BaP oxidation activity is triggered by the PAH injection to malnourished rats. Therefore, a poor nutritional state does not exclude the possibility of BaP oxidation mediated by exposure to environmental pollutants. The increase in BaP oxidation activity and the apparent

TABLE 2

Changes on Benzo(a)pyrene oxidation activity and on the maximal absorption of the CO-reduced cytochrome P-450 complex of liver microsomes from rats in different nutritional status.

Nutritional Status[a]	λ max in CO-P450 spectra	Benzo(a)pyrene oxidation (ηmol x h^{-1} x mg $protein^{-1}$)
(1) Normal fed	450	6.80 ± 0.37[c]
(1) Malnourished	452	0.03 ± 0.007
(2) Re-fed 4 days	451	3.33 ± 0.70[c]
(3) Re-fed 6 days	450	4.88 ± 0.51[c]
(4) Re-fed 15 days	450	5.97 ± 0.30[c]
(1) Malnourished + B(a)P[b]	448	7.34 ± 1.20[c]

[a] Animals in different nutritional status (5 rats for each group) were sacrified at age of (1) 35 d, (2) 39 d, (3) 41 d, (4) 50 d.

[b] Malnourished rats received a single ip injection of B(a)P 40 mg/kg, 48 h prior to sacrifice.

[c] Significantly different from corresponding mean of 35-d-old malnourished rats, $p > 0.001$.

change in cytochrome P-450 after refeeding malnourished rats with a protein diet, may be interpreted in terms of the protein diet triggering the renewed synthesis of cytochrome P-450 isozymes involved in BaP oxidation activity.

Studies on the purification and properties of multiple cytochrome P-450 isozymes have been carried out in several laboratories in order to evaluate the contribution of different isozymes to the oxidation of endogenous and xenobiotics substrates. At least 10 different isozymes have been purified and characterized from rat liver (Waxman, 1986).

Cytochrome P-450c and cytochrome P-450d are major forms of P-450 that are inducible by 3-methylcholanthrene (3-MC), beta-naphthoflavone (B-NF) and by PAH (Thomas *et al.*, 1983; Morville *et al.*, 1983). These two forms show partial immunological cross-reactivity with each other, but they have different substrate specifities and different NH_2 terminal sequences. Benzo(a)pyrene is most efficiently metabolized by P-450c than any other P-450 isozyme (Conney, 1986). Our group has recently purified to homogeneity a P-450 isozyme from protein-energy malnourished rats induced with B-NF (Gil *et al.*, 1988). The purification steps included chromatography on DEAE-Sephadex-A-25, DEAE-cellulose(DE-53), hydroxylapatite and carboxymethyl-sepharose. This isozyme shows a reduced carbon monoxide difference and absolute spectra with a peak at 446.5 nm, appears to have a low spin ferric iron, migrates as a single band of apparent molecular weight 56,000 in sodium dodecyl sulfate poliacrylamyde gels, and has an identical NH_2 terminal composition to cytochrome P-450c. P-446 oxidizes various substrates at different rates in a reconstituted system with NADPH cytochrome c reductase and dilaurylphosphatidylcholine. Thus, benzphetamine and testosterone are poorly oxidized (1.85 and 1.42 nmoles product/min/nmol of cytochrome P-446), whereas BaP is oxidized at a extremely high rate (81.1 nmoles product/min/nmol cytochrome P-446). An interesting finding was observed by HPLC quantitation of various BaP oxidation products and their relative contribution to the total metabolites. Dihydrodiols comprise 15.9%, unknown products 11.9%, phenols 19.5% and quinones 52.8%. A form of P-450 induced by B-NF has been isolated from rabbits exhibiting high BaP oxidation activity with a ratio phenol/quinones of 1 (Vatsis, *et al.*, 1980). Similarly, a form of P-450 has been isolated from rat liver which oxidize BaP with a phenol/quinone ratio of 1.95 (Gozukara *et al.*, 1982), P-446 isolated from protein-energy malnourished rats induced with B-NF, oxidizes BaP with a phenol/quinone ratio of 0.37. Further work is now in progress in our laboratory to establish which are the similarities and differences between this P-446 isozyme and a P-446 isozyme isolated from normal fed rats (Saito and Strobel, 1981; Lau and Strobel, 1982).

CONCLUDING REMARKS

Genetic, dietary and environmental factors have influence in the level of cytochrome P-450 isozymes. PAH and other xenobiotics induce the synthesis of specific cytochrome P-450 isozymes that metabolize the inducer or other chemicals. Many of these compounds can stimulate the metabolism of endogenous substrates and xenobiotics either by detoxification or activation pathways. An important challenge for the future will be to develope methods for quantifying the concentrations of each P-450 isozymes in different individuals and in this way to establish their risk towards chemically induced cancers. It is expected that in the next years medical techniques will allow optimization P-450 levels for the detoxification of environmental chemicals. It is likely that highly selective therapeutic inducers of P-450 isozymes will be found for the treatment of some human diseases. Recombinant DNA technology offers interesting possibilities such as to control diseases involving impaired or excessive expression of cytochrome P-450 genes that participate in the metabolism of endogenous substrates, or to induce cloned P-450 genes in micro-

organisms for metabolizing toxic chemical wastes to nontoxic products in order to improve our environment; or to realize highly stereoselective synthesis of pharmaceutical drugs.

We can not finish this contribution to the memory of our dear professor, colleague and unforgetable friend Osvaldo Cori without strengthening the fact that the mortality rate of the Chilean population to several types of cancer triggered by environmental factors such as tobacco smoking and air pollution, has drastically increased in the last 19 years. Thus, the mortality rate of larynx, trachea, bronchial, and lung cancer was enhanced by 84% between 1966 and 1985 (Anuarios de Demografía, INE, Ministerio de Salud, Chile). Since the production of cigarrettes has diminished in this country in the last years (Indice de Producción y Venta Física de Industrias Manufactureras, INE, Chile, Vol. 1-12), and the number of vehicles, specially those with diesel motors has increased (Anuario Estadístico de Transporte Terrestre, Ministerio de Transportes y Telecomunicaciones, Chile, 1985), we must be concern about smog contamination at least in Santiago. We must be aware of the levels of PAH in urban air and dictate the urgently needed regulations to diminish vehicle contamination. At the same time epidemiological and basic research on this subject should be estimulated.

ACKNOWLEDGMENTS

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REFERENCES

ALLEN, J.A. and COOMBS, M.M. (1980) Covalent binding of polycyclic aromatic compounds to mitochondrial and nuclear DNA. *Nature, 287:* 244-245.

BAKER, J.M. and WEINSTEIN, I.B. (1980) Mitochondrial DNA is a major cellular target for dihydrodiol-epoxide derivative of benzo(a)pyrene. *Science, 209:* 297-299.

CALDWELL, J. (1982) Conjugation reactions in foreign-compound metabolism: Definition, consequences and species variations. *Drug Metab. Rev., 13:* 745-777.

CLINTON, S.L.; TRUEX, C.R. and VISEK, W.J. (1979) Dietary protein aryl hydrocarbon hydroxylase and chemical carcinogenesis in rats. *J. Nutr., 109:* 55-62.

CONNEY, A.H. (1986) Induction of microsomal cytochrome P-450 enzymes: The first Bernard D. Brodie lecture at Pennsylvania State University. *Life Sciences, 39:* 2493-2518.

GELBOIN, H.V. (1967) Carcinogenesis, enzyme induction and gene action. *Adv. Cancer Res., 10:* 1-81.

GELBOIN, H.V. (1980) Benzo(a)pyrene metabolism, activation and carcinogenesis: Role and regulation of mixed function oxidases and related enzymes. *Physiol. Reviews, 4:* 1107-1165.

GIL, L.; VASQUEZ, H.; ORELLANA, M.; SELKIRK, J.; WOLD, F. and STROBEL, J. (1988) Purification and characterization of liver cytochrome P-446 isolated from proteins energy malnourished rats. *Mol. Cell Biochem. 79:* 5-16.

GOZUKARA, E.M.; GUENGERICH, F.P.; MILLER, H. and GELBOIN, H.V. (1982) Different patterns of benzo(a)pyrene metabolism of purified cytochromes P-450 from methylcholanthrene, beta-naphthoflavone and phenobarbital treated rats. *Carcinogenesis, 3:* 129-133.

GUENGERICH, F.P. (1979) Similarity of nuclear and microsomal cytochromes P-450 in the *in vitro* activation of aflatoxin B1. *Biochem. Pharmacol., 28:* 2883-2890.

HAENSZEL, W. and TRAUEBER, K.E. (1964) Lung cancer mortality as related to residence and smoking histories. II. White females. *J. Natl. Cancer Inst., 32:* 803.

HIETANEN, E. (1980) Modification of hepatic drug metabolizing enzyme activities and their induction by dietary protein. *Gen. Pharmacol., 11:* 443-450.

IARC MONOGRAPHS (1986) Evaluation of the carcinogenic risk of chemicals to humans. Tobacco smoking. WHO, *Vol. 38:* 421.

LAU, P.P. and STROBEL, H.W. (1982) Multiple forms of cytochrome P-450 in liver microsomes from beta-naphthoflavone-pretreated rats. Separation, purification and characterization of five forms. *J. Biol. Chem., 257:* 5257-5262.

LESKO, S.A.; LORENTZEN, R.J. and TS O.P.O.P. (1978) Benzo(a)pyrene Metabolism: One electron pathways and the role of nuclear enzymes. In Polycyclic Hydrocarbons and Cancer. (Eds. H.V. Gelboin and P.O.P. Ts O.). *Vol. 1:* 261-269. Academic Press, New York.

LEVIN, W.; WOOD, A.; CHANG, R.; RYAN, D.; THOMAS, P.; YAGI, H.; THAKKER, D.; VYAS, K.; BOYD, C.; CHUG, S-Y.; CONNEY, A. and JERINA, D. (1982) Oxidative metabolism of polycyclic aromatic hydrocarbons to ultimate carcinogens. *Drug Metab. Rev., 13:* 555-580.

LU, A.Y.H. and WEST, S.B. (1980) Multiplicity of mammalian microsomal cytochrone P-450. *Pharmacol. Rev., 31:* 277-295.

MORVILLE, A.L.; THOMAS, P.; LEVIN, W.; REIK, L.; RYAN, D.E.; RAPHAEL, C. and ADESNIK, M. (1983) The accumulation of distinct mRNAs for the immunochemically related cytochrome P-450c and P-450d in rat liver following 3-MC treatment. *J. Biol. Chem., 258:* 3901-3906.

NATIONAL ACADEMY OF SCIENCE REPORTS, USA (1972) Particulate Polycyclic Organic Matter. Committee on Biological Effects of Atmospheric Pollutants, *Div. Med. Sci., Natl. Res. Counc.*, Washington, D.C.

OESCH, F.; BENTLEY, P.; GOLAN, M. and STASIECKI, P. (1985) Metabolism of benzo(a)pyrene by subcellular fractions of rat liver; evidence for similar patterns of cytochrome P-450 in rough and smooth endoplasmic reticulum but not in nuclei and plasma membrane. *Cancer Res., 45:* 4838-4843.

SAFIRMAN, C.; STOICA, G.; LUPU, F.; VOICULETZ, N. and NICULESCU-DUVAZ, I. (1987) *In vitro* screening of carcinogenesis inhibitors acting by inhibition of microsomal polycyclic aromatic hydrocarbon activation. *Neoplasma, 34:* 261-268.

SAITO, T. and STROBEL, H.W. (1981) Purification to homogeneity and characterization of a form of cytochrome P-450 with high specificity for benzo(a)-pyrene from beta-naphthoflavone prretreated rat liver microsomes. *J. Biol. Chem.: 256:* 984-988.

SALAZAR, I.; TARRAGO-LITVAK, L.; GIL, L. and LITVAK, S. (1982) The effect of Benzo(a)pyrene on DNA synthesis and DNA polymerase activity of rat liver mitochondria. *FEBS Letters, 138:* 45-49.

SALAZAR, I.; LITVAK, S. and GIL, L. (1983) Modulation of rat liver aryl hydrocarbon (benzo(a)pyrene) hydroxylase activity by nutritional effects. *Journal of Toxicology and Environmental Health, 11:* 519-533.

SALAZAR, I.; TARRAGO-LITVAK, L.; LITVAK, S. and GIL, L. (1985) Effect of benzo(a)pyrene on DNA synthesis and DNA polymerase activity of rat liver nuclei. *Biochem. Pharmacol., 34:* 755-762.

SIMS, P. and GROVER, P.L. (1981) Involvement of dihydrodiols and diol epoxides in the metabolic activation of polycyclic hydrocarbons other than benzo(a)pyrene. In Polycyclic Hydrocarbons and Cancer (Gelboin, H.V., and Ts O.P.O.P., eds). *Vol. 3:* 117-181. Academic Press, New York.

SLAGA, T.J.; GLEASON, G.L.; MILLS, G.; EWALD, L.; FU, P.O.; LEE, H.M. and HARVEY, R.G. (1980) Comparison of skin tumor-initiating activities of dihydrodiols and diol epoxides of various polycyclic hydrocarbons. *Cancer Res., 40:* 1981-1984.

TAKEMORI, S. and KOMINAMI, S. (1984) The role of cytochromes P-450 in adrenal steroidogenesis. *TIBS, 9:* 393-396.

THAKKER, D.R.; YAGI, H.; AKAGI, H.; KOREEDA, M.; LU, A.Y.H.; LEVIN, W.; WOOD, A.W.; CONNEY, A.H. and JERINA, D.M. (1977) Metabolism of benzo(a)pyrene. VI Stereoselecte metabolism of benzo(a)-pyrene and benzo(a)pyrene-7,8-dihydrodiol to diol epoxides. *Chem. Biol. Interact., 16:* 281-300.

THAKKER, D.R.; LEVIN, W.; BUENING, M.; YAGI, H.; LEHR, R.E.; WOOD, A.W.; CONNEY, A.H. and JERINA, D.M. (1981) Species specific enhancement by 7,8-benzoflavone of hepatic microsomal metabolism of benzo(e)pyrene 9,10-dihydrodiol to bay region diol epoxides. *Cancer Res., 41:* 1389-1396.

THOMAS, P.E.; REIK, L.M.; RYAN, D.E. and LEVIN, W. (1983) Induction of two immunochemically related rat liver cytochrome P-450 isozymes, cytochrome P-450c and P-450d, by structurally diverse xenobiotics. *J. Biol. Chem., 258:* 4590-4598.

VATSIS, K.P.; DEUTSCH, J.; GELBOIN, H.V. and COON, M.J. (1980) Catalytic activity and stereospecificity of phenobarbital-inducible and 5,6-benzoflavone-inducible isozymes of liver microsomal cytochrome P-450 in the oxygenation of benzo(a)pyrene and trans-7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene. In: *Mcrosomes Drug Oxidations and Chemical Carcinogenesis:* 1065-1080, Academic Press, Inc., N.Y.

VYAS, K.P.; LEVIN, W.; YAGI, H.; THAKKER, D.R.; RYAN, D.E.; THOMAS, P.; CONNEY, A.H. and JERINA, D.M. (1982) Stereoselective metabolism of the (+) and (−) enantiomers of trans-1,2-dihydroxy-1,2- dihydrochrysene to bay-region 1,2-diol-3,4-epoxide diastereomers by rat liver enzymes. *Mol. Pharmacol., 22:* 182-189.

WAXMAN, D.J. (1986) Rat Hepatic Cytochrome P-450. Comparative Study of Multiple Isozymic Forms. In: *Cytochrome P-450 Structure, Mechanism, and Biochemistry.* Ortiz de Montellano, P.R. ed. Plenum Press, 525-539.

WHITE, R.E. and COON, M.J. (1980) Oxigen activation by cytochrome P-450. *Annu. Rev. Biochem., 49:* 315-356.

WIEBEL, F.J. and GELBOIN, H.V. (1975) Aryl hydrocarbon (Benzo(a)pyrene) hydroxylases in liver from rats of different age, sex and nutritional status. *Biochem. Pharmacol., 24:* 1511-1515.

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Proteolysis of mitochondrial-coded and nuclear-coded proteins found in yeast mitochondria

Proteolisis en mitocondria de levadura de proteínas codificadas por mitocondria y núcleo

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The rate of degradation of radioactive labeled mitochondrial proteins synthesized both *in vitro* and *in vivo* by isolated yeast mitochondria and growing yeast cells respectively, has been studied. It was found that the *in vitro*-synthesized mitochondrial proteins are rapidly degraded by an energy-dependent proteolytic system. Under the same experimental conditions the *in vivo*-synthesized mitochondrial proteins are slowly degraded to a limited extent by a protease which is slightly inhibited by ATP. During this period, the mitochondria are coupled and metabolically active. It is proposed that mitochondria posses an energy-dependent proteolytic system that recognizes as substrates either "abnormal" proteins or unassembled protein subunits encoded in the mitochondrial genome. An apparently different system, which is independent of energy, seems to be responsible for the slow and limited degradation of "normal" mitochondrial proteins.

INTRODUCTION

Mitochondria (along with chloroplasts) occupy a unique position among cellular organelles because of their possession of a separate genome and all the enzymatic machinery for transcribing and translating the genetic information into functional proteins. The majority of mitochondrial proteins, however, are encoded in the nuclear genome and are synthesized on cytoplasmic ribosomes and therefore need to be transported to one of the four internal compartments of the organelle. Some of these proteins are subunits of the respiratory enzymes or the ATPase complex which form active enzymes when properly assembled with subunits encoded in the mitochondrial genome. The very fact that genetic information for mitochondria biogenesis is distributed among two spatially separated compartments implies the existence of mechanisms for ensuring a coordinate expression of the proteins and/or RNAs encoded in the two genomes. These mechanisms may conceivable regulate gene expression at the level of transcription, processing of RNAs, translation, postranslation, assembling of subunits or degradation of proteins. The latter level appears to be an extensive and highly selective process since proteins, including mitochondrial proteins, are degraded at widely different rates. Mitochondria of different sources have been reported to contain an ATP-dependent proteolytic system that appears to degrade, preferentially, polypeptides with abnormal structure or synthesized by isolated mitochondria or by growing cells in the presence of an inhibitor of the cytoplasmic protein synthesis system. Thus, Luzikov *et al.* (1-4) have characterized an energy-dependent proteolytic activity of yeast mitochondria that cleaves mitochondrial translation products synthesized

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in vivo in the presence of cycloheximide. Goldberg *et al.* (5, 6) have reported an ATP-dependent proteolytic system of liver mitochondria that degrades proteins synthesized by isolated mitochondria and, even more extensively, the polypeptides synthesized in the presence of puromycin. We published previously, that a similar system of yeast seems to be responsible for the rapid elimination of proteins synthesized by isolated mitochondria in the presence of an amino acid analog (7). In the present study we wish to report additional detailed experiments on the rate of degradation of mitochondrial proteins synthesized both *in vitro* and *in vivo* by isolated yeast mitochondria and by growing yeast cells respectively.

MATERIALS AND METHODS

Saccharomyces cerevisiae, strain D273-10B (ATCC 24657), was obtained from the American Type Culture Collection, Rockville, MD, USA. Yeast extract was from Difco, Detroit, MI, USA. Zymolyase 5,000 or 60,000 was purchased from Miles Scientific, Naperville, IL, USA and Scintillation cocktail from Research Products International Corp., Mount Prospect, IL, USA. Mannitol, sorbitol, amino acids, phosphoenolpyruvate, pyruvate kinase (rabbit muscle type II), α-ketoglutarate, ADP and ATP were obtained from Sigma Chemical Co. St. Louis, MO, USA. ^{3}H-Leu (NET-460) and ^{35}S-Met (NEG-009 T) were obtained from New England Nuclear, Boston, MA, USA.

Growth, labeling of cells and isolation of mitochondria

Yeast cells were grown at 30°C in a semisynthetic medium containing per liter: galactose, 10 g; yeast extract, 3 g; NaCl, 0.5 g; $MgSO_4.7H_2O$, 0.7 g; $CaCl_2$, 1 g; $(NH_4)_2SO_4$, 0.8 g and $FeCl_3.6H_2O$, 5 mg. For labeling with $^{35}SO_4^{=}$, the above medium was supplemented with 8 mCi of carrier-free $^{35}SO_4^{=}$/liter. Cell growth was followed turbidimetrically at 540 nm. When cell density reached 8 x 10^7 cells/ml (OD_{540} = 2.25) they were harvested by centrifugation (10 min at 2,500 x g). The mitochondrial fraction was obtained by a previously described procedure (7, 8). The oxidation capacity and the degree of coupling of mitochondria were checked according to Téllez *et al.* (7). The method of Lowry (9) was used to determine proteins.

In vitro labeling of mitochondrial proteins

Isolated mitochondria (0.25 mg) (labeled or not labeled *in vivo* with $^{35}SO_4^{=}$) in a final volume of 0.5 ml were incubated according to McKee *et al.* (10, 11) except that the Tris-HCl buffer concentration was 50 mM, pH 7.2 and the amino acids tyrosine, asparagine and glutamine were not included in the protein synthesizing mixture. Either, 40 μCi of ^{3}H-leucine (123 Ci/nmol) or 110 μCi of ^{35}S-methionine (1,223 Ci/mmol) were used a radioactive precursors. The kinetics of incorporation of radioactivity into mitochondrial proteins were followed by spotting, every 5 min, 20 μl aliquots of the incubation mixture onto a Whatman 3 MM filter paper grid (2.5 cm diameter) which was followed by washing with 5% trichloroacetic acid (TCA), drying with a mixture of ethanol-ether 3:1 (v/v) and counting as previously described (8).

Degradation of in vivo-labeled mitochondrial proteins

In vivo $^{35}SO_4^{=}$-labeled mitochondria (0.5 mg of mitochondrial protein with a radioactivity of 1.3 x 10^6 dpm/mg protein) was incubated at 30°C, with shaking, in a medium containing in a final volume of 1.5 ml: mannitol, 0.6 M; potassium phosphate, 10 mM pH 7.2; α-ketoglutarate, 6 mM; ADP, 1 mM; phosphoenolpyruvate, 5 mM; ATP, 4 mM; pyruvate kinase, 10 U/ml, and carrier nonradioactive mitochondrial protein, 2 mg (degradation suspending medium). At zero time and at 30 min intervals two aliquots of 100 μl each were withdrawn and centrifuged (Eppendorf Centrifuge, Model 5414) for 4 min at room temperature. The use of 10% TCA did not increase the radioactivity recovered in the pellet, therefore it was decided not to use this reagent (data not shown). 80 μl from each supernatant were carefully removed and an aliquot counted in a scintillation spectrometer using a commercially prepared scintillation cocktail. A control was prepared in the same way except that α-ketoglutarate, ADP, ATP and the ATP regenerating system were not added. Results were expressed as percentage of the radioactivity released to the supernatant with respect to the total radioactivity present in the incubation mixture at zero time.

Degradation of in vitro-labeled mitochondrial protein

^{3}H-leucine or ^{35}S-methionine *in vitro*-labeled mitochondria (0.5 mg of mitochondrial protein with a radioactivity of 2-6 x 10^6 dpm/mg protein) were suspended in 1.5 ml of the degradation suspending medium, incubated at 30°C and treated like the *in vivo* labeled mitochondria as described in the preceding paragraph.

RESULTS

Degradation of mitochondrial proteins synthesized in vivo by growing cells

When *in vivo* $^{35}SO_4^{=}$-labeled mitochondria are resuspended in the degradation suspending medium (see Methods) and incubated at 30°C, aproximately 40% of the radioactivity was released to the medium as a TCA soluble material after 3 hours of incubation. The addition of ATP lowered this amount to about 30%. Thus, the degradation of mitochondrial proteins synthesized *in vivo* was slightly protected by ATP as illustrated in Fig. 1. This effect of ATP, however, did not depend on its concentration since (ATP) ranging from 2 to 20 mM gave similar results. The same figure shows that nearly 20% of the initial total radioactivity was found at zero time. It was found that no radioactive oligopeptides were present in the supernatant and that practically all the radioactivity was associated with free amino acids (data not shown). Moreover, the amount of this extremely rapid initially released radioactivity was not affected by the presence or absence of ATP as it was the slow release of radioactivity that followed for three hours after the initial burst. These facts strongly suggest that the initially released radioactivity does not represent real proteolysis. A possible explanation for this result is given in the Discussion.

In order to assess the state of the mitochondria during the period of proteolysis, an aliquot of the *in vivo* ^{35}S-labeled mitochondria was resuspended in the protein synthesizing medium containing ^{3}H-leucine as radioactive precursor and incubated at 30°C (see Methods). As shown in Fig. 2,

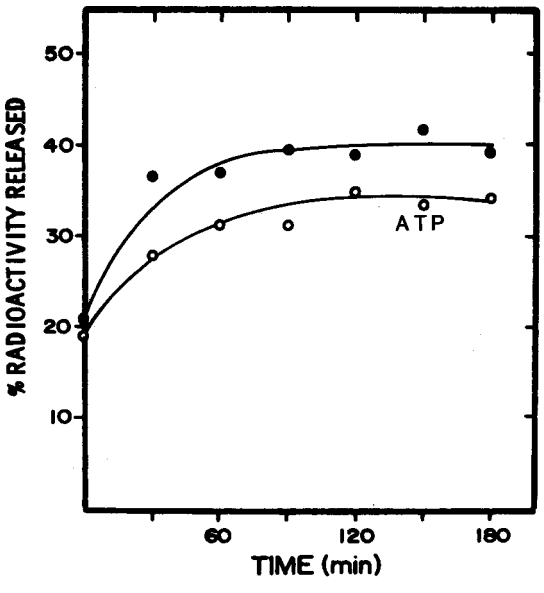

Fig. 1: Release of radioactivity from in vivo ^{35}S-labeled mitochondrial proteins as a function of time. *In vivo* ^{35}S-labeled mitochondrial proteins were resuspended in the degradation suspending medium an incubated at 30°C. At the time indicated, duplicate aliquots were taken and the radioactivity released to the medium was determined as described in Methods. No ATP: •———•; ATP, 4 mM plus the ATP regenerating system, o———o.

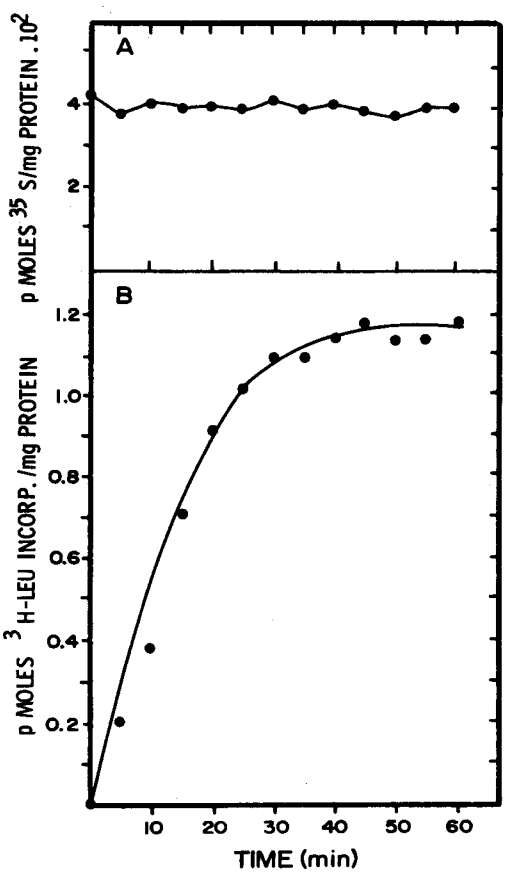

Fig. 2: Time course of ^{3}H-leucine incorporation and concurrent release of radioactivity of isolated mitochondria labeled in vivo with $^{35}SO_4^{=}$. Isolated mitochondria labeled *in vivo* with $^{35}SO_4^{=}$ were resuspended in the protein synthesizing medium and incubated with ^{3}H-leucine. Duplicate aliquots were taken and the radioactivity in the acid insoluble fraction was measured as described in Methods. Panel A: radioactivity of the *in vivo* ^{35}S-labeled proteins remaining in the acid insoluble fraction. Panel B: radioactivity of ^{3}H-leucine incorporated into the acid insoluble fraction.

panel B, the mitochondria incorporated amino acids for at least 40 min, meaning that they were perfectly coupled and metabolically active. Interestingly, panel A shows that during this period all the ^{35}S radioactivity remained attached to the mitochondria indicating that under these experimental conditions the *in vivo* synthesized mitochondrial proteins are completely stable.

Degradation of mitochondrial proteins synthesized in vitro by isolated mitochondria

For this purpose, isolated mitochondria were resuspended in the protein synthesizing mixture and allowed to incorporate either ^{3}H-leucine or ^{35}S-methionine for 60 min at 30°C (see Methods). After this period they were washed 3 times with 2 mM sodium phosphate buffer, pH 7, containing 0.6 M mannitol and 5 mM of either leucine or methionine. The washed mitochondria were resuspended in the degradation suspending medium and incuvated at 30°C. Fig. 3 shows the time course of the release of radioactivity from the *in vitro*-synthesized mitochondrial proteins labeled with ^{3}H-leucine (panel A) or ^{35}S-methionine (panel B). In both instances, and in the absence of ATP, less than 15% of the radioactivity was liberated to the medium after 3 hours of incubation. By contrast, the addition of ATP and ATP regenerating system markedly stimulated the degradation of these proteins. Approximately 40% degradation was observed in the case of the ^{3}H-leucine and 50% in the case of the ^{35}S-methionine labeled proteins. The radioactivity measured at zero time was relatively low and was partially affected by ATP, indicating that at least part of the radioactivity found at this time was due to a real breakdown of proteins.

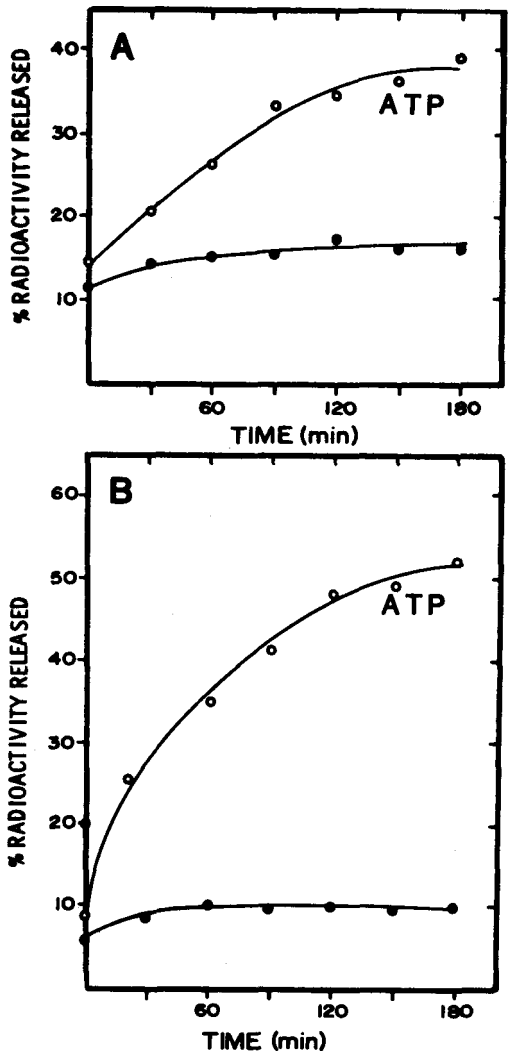

Fig. 3: **Release of radioactivity from *in vitro* labeled mitochondria as a function of time.** Isolated mitochondria labeled *in vitro* with either ^{3}H-leucine or ^{35}S-methionine were resuspended in the degradation suspending medium and incubated at 30°C. The release of radioactivity was followed as described in Methods. Panel A: ^{3}H-leucine labeled proteins. Panel B: ^{35}S-methionine labeled proteins. No ATP: •———•; ATP 4 mM plus the ATP regenerating system; o———o.

DISCUSSION

We have studied the rate of degradation of the mitochondrial proteins synthesized both *in vivo* by growing cells and *in vitro* by isolated mitochondria. The first ones represent normally assembled proteins, made with polypeptides encoded in both, nuclear and mitochondrial genome. On the other hand, *in vitro* synthesized proteins represent unassembled proteins encoded by the mitochondrial genome. It is known that some of these proteins are subunits of multimeric enzymes made with polypeptides originated from both nuclear and mitochondrial genomes (for a review see ref. 12). Under identical

experimental conditions the rate of degradation of both types of proteins is quite different. *In vivo*-made mitochondrial proteins are slowly degraded to a limited extent by a protease which is not dependent on energy. In fact, ATP shows a slight inhibitory effect, however, different concentrations of ATP ranging from 2 mM to 20 mM gave essentially the same result. As shown in Fig. 1, the extent of degradation after 3 hours of incubation reached a maximum of 40% in the absence of ATP and 30% in its presence. However, in both cases 20% of the total initial radioactivity was found at zero time, an unexpected result which, most probably, does not represent proteolysis. We are inclined to believe that this radioactivity is due to the presence of weakly bound radioactive precursors that are released when the mitochondria are resuspended in the degradation suspending medium. Supporting this interpretation is the fact that no radioactive oligopeptides were found in the supernatant, and practically all radioactivity was found associated with free amino acids. Moreover, ATP did not have any effect on the amount of the initially released radioactive material. Further experiments are obviously needed to elucidate this point. Assuming that the radioactivity found at zero time is not due to proteolysis then the actual extent of proteolysis of the *in vivo*-synthesized proteins are, indeed, very stable. On the other hand, when isolated mitochondria are first labeled with ^{3}H leucine or ^{35}S-methionine and then resuspended in the degradation suspending medium supplemented with ATP, the radioactivity is readily released to the medium. By contrast, if ATP is omitted, less than 5% of the radioactivity is liberated as acid-soluble material. The radioactivity found at zero time varied between 5 and 15% and was partially stimulated by ATP indicating that at least part of it was due to a real breakdown of proteins. At three hours of incubation, around one half of the initial radioactive protein had been degraded. After this period the degradation continued although at a much slower rate (data not shown).

The energy-dependent proteolytic activity described in this paper appears to be similar, if not identical, with the one reported by Kalnov *et al.* (2). These authors described an energy-dependent proteolytic system of yeast that recognized as substrates mitochondrial proteins synthesized *in vivo* in the presence of cycloheximide. These proteins are equivalent to those synthesized by isolated mitochondria. A similar system has been described by Desautels and Goldberg (5) in liver mitochondria. This system is stimulated by exogenous ATP and is inhibited by cyanide, oligomycin, dinitrophenol and vanadate, the vanadate also being an inhibitor of ATPases.

ATP-dependent proteases have been described in several biological systems including procaryotes (14, 15), liver (16, 17) and reticulocytes (18). Extensive studies performed with reticulocytes led to the discovery that ubiquitin, a small heat stable peptide present in all tissues, combines with proteins in an ATP requiring reaction, converting them into substrates for intracellular proteases. This is the only case in which the role of ATP is fairly well understood. Proteolysis per se is an exergonic reaction and therefore does not require energy to proceed. Energy is needed to make proteolysis a specific process. It is not believed that all intracellular proteolysis depends on energy, since the intracellular degradation of proteins is involved in the fine regulation of the half-lifes of enzymes which control, in turn, all the metabolic activity of cells (for a review see 13 and 19). Mitochondria seem to use the energy-dependent proteolytic system to eliminate abnormal as well as normal but not properly assembled peptides. The mechanism of recognition of these peptides and the role of ATP remains to be elucidated. The molecular characterization of the mitochondrial proteolytic systems have not been accomplished, although lately, Zubatov *et al.* (20) have been able to isolate and partially characterize three mitochondrial enzymes which are capable of cleaving exogenous cytochrome c. Whether these proteases bear some relation to the ATP-dependent

proteolytic activity described in this report remains to be seen.

REFERENCES

1. LUZIKOV, V.N.; MAKHLIS, T.A. and GALKIN, A.V. (1976) *FEBS Lett. 69:* 108-110.
2. KALNOV, S.L.; NOVIKOVA, L.A.; ZUBATOV, A.S. and LUZIKOV, V.N. (1979) *Biochem. J. 182:* 195-202.
3. KALNOV, S.L.; NOVIKOVA, L.A.; ZUBATOV, A.S. and LUZIKOV, V.N. (1979) *FEBS Lett. 101:* 355-358.
4. GALKIN, A.V.; TSOI, T.V. and LUZIKOV, V.N. (1979) *FEBS Lett. 105:* 373-375.
5. DESAUTELS, M. and GOLDBERG, A.L. (1982) *Proc. Natl. Acad. Sci. (USA) 79:* 1869-1873.
6. DESAUTELS, M. and GOLDBERG, A.L. (1982) *J. Biol. Chem. 257:* 11673-11679.
7. TELLEZ, R.; JACOB, G.; BASILIO, C. and GEORGE-NASCIMENTO, C. (1985) *FEBS Lett. 192:* 88-94.
8. POYTON, R.O. and KAVANAGH, J. (1976) *Proc. Natl. Acad. Sci. (USA) 73:* 3947-3951.
9. LOWRY, O.H.; ROSEBROUGH, N.J.; FARR, A.L. and RANDALL, R.J. (1951) *J. Biol. Chem. 199:* 265-275.
10. McKEE, E.E. and POYTON, R.O. (1984) *J. Biol. Chem. 259:* 9320-9331.
11. McKEE, E.E.; McEWEN, J.E. and POYTON, R.O. (1984) *J. Biol. Chem. 259:* 9332-9338.
12. TZAGALOFF, A. and MYERS, A.M. (1986) *Ann. Rev. Biochem. 55:* 249-285.
13. CIECHANOVER, A.; FINLEY, D. and VARSHAVSKY, A. (1984) *J. Cell. Biochem. 24:* 27-53.
14. MURAKAMI, K.; VOELLMY, R. and GOLDBERG, A.L. (1979) *J. Biol. Chem. 254:* 8194-8200.
15. ROBERTS, J.W. and ROBERTS, C.W. (1975) *Proc. Natl. Acad. Sci. (USA) 72:* 147-151.
16. DeMARTINO, G.N. and GOLDBERG, A.L. (1979) *J. Biol. Chem. 254:* 3712-3715.
17. ROSE, I.A.; WARMS, J.V.B. and HERSHKO, A. (1979) *J. Biol. Chem. 254:* 8135-8138.
18. SCHWEIGER, H.G.; RAPOPORT, S. and SCHOLZEL, E. (1956) *Nature. 178:* 141-142.
19. HERSHKO, A.J. and CIECHANOVER, A. (1982) *Ann. Rev. Biochem. 51:* 335-364.
20. ZUBATOV, A.S.; MIKHAILOVA, A.E. and LUZIKOV, V.N. (1984) *Biochem.. Biophys. Acta 787:* 188-195.

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Temperature acclimatization of the carp. Cellular and molecular aspects of the compensatory response* **

Aclimatización de la carpa a la temperatura. Aspectos celulares y moleculares de la respuesta compensatoria

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INTRODUCTION

Reprogramming of molecular and cellular functions appears to be a fundamental mechanism in the strategies that eurythermal poikilotherms use to compensate environmental changes. From the various ambient factors known to influence the adaptative response, changes that follow thermal shifts have been the most extensively investigated. Several studies have suggested that protein synthesis plays a role in the acclimation and acclimatization of ectothermal organisms (1-3).

Searching for the molecular processes committed in the compensatory response which allow eurythermal fish to survive within the cyclic seasonal temperature changes of their habitat, we have found that in the carp, hepatocyte reprogramming of gene expression might be a prime mechanism of the adjustment that the environmental conditions demands (3-7).

Carp liver cellular phenotypes depend on the adaptative state

Profound cytoplasmic and nuclear changes in the cytoarchitecture of *Cyprinus carpio* hepatocytes are associated with the adaptative process. The amount and distribution of glycogen in the cytoplasm of liver cells from the cold acclimatized fish clearly differs from that found in summer where glycogen particles decrease and appear mainly around the numerous lipid droplets. In winter, mitochondria are scarce and in close association with the rough endoplasmic reticulum surrounding the nucleus (5).

Although several morphological features reveal that macromolecular synthetic activity may be clearly diminished in winter hepatocytes, the most prominent indication is found at the nucleolar level. As shown in Figure 1a, three components can be distinguished in the nucleolus. The fine filaments (A) in the winter carp liver cell are densely packed (Figure 1a). The granular component B appears in this season as a homogeneous mass that is not mixed with the other two components: the A filaments and component C regarded as nucleolar chromatin (5, 8). Thus, acclimatization to cold leads to a classic segregation of the nucleolar components. Actinomycin D which inhibits DNA-dependent RNA synthesis induces a similar effect in the nucleoli of a wide variety of cells (5, 8-10). In contrast, in the nucleolus of summer carp hepatocytes components A, B, and C are highly intermingled (Figure 1b). Furthermore, in this season the content of the granular component B diminished, whereas component C increased. This would suggest an increased need for ribonucleoproteins (granular component) and also an en-

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hancement in the number of DNA molecules transcribing rRNA, features characteristic of cells active in gene expression (5). Nevertheless, the cyclic seasonal reprogramming of the cellular arrangement can be altered, when winter carps are treated with insulin (6). In these animals the hepatocytes exhibit all the features of the summer-adapted fish (6). Figure 1c shows the nucleolus of an hepatocyte from a winter-acclimatized carp treated with insulin (6). As in the summer liver cell, the three nucleolar components are completely intermingled.

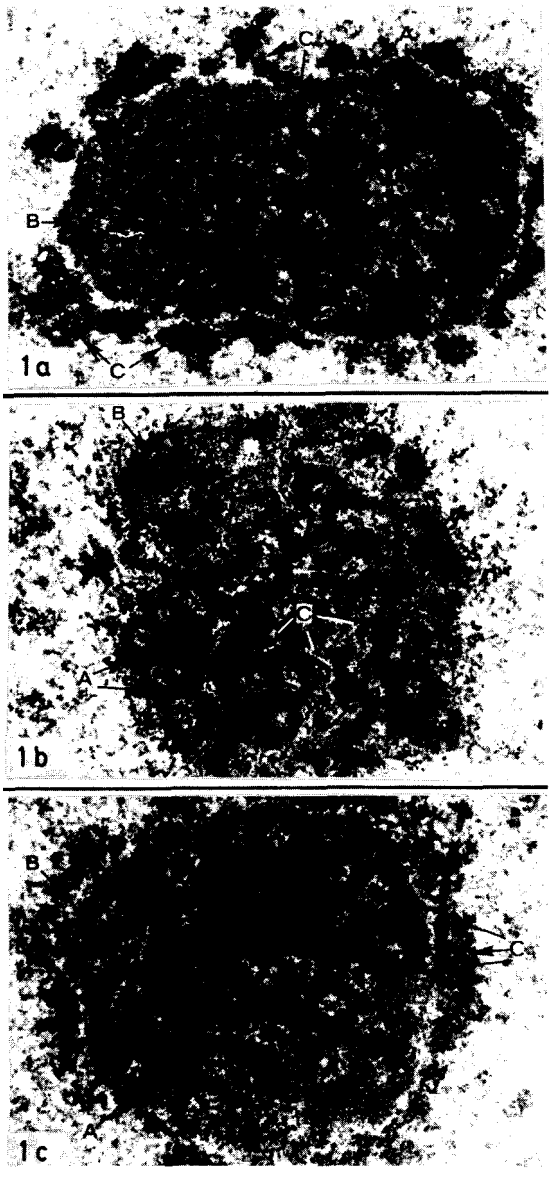

Fig. 1: **Nucleolus of carp hepatocytes.** Small pieces of carp liver were fixed in 2.5% glutaraldehyde, 4% formaldehyde and 4% acrolein buffered with 200 mM sodium phosphate pH 7.5. After embedding in a mixture of epon-araldite, ultrathin sections were prepared and stained with uranyl acetate and lead citrate (5). a) Nucleolus of winter carp hepatocytes. b) Nucleolus of summer carp hepatocytes. c) Nucleolus of winter carp hepatocytes treated with insulin (2.5 I.U./100 g body weight for three days). A. fine filaments. B. Granular component. C. Nucleolar chromatin. Reprinted with permission from Sáez *et al.* (5, 6).

Insulin induces other profound effects when injected in winter carps (6). Glycogen depletion to levels comparable to those found in the livers of summer-adapted fish and rRNA enhancement in the hepatocytes of hormone-treated, cold-adapted fish are further features of the liver cell differentiated state which resembles the summer phenotype (5, 6).

Changes in carp liver RNA and protein synthesis during acclimatization

Early work performed in our laboratory has shown that transcription in carp hepatocytes is profoundly influenced by acclimatization (4). The rate of total RNA synthesis in isolated liver cells is one order of magnitude higher in summer than in winter, when both are compared with regard to the physiological temperature of the corresponding adaptative state. Overall protein synthesis is also enhanced in carp isolated hepatocytes from summer-adapted fish compared to the activity present in the cold-acclimatized fish. Whereas transcription appears to be repressed during the cold season, protein synthesis decreases mainly by the Q_{10} effect (4).

The tRNA content in the liver of summer-acclimatized carps is 70% higher than that from the liver of cold-adapted fish and the *in vivo* intracellular levels of aminoacyl-tRNAs decreases significantly in the warm season (7) which is consistent with a state of increased protein synthesis (7, 11). In addition, changes in the distribution of the tRNA isoacceptor species occur between the two reversible adaptative states of the carp (12).

Carp liver cell-free protein synthesis is affected by insulin. Amino acid incorporation decreases in the insulin-treated carp liver system when compared to the un-

treated cold-acclimatized fish (6). Moreover, soluble factors obtained from livers of winter control carps when assayed with polysomes from hormone-injected fish increase both the rate and extent of amino acid incorporation by up to 85% over the levels attained with the cell sap and polysomes from the summer resembling insulin-treated carp (6). If winter adapted-carps treated with insulin represents molecular processes featured by the summer-acclimatized fish, it is possible to postulate that during the cold season carp liver elongation factors behave in a different manner, exhibiting higher activities. Working with fish adapted to natural environmental changes has made the possibility to confirm this difficult. However, acclimation studies with the toadfish *Opsanus tau, i.e* laboratory regulated adaptation of the ectotherm, have revealed that cold acclimation at 10°C results in an 60-70% increase in the amino acid incorporation into liver proteins compared to fish adapted at 20°C (1, 13). During the increased protein synthetic activity in cold acclimated toadfish, differences in the elongation factors have been detected, specifically in the low molecular weight forms of EF-1 (13, 14). Furthermore, correlation of EF-1 activity with elongation rate *in vivo* was shown to occur (14). Therefore, the compensatory response to change in temperature might implicate modulations at the level of both transcription and translation of the available mRNAS.

It is well established that cold acclimation of ectotherms involves adaptative responses at the level of membrane fluidity (15). In the carp Wodtke *et al.* (15) observed increased membrane lipid unsaturation as the result of part of an adjustment of the activity and amount of Δ^9 desaturases. This suggests that the expression of this enzyme might be associated with the acclimatization process.

Carp insulin synthesis

Biosynthesis of proinsulin in islets of Langerhans of the carp has been studied by Lukowsky *et al.* (16). About 3-4% of the newly synthesized protein was proinsulin upon incubation of the Brockmann bodies with ^{14}C-Leucine at 15°C for 2 h (16). No conversion of proinsulin into insulin was observed. The optimal temperature for the carp used in this study was 26°C (16). Carp insulin has some unusual features as compared with known mammalian and fish insulin sequences (17). Because cellular phenotype and molecular processes associated with gene expression seem to be affected by exogenous insulin administration in winter adapted carp (6), it is of particular interest to examine the level of this hormone during the year round acclimatization of the fish. Although the biological and physical properties of carp insulin are very similar to those of other mammalian and fish species (17), we were unable to detect the hormone in the sera using a radioimmunoassay with antibodies which crossreacted with human (100%) and porcine insulin (92%), with a lower limit of sensitivity situated between 2 and 3 μIU/ml. Thus, we have synthetized the oligonucleotide OH-GGTCCAACAGGTTTCTTCTATAACCCC AAG-OH with the aim of using it as a probe to detect transcription in the islets of Langerhans in fish adapted to the warm and cold season. The sequence of the 30mer corresponds to amino acids 20-29 from the carp insulin B-chain, and was taken from Hahn, *et al.* (18) who deduced the primary structure from a cDNA clone.

The search for protein markers to probe reprogramming during the acclimatization process

If qualitative reprogramming of gene expression is part of the compensatory response towards the change of temperature of the fish habitat, differences in the nature of proteins being synthetized at a given acclimatized state can be expected. To study the kinds of protein synthesized, isolated hepatocytes from winter and summer-adapted carp were incubated with ^{35}S-methionine and proteins of the lysed cells were analyzed by polyacrylamide gel electrophoresis. The typical densitometric scanning obtained from the cor-

responding fluorographies is shown in Figure 2. Clearly, winter and summer liver cells exhibit distinctive protein products when amino acid incorporation is examined at the physiological temperature of the corresponding acclimatized state. In 3 hours of incubation at 20°C, the warm-season carp hepatocytes synthesize not only more proteins (4) but also additional kinds of proteins with respect to those observed in the winter-carp liver cells at 10°C, at the level of sensitivity of the experimental procedure used.

Because, as seen in Figure 2, one of the predominant proteins synthesized in the liver of summer-adapted carp featured an M_r of 66000, we hypothesized that it could be similar to albumin. Sorvachev (19) had previously reported changes in proteins of carp blood serum during hibernation. The changes affected mainly the albumin fraction, which decreased gradually during the fasting that accompanies hibernation. In addition, Saito (20) found that the concentrations of serum protein and albumin of carp are lower in winter than in the other seasons. The temperature of cold acclimatization seems to be relevant for proper comparisons. In Sorvachev's study (19) this parameter is not clearly stated. "Physiological fasting" seems to occur only at temperatures which depress metabolism to extremely low levels. Gas and Serfaty (21) studied the morphological modifications of the carp hepatocyte in fish subjected to total winter starvation. During the latter, the glycogen decreases and after 6 month of denutrition, glycogenosomes appear in the hyaloplasm. Under the natural conditions of acclimatization to which the carps of our studies are subjected (8-10°C in winter and 19-20°C in summer), we do not find these structural changes suggesting that they are only indicative of the pathological state that follows total winter starvation (5). Gut content analyses (22) have shown that the food intake decreases significantly upon changes in the temperature habitat of these carps.

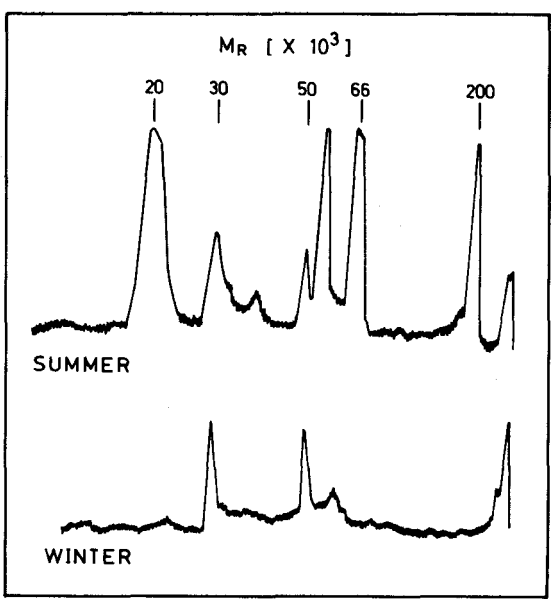

Fig. 2: **Proteins synthesized by winter and summer isolated hepatocytes.** Isolated hepatocytes were prepared by treatment with collagenase(4), and incubated with 100 μCi of ^{35}S-methionine for 3 h at 10 or 20°C for winter or summer acclimatized fishes, respectively. The hepatocyte proteins containing an appropriate amount of counts, were separated on a linear gradient (10-15%) polyacrylamide-SDS slab gel. After fluorography of the gel, the X-ray film was scanned in a microdensitometer.

In order to ascertain whether carp albumin changes were found during the acclimatization process, it was necessary to examine its isolation and characterization. Description of carp albumin in the literature was found to be controversial (23). Nakagawa *et al.* (24) characterized a carp plasma albumin of about 150,000 daltons, with no cysteine in its polypeptide structure (25). Nagano *et al.* (26) identified an albumin-like protein from carp serum with a molecular weight of 59,000.

During the course of our experiments to isolate and characterize carp plasma albumin, we realized that the protein that was considered carp albumin by all previous reports (19, 20, 24-26) corresponded to high density lipoproteins (HDLs) (27). We isolated the apoliproteins A-I and A-II by a novel procedure method which uses affinity chromatography. The structural characterization of these proteins is being completed. Details will be published elsewhere. The one-step affinity chromatography procedure based on properties of these apolipoproteins to bind dyes was used to quantify the level of these protein fraction in carp plasma from fish adapted to the warm and cold season.

Concommitantly, the plasma levels of total proteins, HDL-cholesterol, total cholesterol, lipids and triglycerides were measured. It was estimated that these components could reveal changes in the main constituents of HDLs upon acclimatization.

As shown in Table I, with the exception of the protein content, no other significant variations in the absolute level values were observed in the plasma of carps acclimatized to the warm and cold season. Surprisingly, the protein fraction of plasma increases notoriously during the cold season. This contrast with earlier observations (19, 20) might be the result of different adaptative strategies towards the degree of temperature coolness. Adaptation to temperature extremes affects protein synthesis in a different manner (28). Our eurythermal fish model system lives under natural environmental conditions which do not demand acclimatization to extreme temperatures. Of particular interest is the change in relative HDL concentration between summer and winter. While in the cold season HDL constitutes 30% of the total plasma protein content, in summer this fraction corresponds to 40% of the total proteins.

It has been established that apo A-I mRNA codes for a protein 24 amino acids longer than mature apo A-I. The first 18 amino acids are removed cotranslationally during translocation of the nascent peptide chain across the rough endoplasmic reticulum, yielding a proapo A-I protein with a hexapeptide in its NH_2 terminus (29). It has been reported that the hexapeptide is removed only after the proprotein is secreted from the cell (30). According to Scanu (29), in addition to the proteolytic system responsible for the conversion of proapo A-I to mature apo A-I, other enzymes in the plasma may be involved in the modulation of the concentration and distribution of mature apo A-I in plasma. If this is true for the carp, then the plasmatic apo A-I levels observed in Table I for the cold and warm acclimatized fish does not necessarily correlate with its rate of synthesis. While apo A-I expression could

TABLE I

Carp plasma levels of proteins and lipids during acclimatization

	Season		
	Warm[a]	Cold[b]	Warm[c]
Temperature (°C)	19.40 ± 0.75	10.00 ± 0.63	20.30 ± 0.29
Total protein (g/dL)	2.56 ± 0.81 (16) p = 0.0328	3.09 ± 0.65 (21) p = 0.0119	2.56 ± 0.50 (15)
Apoproteins (HDL) (g/dL)	0.94 ± 0.25 (17)	1.06 ± 0.22 (21)	1.02 ± 0.27 (15)
HDL-cholesterol (mg/dL)	63.90 ± 27.50 (16)	43.10 ± 22.60 (21)	42.40 ± 14.50 (15)
Total cholesterol (mg/dL)	77.80 ± 28.30 (17)	89.30 ± 23.70 (21)	90.73 ± 20.30 (15)
Total lipids (g/L)	8.76 ± 2.93 (17)	8.83 ± 2.17 (21)	8.21 ± 2.84 (15)
Triglycerides (mg/dL)	123.65 ± 51.30 (17)	116.81 ± 35.25 (21)	154.73 ± 42.31 (15)

(a) November 1985 to February 1986.
(b) June to September 1986.
(c) January to March 1987.

Total plasma protein was determined by Biuret. Apoproteins were cuantified utilizing an affinity chromatography procedure (27). HDL-cholesterol, total cholesterol, total lipids and tryglycerides in carp plasma were cuantified utilizing commercial kits from Merck.
The number of individual determinations are indicated in parenthesis.

be affected as part of the acclimatization process, its plasmatic levels could remain unaltered between the natural temperatures that the seasonal cycle impose on the fish of our study, as a result of changes in the processing and turnover of the mature protein. Therefore, examination of pre-proapo A-I synthesis is deemed of particular importance.

In all species studied so far, liver and intestine appear to be the major sites of synthesis of apo A-I (31). We have raised specific antibodies against carp apo A-I in both rabbit and goat, and we were able to confirm by immunohistochemistry the presence of this protein in the liver and intestine of the fish. We are currently studying the synthesis of preproapo A-I in isolated carp hepatocytes and perfused intestine to ascertain whether its expression is reprogrammed upon seasonal acclimatization.

REFERENCES

1. HASCHEMEYER, A.E.V. (1969) Rates of polypeptide chain assembly in liver *in vivo:* Relation to the mechanism of temperature acclimation in *Opsanus tau. Proc. Nat. Acad. Sci. USA., 62:* 128-135.
2. HAZEL, R.J. and PROSSER, C.L. (1974) Molecular mechanisms of temperature compensation in poikilotherms. *Physiol. Rev., 54:* 620-677.
3. KRAUSKOPF, M.; AMTHAUER, R.; SAEZ, L. and ZUVIC, T. (1981) On the role of protein synthesis in the strategies of adaptation to environmental changes. In *Molecular Approaches to Gene Expression and Protein Structure.* (M.A.Q. Siddiqui, M. Krauskopf and H. Weissbach, eds.). Academic Press, New York, pp. 197-214
4. SAEZ, L.; GOICOECHEA, O.; AMTHAUER, R. and KRAUSKOPF, M. (1982) Behaviour of RNA and protein synthesis during the acclimatization of the carp. Studies with isolated hepatocytes. *Comp. Biochem. Physiol. 72B:* 31-38.
5. SAEZ, L.; ZUVIC, T.; AMTHAUER, R.; RODRIGUEZ, E. and KRAUSKOPF, M. (1984) Fish liver protein synthesis during cold acclimatization: Seasonal changes of the ultrastructure of the carp hepatocyte. *J. Exp. Zool. 230:* 175-186.
6. SAEZ, L.; AMTHAUER, R.; RODRIGUEZ, E. and KRAUSKOPF, M. (1984) Effects of insulin on the fine structure of hepatocytes from winter-acclimatized carps: Studies on protein synthesis. *J. Exp. Zool., 230:* 187-197.
7. ZUVIC, T.; BRITO, M.; VILLANUEVA, J. and KRAUSKOPF, M. (1980) *In vivo* levels of aminoacyl-tRNA species during acclimatization of the carp *Cyprinus carpio. Comp. Biochem. Physiol. 67B:* 167-170.
8. GHOSH, S. (1976) The nucleolar structure. *Int. Rev. Cytol., 44:* 1-24.
9. GOLDBLATT, P.J.; SULLIVAN, R.J. and FARKER, E. (1969) Morphologic and metabolic alterations in hepatic cell nucleoli induced by varying doses of actinomycin D. *Cancer Res., 29:* 124-135.
10. RECHER, L.; BRIGGS, L.G. and PARRY, N.T. (1971) A reevaluation of nuclear and nucleolar changes induced *in vitro* by actinomycin D. *Cancer. Res., 31:* 140-151.
11. LITT, M. and WEISSER, K. (1978) Histidine transfer RNA levels in Freund Leukemia cells: stimulation by histidine deprivation. *Science 201:* 527-529.
12. OÑATE, S.; AMTHAUER, R. and KRAUSKOPF, M. (1987) Differences in the tRNA population between summer and winter acclimatized carp. *Comp. Biochem. Physiol. 86B:* 663-666.
13. PLANT, P.W.; NIELSEN, J.B.K. and HASCHEMEYER, A.E.V. (1977) Control of protein synthesis in temperature acclimation. I. Characterization of polypeptide elongation factor 1 of toadfish liver. *Physiol. Zool., 50:* 11-21.
14. NIELSEN, J.B.K.; PLANT, P.W. and HASCHEMEYER, A.E.V. (1977) Control of protein synthesis in temperature acclimation. II. Correlation of elongation factor 1 activity with elongation rate *in vivo. Physiol. Zool., 50:* 22-30.
15. WODTKE, E.; TEICHERT, T. and KONING, A. (1986) Control of membrane fluidity in carp upon cold stress: Studies on fatty acid desaturases. In *Living in the Cold: Physiological and Biochemical Adaptations* (H.C. Heller *et al.*, eds.). Elsevier Publishing Co., New York, pp. 35-42.
16. LUKOWSKY, A.; PREHN, S. and RAPOPORT, T.A. (1974) Biosynthesis of proinsulin in islets of Langerhans of the carp *Cyprinus carpio. Biochim. Biophys. Acta. 359:* 248-252.
17. MAKOWER, A.; DETTMER, R.; RAPOPORT, T.A.; KNOSPE, S.; BEHLKE, J.; PREHN, S.; FRANKE, P.; ETZOLD, G. and ROSENTHAL, S. (1982) Carp insulin: amino acid sequence, biological activity and structural properties. *Eur. J. Biochem., 122:* 339-345.
18. HAHN, V.; WINKLER, J.; RAPOPORT, T.A.; LIEBSCHER, D.H.; COUTELLE, Ch. and ROSENTHAL, S. (1983) Carp preproinsulin cDNA sequence and evolution of insulin genes. *Nucleic Acid Res. 11:* 4541-4552.
19. SORVACHEV, K.F. (1957) Changes in proteins of carp blood serum during hibernation. *Biokhimiya, 22:* 822-827.
20. SAITO, K. (1957) Cited in reference 18. *Bull. Japan. Soc. Sci. Fish., 22:* 768-772.
21. GAS, N. and SERFATY, A. (1972) Cytophysiologie du foie de carpe (*Cyprinus carpio L.*) Modifications ultrastructurales consecutives au maintien dans de conditions de jeûne hiveral. *J. Physiol. Paris, 64:* 57-67.
22. PROCHELLE, O.G. (1982) Estudio de la biología de *Cyprinus carpio L.*, en el río Cayumapu, Valdivia. Licenciado en Medicina Veterinaria. Thesis. Universidad Austral de Chile, Valdivia.
23. DOOLITTLE, R.F. (1984) Evolution of the vertebrate plasma proteins. In *The Plasma Proteins* (F.W. Putnam, ed.) Academic Press, New York, pp. 317-360.
24. NAKAGAWA, H.; KAYAMA, M. and ASAKAWA, S. (1976) Biochemical studies on carp plasma protein. I. Isolation and nature of an albumin. *Bull. Japan. Soc. Sci. Fish., 42:* 677-685.

25. NAKAGAWA, H. and KAYAMA, M. (1978) Biochemical studies on carp plasma protein. II. Amino acid composition of an albumin. *Bull. Japan. Soc. Sci. Fish., 44:* 259-262.
26. NAGANO, H.; HOSAKA, K. and SHUKUYA, R. (1975) Comparative biochemistry of serum albumin. A serum albumin-like protein from carp, *Cyprinus carpio, Comp. Biochem. Physiol., 50B:* 573-578.
27. AMTHAUER, R.; CONCHA, M. and VILLANUEVA, J. (1987) Characterization of the high density lipoprotein of carp serum. *Arch. Biol. Med. Exp., 20:* R174.
28. HASCHEMEYER, A.E.V. and MATHEWS, R. (1982) Effects of temperature extremes on protein synthesis in liver of toadfish, *Opsanus tau, in vivo. Biol. Bull. 162:* 18-27.
29. SCANU, A.M. (1987) Proteolytic enzymes in the biogenesis and metabolism of plasma HDL. *Atherosclerosis Rev. 16:* 9-18.
30. GORDON, J. I.; SIMS, H.F.; LENTZ, S.R.; EDELSTEIN, C.; SCANU, A.M. and STRAUSS, A.W. (1985) Proteolytic processing of human preproapoliprotein A-I a proposed defect in the conversion of proapo A-I to A-I in Tangier's disease. *J. Biol. Chem., 258:* 4037-4044.
31. EISENBERG, S. (1984) High density lipoprotein metabolism, *J. Lipid. Res. 25:* 1017-1058.

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Catepsina D de próstata humana*

Cathepsin D from human prostate

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En este trabajo se ha desarrollado una metódica simple que ha permitido la obtención de catepsina D de próstatas humanas en cantidades apreciables para estudios enzimáticos y químicos, empleando cromatografías combinadas de intercambio en DEAE celulosa y cromatoenfoque en gel PBE-94.

La síntesis química de un nuevo sustrato sintético permitió comparar la actividad hidrolítica de la catepsina D con las gastricsinas de próstata y líquido seminal humano, así como con pepsina y gastricsina de mucosa gástrica. La actividad de la catepsina prostática sobre el sustrato sintético N-acetil-L-fenilalanil-L-diyodotirosil-L-valina metil éster (APDTV) fue similar a la de las gastricsinas y mucho mayor con respecto a la pepsina.

Las relaciones ácido glutámico/ácido aspártico (Glu/Asp) y leucina/isoleucina (Leu/Ile) de la catepsina D son semejantes a las presentes en las gastricsinas y no en las pepsinas, en cuyo caso estos aminoácidos se encuentran en una razón inversa.

INTRODUCCION

La catepsina D es una proteinasa lisosomal activa a pH ácido que se encuentra en células eucariónticas y procariónticas (1) y cuya función es la de degradar proteínas en el interior de las células. En trabajos anteriores (2, 3, 4) tendientes a establecer el origen del zimógeno de una proteinasa acídica secretada en el líquido seminal (5, 6), nos permitieron separar este zimógeno y catepsina D desde próstatas humanas. El hallazgo de que la catepsina D hidrolizaba hemoglobina a pH 3.0, pero no a pH 1.0, nos permitió desarrollar un método simple para determinar gastricsina y catepsina D en tejido prostático (7). Debido a que el estado funcional de la próstata de mamíferos depende de la presencia de testosterona se procedió al estudio del contenido de estas dos enzimas en próstatas de personas normales y con hiperplasia prostática benigna con el fin de utilizar la determinación de estas enzimas como posibles marcadores de la función prostática (8). La influencia de la castración y de la administración posterior de testosterona sobre el contenido de gastricsina y de catepsina D fue comunicado por Chiang y col. (9) y la posible participación de la catepsina D lisosomal durante la involución de la próstata ha sido sugerida por Tanabe y col. (10).

En este trabajo se describe la purificación de catepsina D de próstata humana y se comparan sus propiedades con las de pepsina y gastricsina obtenidas de mucosa gástrica humana (11). Además se describe la síntesis de un sustrato sintético hidrolizable por la catepsina D.

MATERIALES Y METODOS

Materiales. Las mucosas gástricas y las próstatas humanas fueron obtenidas de muestras de autopsias que se guardaron a −18°C hasta su utilización. N-acetil-L −fenilalanil-L-3,5− diyodotirosina (APDT), L-valina-metil ester, L-valina, diciclohexilcarbodiimida, hemoglobina y DEAE-celulosa eran productos Merck-Darmstadt. DEAE-Sephadex A-50, Sephadex G-75, Gel PEE 94, Polybuffer 74 se obtuvieron de Pharmacia Fine Chemicals.

La actividad proteolítica y la concentración de proteínas de las diferentes fracciones sometidas a purificación se determinaron de acuerdo a lo descrito anteriormente (2). La unidad de actividad enzimática se definió como la cantidad de enzima capaz de producir un cambio de absorbancia de 1,0 medido a 280 nm, después de incubar 10 min a 37°C.

* En memoria de nuestro maestro, profesor Dr. Osvaldo Cori.

El peso molecular de la catepsina D se determinó igual que para la gastricsina de próstata humana (4).

Síntesis de sustrato peptídico N-acetil L-fenilalanil-L-diyodotirosina-L-valina metil éster (APDTV). La síntesis se realizó según la técnica descrita por Inouye (12). Se suspendió APDT (235 mg) y L-valina metil éster (50 mg) en 50 ml de tetrahidrofurano (THF). La reacción comenzó con la adición de un leve exceso de N-N' diciclohexil carbodiimida (90 mg) y se dejó reaccionar por 120 h a temperatura ambiente. El producto de síntesis se recristalizó, posteriormente, en éter dietílico y se le efectuó el análisis elemental y la composición de aminoácidos.

Análisis de aminoácidos. Muestras de proteínas y de APDTV se hidrolizaron en 1 ml de HCl 6M en tubos sellados al vacío, durante 24 y 72 h, a 110°C. El análisis se realizó en un analizador de aminoácidos Beckman 119, de acuerdo esencialmente a Spackman y col. (13).

Hidrólisis del péptido sintético APDTV. Para determinar la actividad de las proteasas sobre el tripéptido sintético se utilizó la técnica descrita anteriormente (14).

Pepsina y gastricsina se purificaron de mucosa gástrica humana siguiendo el método descrito por Tang y col. (11). Gastricsina de líquido seminal se preparó de acuerdo a los métodos descritos anteriormente (6, 7).

Purificación de catepsina D. Próstatas humanas, normales, se homogeneizaron en cuatro veces su peso de tampón 0,05M TRIS-HCl, pH 7,2 y se centrifugó a 10.000 g, durante 20 min, a 4°C. El sobrenadante se fraccionó con sulfato de amonio (30-65% saturación) y el precipitado resultante se disolvió en el menor volumen de tampón TRIS-HCl y se dializó exhaustivamente con el mismo tampón. El dializado se cromatografió en una columna de DEAE-celulosa (4,5 x 45 cm) y se eluyó, primero, con el tampón TRIS-HCl y luego mediante una gradiente de NaCl. Las fracciones (B) que eluyeron con la gradiente salina y que eran activas, a pH 1,0 y 3,0 sobre el sustrato hemoglobina, se utilizaron en la obtención de la gastricsina prostática (4). Las fracciones (A) que eluyeron antes de aplicar la gradiente y que eran activas a pH 3,0 solamente, se juntaron y se dializaron contra tampón Imidazol-HCl 0,025M, pH 7,0. Una alícuota de este dializado se aplicó a una columna de cromatoenfoque, PBE 94, de 0,9 x 10 cm, equilibrada con el tampón Imidazol-HCl. La columna se eluyó, primero, con el mismo tampón (10 ml) y la elución final se realizó con polybuffer 74 = HCl, pH 4,0. Las fracciones activas a pH 3,0 se recolectaron separadamente (A y B), se concentraron a un volumen menor y se cromatografiaron en columnas de Sephadex 6-75 (1,2 x 200 cm). La purificación final de la catepsina D se alcanzó después de recromatografiar, en la columna de cromatoenfoque, la fracción activa obtenida de Sephadex G-75.

RESULTADOS

La Figura 1 muestra el perfil cromatográfico en DEAE-celulosa del homogeneizado de próstata humana parcialmente purificado por tratamiento con sulfato de amonio (30 - 65% saturación). El primer pico de proteínas (A) que emergió de la columna se mantuvo a −18°C para su posterior purificación. El segundo pico de proteínas que eluyó con la gradiente salina se utilizó para purificar la gastricsina prostática (4). El pico de proteínas A, que no fue retenido por la columna, después de concentrado y dializado se sometió a cromatografía en gel PBE 94, como se muestra en la Figura 2. Los dos picos de proteínas (A y B) activos a pH 3,0 se recolectaron separadamente y después de concentrarlos a un volumen pequeño se cromatografiaron en columnas de Sephadex G-75, como se describe en la Figura 3. La purificación final de los picos de proteínas activos se obtuvo por recromatografía de éstos en gel de PBE 94 (Fig. 2).

En la Figura 4 se observa el peso molecular de 40.740 daltons, determinado para la catepsina D por el método de Andrews (16), que está de acuerdo con el comunicado por otros autores (17). Lo mismo se observa con la gastricsina prostática (32.740 daltons) cuando se le com-

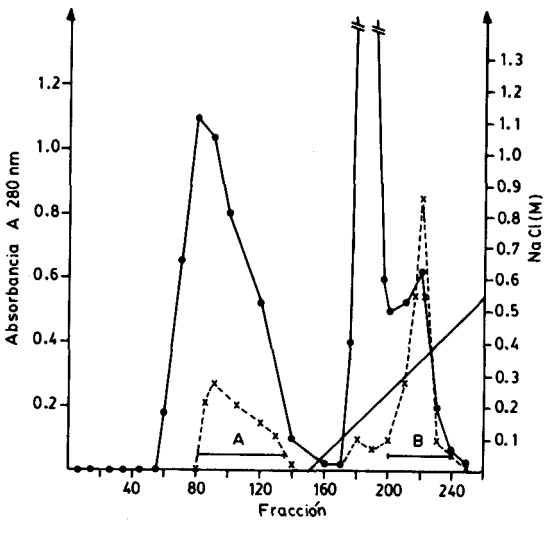

Fig. 1: Cromatografía en columna de DEAE-celulosa del extracto fraccionado con sulfato de amonio (4,8 g proteína en 250 ml de tampón). Flujo: 60 ml/h. Tamaño de la fracción: 11 ml. Actividad proteolítica: 0,3 ml incubado por 60 min (x- - - - x). Proteínas (●——●),

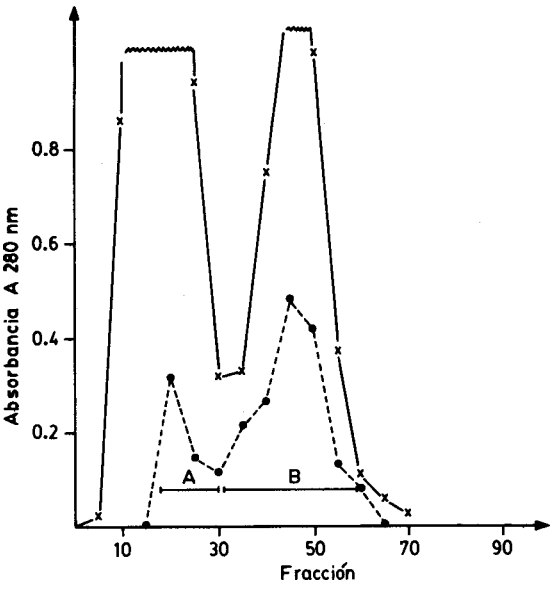

Fig. 2: Cromatografía de cromatoenfoque en el gel PBE de la fracción A. Muestra 120 mg proteína en 1,20 ml de 0,025M Imidazol-HCl, pH 7,0. Flujo: 12 ml/h. Tamaño de la fracción: 2,0 ml. Actividad proteolítica: 0,20 ml por 60 min (●----●). Proteínas (x——x).

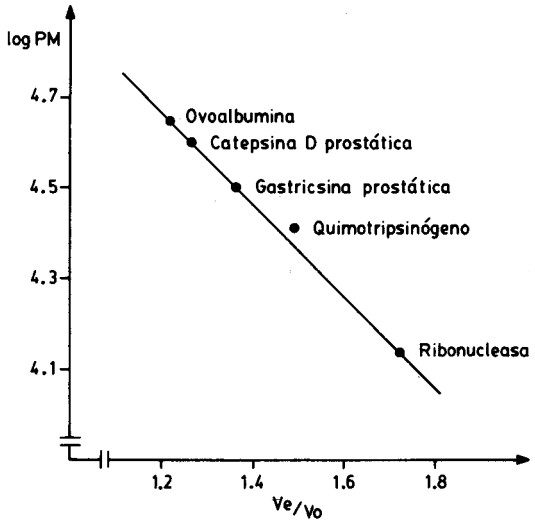

Fig. 4: Determinación de peso molecular por filtración en Sephadex G-75 de catepsina D y gastricsina prostática. Flujo: 9,0 ml/h. Tamaño de la fracción: 2,0 ml.

para con los 32.840 daltons de la gastricsina de porcino (18).

La catepsina D de próstata comparte varias propiedades enzimáticas de las gastricsinas tanto de próstata como gástrica. El pH óptimo de las gastricsinas y de las catepsinas D difieren del pH más ácido de las pepsinas. La composición de aminoácidos de las catepsinas se asemejan a la composición de las gastricsinas especialmente en la relación entre los aminoácidos Glu/Asp y Leu/Ile, que es mayor en estos grupos, que las que presenta la clásica pepsina. En la Tabla 1 se compara la composición de

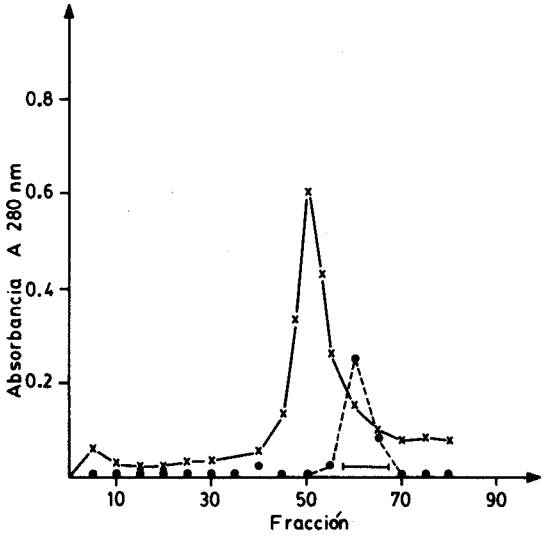

Fig. 3: Cromatografía en columna de Sephadex 6-75 de la fracción B (Fig. 2) (25 mg proteína en 1,0 ml solución). Flujo: 18 ml/h. Tamaño de la fracción: 2,0 ml. Actividad proteolítica: 0,20 ml por 60 min (●----●). Proteínas (x——x).

TABLA 1

Composición de aminoácidos de catepsina D de próstata y de hígado humanos

	Catepsina D de próstata	Catepsina D de hígado
	Nº residuo / mol de proteína	
Lys	19	25
His	7	5
Arg	11	1
Asp	38	32
Thr	23	2
Ser	32	26
Glu	39	36
Pro	26	23
Gly	46	41
Ala	37	2
Cys	5	8
Val	21	32
Met	6	14
Ile	17	22
Leu	27	35
Tyr	16	21
Phe	19	15
Trp	ND	4
Nº residuos	389	389
P.M.	40.740	42.000

ND = No determinado PM = Peso Molecular

aminoácido de la catepsina D de próstata con otra de hígado (17). El APDT, buen sustrato de las pepsinas de diferentes especies, no es hidrolizado por las gastricsinas ni por la catepsina D de próstata; en cambio, el nuevo sustrato sintetizado a partir de APDT y L-valina metil éster es rápidamente hidrolizado por la catepsina D y las gastricsinas, como se indica en la Tabla II, a diferencia de la pepsina gástrica que posee tan sólo un 20% de la actividad de las anteriores.

TABLA 2

Hidrólisis del sustrato sintético APDTV por proteasas acídicas, a pH 3.0 y 37°C

	ΔDO*	%
Pepsina gástrica humana	0,023	18,9
Catepsina D prostática humana	0,116	95,1
Gastricsina prostática humana	0,122	100,0
Gastricsina de líquido seminal humano	0,114	93,2
Gastricsina gástrica humana	0,114	93,4

* ΔDO producido por 1 unidad de actividad enzimática (hemoglobina) en 60 min.

DISCUSION

La combinación de las cromatografías de intercambio en DEAE-celulosa y de cromatoenfoque en gel PBE 94 ha permitido una fácil separación de catepsinas D, posiblemente del tipo isoenzimas (Fig. 2), como lo sugiere el estudio de las catepsinas D de cerdo,descrito por Huang y col. (1).

La destacable similitud entre la catepsina D de próstata y las gastricsinas se puede observar si se compara el pH óptimo de actividad sobre sustratos proteicos, la resistencia a la hidrólisis del dipéptido APDT, la facilidad con que es hidrolizado el nuevo tripéptido sintético APDTV, y la típica relación de los aminoácidos Glu/Asp y Leu/Ile sólo es comparable con lo observado en la catepsina D-II de monos (20) cuya similitud con las gastricsinas no está circunscrita solamente a las actividades enzimáticas, sino que presenta la misma antigenicidad y secuencia aminoterminal de las gastricsinas.

Aunque la catepsina D de próstata presenta, sobre las uniones peptídicas, una amplia especificidad similar a gastricsina y pepsina, a diferencia de ellas funcionan dentro de las células. Por lo tanto, es de mucho interés determinar la estructura primaria de este tipo de catepsinas D que permita establecer una relación estructura-función con el fin de compararlas con la de otras proteasas aspárticas.

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REFERENCIAS

1. HUANG, J.S.; HUANG, S.S.; TANG, J. (1979) *J. Biol. Chem., 254:* 11405-11417.
2. WARD, P.H.; NEUMANN, V.K.; CHIANG, L. (1978) *Comp. Biochem. Physiol. B. 61:* 491-498.
3. MALDONADO, M.; VIVALDI, E.; WARD, P.H.; CHIANG, L. (1980) *Arch. Biol. Med. Exp. 13.* 117.
4. CHIANG, L.; CONTRERAS, L.; CHIANG, J.; WARD, P.H. (1981) *Arch. Biochem. Biophys., 210:* 14-20.
5. SAMLOFF, I.M.; LIEBMAN, W.M. (1972) *Clin. Exp. Immunol., 11:* 405-414.
6. RUENWONGSA, P.; CHULAVATNATOL, M. (1975) *J. Biol. Chem., 250:* 7574-7578.
7. WARD, P.H.; CONTRERAS, L.; MALDONADO, M.; CHIANG, L. (1980) *IRCS Med. Sci. Biochem., 8:* 830-831.
8. WARD, P.H.; CONTRERAS, L.; MALDONADO, M.; BAEZA, H.; CHIANG, L. (1982) *J. Urology 127:* 1027-1030.
9. CHIANG, L.; MALDONADO, M.; VIVALDI, E.E.; WARD, P.H. (1982) *Biochem. Int. 4:* 161-168.
10. TANABE, E.T.; LEE, CH.; GRAYHACK, J.T. (1982) *J. Urol., 127.* 826-828.
11. TANG, J.; MILLS, J.; CHIANG, L.; DE CHIANG, L. (1967) *Ann. N. Y. Acad. Sci., 140:* 688-696.
12. INOUYE, K.; VOYNICK, I.M.; DEPIERRE, G.R.; FRUTON, J.S. (1966) *Biochemistry, 5:* 2473-2478.
13. SPACKMAN, D.H.; STEIN, W.H.; MOORE, S. (1959) *Anal. Chem. 30:* 1190-1206.
14. CHIANG, L.; SANCHEZ-CHIANG, L.; WOLF, S.; TANG, J. (1966) *Proc. Soc. Exp. Biol. Med. 122:* 700-704.
15. WARD, P.H.; DIAZ, M.; MERINO, V.; CHIANG, L. (1978) *Arch. Biol. Med. Exp. 11:* 222.
16. ANDREWS, P. (1964) *Biochem. J., 91:* 222-233.

17. BARRETT, A.J. (1977) Proteinases in Mammalian Cells and Tissues North Holland, Biomedical Press, Amsterdam.
18. CHIANG, L.; SANCHEZ-CHIANG, L.; MILLS, J.N.; TANG, J. (1967) *J. Biol. Chem., 242:* 3098-3102.
19. KAGEYAMA, T.; TAKAHASHI, K. (1986) *J. Biol. Chem., 261:* 4406-4419.
20. MORIYAMA, A.; KAGEYAMA, T.; TAKAHASHI, K. (1983) *Eur. J. Biochem., 132:* 687-692.

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A rapid decline in external Ca^{2+} induces Ca^{2+} mobilization in bovine adrenal glomerulosa cells

Una disminución rápida en el Ca^{2+} externo, induce una movilización de Ca^{2+} en células glomerulosas adrenales de bovino

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Bovine adrenal glomerulosa cells perifused with M 199 containing 1 mM Ca^{2+} showed a transient increase in $^{45}Ca^{2+}$ when the external Ca^{2+} was decreased. The efflux observed in the presence of 100 nM angiotensin II was similar to that observed when the external Ca^{2+} was changed from 1 mM to 50 μM. This efflux is the result of the transient activation of the PIP_2 hydrolisis with the subsequent production of inositol-trisphosphate.

INTRODUCTION

The synthesis and secretion of aldosterone in adrenal glomerulosa cells activated by Angiotensin II (AII) is thought to be initiated by the phospholipase C mediated hydrolisis of phosphatidylinosol 4,5 bisphosphate (PIP_2) through a receptor dependent mechanism, presumably involving the participation of a G protein (1, 3). Consequently, two intracellular messengers are formed (4, 5): inositol-trisphosphate (IP_3) and diacyl glicerol (DAG). The first mobilize Ca^{2+} from intracellular stores, presumably located in the endoplasmic reticulum and the second activates the phospholipid and Ca^{2+} dependent enzyme protein kinase C, phosphorylating intracellular receptor proteins (6, 7).

Glycogenolisis activated by agonists that mobilize intracellular Ca^{2+} is mediated by a similar mechanism (8, 10, 11). Experimental evidence supports the concept that, as in the adrenal cells, the first intracellular event coupling stimulus and initiation of the respose is the IP_3-induced increase in cytosolic Ca^{2+} concentration. Koide *et al.* reported (12) that a perturbation in the Ca^{2+} equilibrium between the external space and the cytosol, created by the presence of milimolar concentrations of EGTA, triggered the output of glucose in the liver. This response was paralleled with an increase in efflux of $^{45}Ca^{2+}$ from preloaded cells, which was similar to that observed when. All or vasopresin is used (10). Whether this efflux was related to the methabolism of phosphoinositides has never been adressed. The aim of the present work was to study the possibility that a perturbation generated by the presence of EGTA might activate the accumulation of IP_3 explaining the observation that ^{45}Ca efflux is activated under this condition.

MATERIALS AND METHODS

Cell preparation

Adrenals glomerulosa cells were prepared as described (15) from calf adrenal glands obtained at a local slaughter house. The glands, freed of fat and connective tissue, were sliced in thin sections of approximately 1 mm. The slices were washed twice with medium M199 (Earle's salt solution; Gibco) which unless indicated, contained 1 mM $CaCl_2$ and 4 mM KCl and later incubated in a medium containing 2 mg/ml collagenase (Cappel Biochemical Co) and 4 mg/ml bovine serum albumin (sigma), for 45 min, under 95% O_2 and 5% CO_2, at 37°C, and at a ratio of 1 g of tissue per 10 ml of the described solution. After his incubation period, the mixture of cells and digested tissue was filtered through nylon mesh (125 microns). The supernatant was harvested and the remaining tissue was passed 5 times through Tygon tubing (10 cm long, 0.5 cm inside diameter) attached to a 30 ml plastic syringe. The cells were separated again following the same procedure until a clear solution was obtained. The dispersed cells were taken up in a soft pellet and resuspended in M199, at a concentration of 10^7 cells/ml.

Measurement of [^3H] Inositol-Phosphate Production

Cells at the above described concentration were incubated, at 37°C, with 25 μCi/ml of myo (^{3}H) inositol (American Radiolabelled Chemicals, specific activity 15 mCi/mmol) in 10 ml of M199. After incubation, the cells were washed with 25 ml of medium M199 containing 10 mM inositol and 20 mM LiCl and, where indicated, twice with medium M199 containing 20 mM LiCl and 1 mM EGTA in nominaly Ca-free medium. Free Ca^{2+} concentrations were calculated from the Ca-EGTA dissociation constant given by Burgess *et al.* (9).

Cells were taken up in the same solution and aliquoted into plastic tubes. After a preincubation of 15 min at 37°C under 95% O_2, 5% CO_2 and varying concentration of $CaCl_2$. Cells were then treated with 100 nM AII or 2 mM EGTA (Sigma Chemical Company) as indicated. Incubations were terminated by addition of cold trichloroacetic acid (TCA) at 5% final concentration. The TCA extracts were washed with ether, neutralized and the inositol-phosphates therein were eluted sequentially from Dowex-1 (formate form) anion exchange columns as described by Berridge *et al.* (5).

Cytosolic Ca^{2+} concentration measurement

Cytosolic Ca^{2+} concentration were estimated by monitoring the fluorescence of Quin 2 trapped in adrenal cells, as described by Capponi *et al.* (13). The calibration of Quin 2 fluorescence in relation to the intracellular Ca^{2+} concentration was performed as described (13).

Ca^{2+} efflux experiments

Cells were incubated with 10 μCi/ml of [^{45}Ca] $CaCl_2$ (Amersham international for 2 hrs. After this period cells were centrifugated at 100 x g for 5 min. The soft pellet was taken up in a small volume of fresh incubation solution and aliquots of 300 μl of cell suspension were placed in each of four flow through chambers in a single Lucite block. The block containing the cells was placed in a water bath at 37°C and each chamber perifused with M199 with or without Ca^{2+} were indicated. The fractional efflux was calculated as described previously (15).

RESULTS AND DISCUSSION

Figure 1 (Panel A and B) shows the transient increase in $^{45}Ca^{2+}$ efflux, expressed

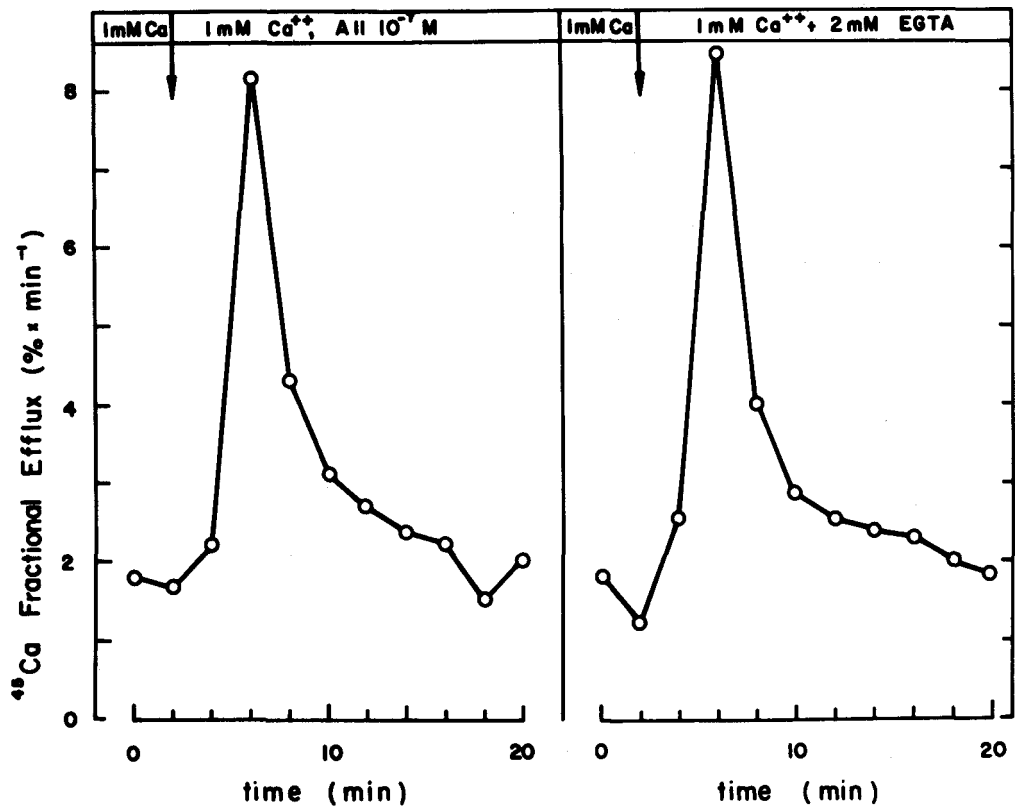

Fig. 1: Efflux of $^{45}Ca^{2+}$ from preloaded bobine adrenal glomerulosa cells. Panel A, cells exposed to 100 nM AII; Panel B, cells exposed to 2 mM EGTA. Cells were obtained and treated as described in Methods. This experiment is representative of several experiments with similar results.

as fractional efflux, when adrenal glomerulosa cells incubated with $^{45}Ca^{2+}$, for 120 min, were perifused with M199 in the presence of AII at a concentration of 100 nM (Panel A) or when the extracellular concentration Ca^{2+} was decreased in the perifusion medium (Panel B). When the external Ca^{2+} concentration was abruptly changed from 1 mM to 1 μM (in the presence of EGTA) a transient increase was observed which is essentially similar to that observed in the presence of AII. In both cases $^{45}Ca^{2+}$ efflux increased rapidly reaching its maximum value at 6 min, which is approximately 5 fold the basal value just before the treatment and decreasing thereafter to a basal $^{45}Ca^{2+}$ efflux. The efflux of radioactive Ca^{2+} is also increased in the presence of verapamil or when the external Ca^{2+} concentration was changed from 1 mM to 0, instead of adding EGTA to the perifusion solution (data not shown). This observation was originally described by Koide *et al.* (12) and is extended to the adrenal cells, and as in the liver, the $^{45}Ca^{2+}$ efflux is also observed in the presence of verapamil or when the external Ca^{2+} concentration was changed from 1 mM to 0, instead of adding EGTA to the perifusion medium (data not shown).

We know now that the mobilization of Ca^{2+} caused by AII in adrenal glomerulosa cells (4) and rat liver cells (10) is the result of the generation of IP_3 from the hydrolysis of PIP_2 induced by the activation of phospholipase C and one likely possibility is that rapid decline in the external Ca^{2+} concentration bypasses the receptor and activates the production of IP_3. Figure 2 shows the effect of decreasing the external Ca^{2+} concentration in adrenal glomerulosa cells previously incubated with [^{3}H]myoinositol for 120 min, treated with 2 mM EGTA, the IP_3 measured later. When EGTA is added to the cell suspension a small, transient increase in IP_3 is observed at 5, 10 and 20 seconds. Under these conditions, AII does not have any effect in the production of IP_3, at 10, 20 or 40 secs, the values observed in the presence of AII are similar to those observed in the control. However, if AII is added to the cell suspension simultaneous with enough Ca^{2+} to

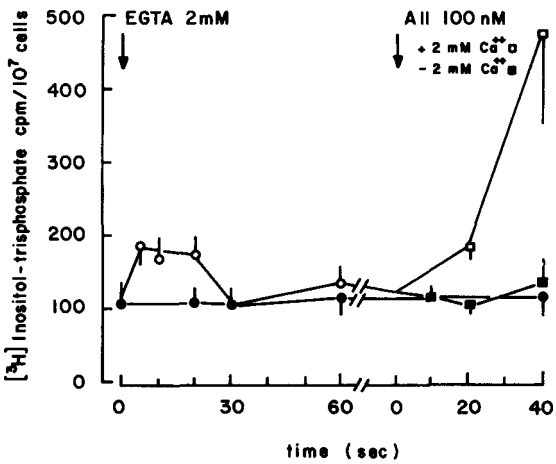

Fig. 2: Time course of Ca^{2+} effects on [^{3}H] inositol-trisphosphate production in EGTA treated cells. [^{3}H] inositol-labeled cells were resuspended in M 199 1 mM Ca^{++} concentration. EGTA 2 mM was added at time 0 and aliquots were taken at the indicated times (○ - ○). Control values (● - ●) were obtained at 0, 60 and 100 sec. 100 nM AII was added simultaneously with (□ - □) or without (■ - ■) $CaCl_2$ and aliquots were taken as indicated in the figure. Results are mean SE of three determinations.

achieve a ratio of 1, a rapid increase in the production of IP_3 was observed.

The dependency on the external Ca^{2+} concentration in the inositolpolyphosphates accumulation is studied in more detail in Figure 3. A range of external Ca^{2+} concentrations is used in the incubation medium when the cells are incubated for 30 min in the presence of 100 nM AII. As shown, when the external Ca^{2+} concentration is below 0.2 μM, AII failed to increase the inositolpolyphosphates formation –above this value the IP_3 accumulation increases. This dependency on external Ca^{2+} has also been documented in liver cells (9) and, as in the adrenal glomerulosa cells, the hydrolysis of PIP_2 seems to require the presence of external Ca^{2+} (Figure 2). When the fluorescence of Quin II, trapped in adrenal glomerulosa cells, is monitored during the action of AII, under conditions in which the external Ca^{2+} concentration is low, the effect of AII is severely impaired. However when the Ca^{2+} concentration increases to 50 μM in the incubation medium, a prompt and clear response is observed (Figure 4).

The experimental evidence documented here seems to be unusual, since the classical view supports the concept that normal

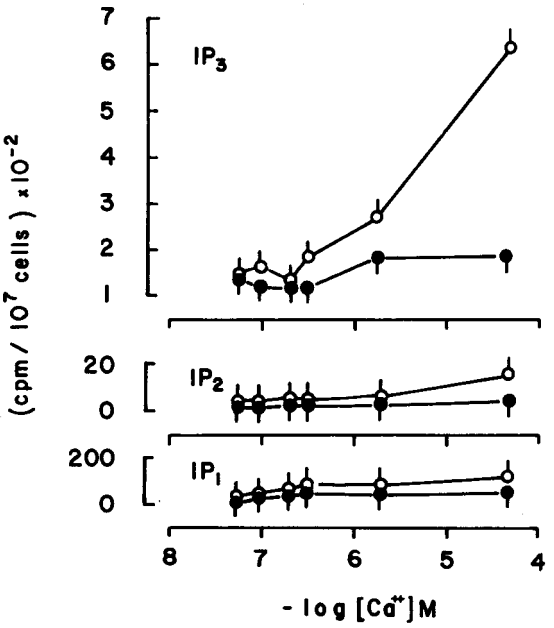

Fig. 3: Effects of AII on inositol-phosphates production at different Ca^{2+} concentration in the incubation medium. [^{3}H] inostol-labeled EGTA washed cells were resuspended in Ca^{2+} free M199 containing 1 mM EGTA. $CaCl_2$ was added to obtain the indicated Ca^{2+} concentration and expressed as-log $[Ca^{2+}]$M. After 15 min preincubation at the indicated Ca^{2+} concentrations, cells were incubated for 30 min with (○ - ○) or without (● - ●) 100 nM AII. Results are expressed as mean ± SE of three determinations.

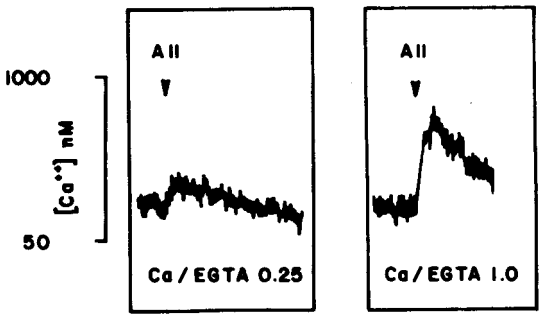

Fig. 4: Effect of different Ca^{2+} concentrations on AII-induced changes in the fluorescence of Quin 2 in EGTA treated adrenal glomerulosa cells. Cells in M 199 1 mM EGTA were resuspended in Ca^{2+} containing medium at the indicated ratios.

physiological Ca^{2+} concentration is required for cellular response. The rapid decline in the external Ca^{2+} concentration has not been described playing a role in the regulation of the metabolism of liver or adrenal glomerulosa cells, but secretion of PTH by the parathyroid cells is increased by a decrease in the external Ca^{2+} concentration and inhibited at normal, physiological Ca^{2+} concentration. Oetting *et al.* suggested (16) that Ca^{2+} may interact with a "membrane receptor" or "sensor" on the plasma membrane, although how this signal is transduced to the cell interior is totally obscure. Two possibilities may account for these results: 1) If a G protein is part of the transducer system in the adrenal glomerulosa cell, it is possible that the rapid decrease in external Ca^{2+} concentration provokes a conformational change in the G protein, causing a transient activation of phospholipase C with the subsequent transient production of IP_3; and 2) That the rapid decrease in external Ca^{2+} concentration is "sensed" directly by the phospholipase C, thus initiating the hydrolysis of PIP_2. Why is the increase in IP_3 production transient? It is possible that IP_3 (1, 4, 5) is produced by EGTA treatment and that it releases Ca^{2+} from the endoplasmic reticulum, however because of the Ca^{2+} sink created in the extracellular space by EGTA, the increase in the cytosolic Ca^{2+} concentration is not observed as shown in Figure 4, but conversely, an efflux of radioactive Ca^{2+} would be observed as shown in Figure 1.

If the cytosolic Ca^{2+} concentration does not increase, this may have several consequences. First, it has been shown that the enzyme PIP kinase of skeletal muscle (18) is activated by an increase in the cytosolic Ca^{2+} concentration and under conditions in which the increase in cytosolic Ca^{2+} concentration is impaired it is likely possible that the PIP_2 pool will not be replenished. Second, if the cytosolic Ca^{2+} concentration remains at a basal level, the production of IP_3 (1, 3, 4) does not take place, because the enzyme inositol-trisphosphate kinase is not activated (17) and a constant activation would not be supported. Third, as demonstrated in Figures 2 and 3, Ca^{2+} is required to maintain a sustained hydrolysis of PIP_2 which is a requirement to support a sustained activation of protein kinase C.

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REFERENCES

1. FARESE, R.V., LARSON, R.E. and DAVIS, J.S. (1984) Endocrinology *114:* 302-304.
2. ENYEDL, P.; BUKL, B.; MUCSI, I. and SPAT, A. (1985) Mol. Cell. Endocrinol. *41:* 105-112.
3. COKCROFT, S. and GOMPERTS, B.D. (1985) Nature *314:* 534-535.
4. KOJIMA, I.; KOJIMA, K.; KREUTER, D. and RASMUSSEN, H. (1984) J. Biol. Chem. *259:* 14448-14457.
5. BERRIDGE, M.J. (1984) Biochem. J. *220:* 345-360.
6. BAUKAL, A.J.; GUILLEMETTE, G.; RUBIN, R.; SPAT, A. and CATT, K. (1985) Biochem. Biophys. Res. Comm. *133:* 532-538.
7. NISHIZUKA, Y.; TAKAI, Y.; KISHIMOTO, A.; KIKKAWA, V. and KAIBUBUCHI, K. (1984) Rec. Progr. Hom. Res. *40:* 301-341.
8. JOSEPH, S.K.; COLL, K.E.; THOMAS, A.P.; RUBIN R. and WILLIAMS, J.R. (1985) J. Biol. Chem. *260:* 12508-12515.
9. BURGUESS, G.M.; MCKINNEY, J.S.; FABIATO, A.; LESLIE, B.A. and TUTNEY, J.W. (1983) J. Biol. Chem. *258:* 15336-15342.
10. PRPIC, V.; BLACKMORE, P.F. and EXTON, J.M. (1982) J. Biol. Chem. *257:* 11323-11331.
11. CHAREST, A.; PRPIC, V.; EXTON, J.M. and BLACKMORE, P.F. (1985) Biochem. J. *227:* 79-90.
12. KOIDE, Y.; KIMURA, S.; TADA, A.; KUGAL, N. and YAMASHITA, K. (1983) J. Pharmacol. *32:* 517-522.
13. CAPPONI, A.M.; LEW, P.D.; JONNOT, L. and VALLOTON, M.B. (1984) J. Biol. Chem. *259:* 8862-8869.
15. FOSTER, R. and RASMUSSEN, H. (1983) Am. J. Physiol. *245:* E281-E287.
16. OETTING, M.; LEBOFF, M.; SWISTON, L.; PRESTON, J. and BROWN, E. (1986) FEBS Lett. *208:* 99-104.
17. PUTNEY, J.W. (1987) Jr. Am. J. Physiol. *252:* G149-G157.
18. MAGENDZO, K. (1986) Ann. Fac. Sci. University of Chile.
19. CARRASCO, M.A.; MAGENDZO, K.; JAIMOVICH, E. and HIDALGO, C. Arch. Biochem. Biophys. (In press).

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Steroidogenesis and ionic permeability in adrenal glomerulosa cells

Esteroidogénesis y permeabilidad a iones en células glomerulosas adrenales

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Over the past several years it has become possible to study some of the electrical properties of excitable and endocrine cells by measuring fluxes of radioactive tracer ions; ^{86}Rb fluxes has been widely used to study potassium permeability. We have validated this approach in adrenal glomerulosa cells, in which we demonstrated the presence of a Ca-dependent K channel that is activated by angiotensin II, ATP, the ionophore A23187 and external K. Here, we present evidence that the steroidogenic response of the bovine adrenal glomerulosa cells is related, in the case of angiotensin II, to the inhibitory effect to the hormone on the coefficient rate of ^{86}Rb efflux that occurs after the initial transient increase. This inhibition of the potassium permeability is probable responsible of the depolarization of the cells. Apamin, that blocks the initial transient raise on ^{86}Rb efflux mediated by angiotensin II, has a minor stimulatory action on the hormone induce steroidogenesis; whereas the opposite is true for the steroidogenic action of potassium ions in the presence of apamin.

The second groups of experiments examined the effect of angiotensin II on ^{86}Rb fluxes when the Ca in the medium was increased from 0.6 to 1.25 mM in the case of bovine glomerulosa cells or angiotensin was assayed in rat glomerulosa tissue perifused with 0.6 mM Ca; in both conditions only the inhibitory effect in ^{86}Rb efflux was observed. When the effect of external ATP on steroidogenesis was examined a significant increase on aldosterone secretion occured probable by a similar mechanism. These results are indicative that Ca-mediated K efflux in adrenal glomerulosa cells may provide a modulatory mechanism for agonist action.

INTRODUCTION

Evidence has been rapidly growing to indicate that a characteristic of agents acting as agonists in endocrine glands is a dramatic effect on membrane permeability. Putney (1), has suggested the term "stimulus-permeability coupling" to describe the sequence of events involved in these response.

As shown by us (2), a variety of agonists increase adrenal glomerulosa membrane permeability to K by inducing a transient increase in ^{86}Rb efflux, that it is apamin sensitive. Among these agents, angiotensin II has a dual effect on K permeability (3) that correlates with changes in membrane potential (4, 5). Also, an increment on external K is capable of increasing ^{86}Rb efflux (3). As it is well known, angiotensin II and potassium ions are the main secretagogues of adrenal glomerulosa cells (6, 8). The purpose of this work has been to correlate the changes in K permeability, already described, with steroidogenesis in bovine adrenal glomerulosa cells. One of the aims of the present work was determine wether agents known to inhibit Ca-dependent potassium permeability could have an effect on the agonist action of angiotensin II and potassium ions on aldosterone production. Also, the effect of ATP on steroidogenesis was measured.

The results indicate that calcium-induced K release in glomerulosa cells is not essential to trigger steroidogenesis but may provide a modulatory mechanism to prevent deleterious effect of abnormally high calcium levels.

MATERIALS AND METHODS

Preparation of dispersed beef adrenal glomerulosa cells. Dispersed bovine adrenal glomerulosa cells were prepared from adrenal glands obtained from

animals slaughtered at a local abattoir (9). The glands were freed of fat and the glomerulosa zone was obtained by slicing thin sections from the outermost portions of the glands. The tissue corresponding to the zona glomerulosa was briefly minced with scissors and washed with standard Krebs Ringer Bicarbonate-glucose (KRBG). The washed tissue was taken up into fresh medium containing 2 mg/ml bovine albumin and 1 mg/ml collagenase (Worthington Biochemical, St. Louis, MO). The tissue was incubated at 37°C for 30 min under an atmosphere of 95% O_2-5% CO_2 in a shaking water bath. Isolated cells were obtained according to the method described by Fredlund *et al.* (10). The cells were collected by centrifugation at 200 g for 5 min at room temperature.

After washing, the cells were incubated in medium containing 0.6 mM Ca^{2+}, unless stated to the contrary, supplemented with 2% albumin. The viability of the cells was checked by their ability to exclude trypan blue (4%). When rat tissue was used, the glands were freed of fat and squezed to obtain capsules with the glomerulosa zone.

Measurements of radiolabeled fluxes. Aliquots of the cell pellet (1 ml) were placed in a volume of 5-10 ml of medium containing 1 μCi/ml or [^{86}Rb] RbCl (Chilean Atomic Energy Commission) and incubated for 60 min at 37°C. At the end of this period, the cells were centrifuged at 500 g for 30 s, the supernatant discarded, and the pellet resuspended in a mixture medium with Sephadex G-10. Aliquots with of cell suspension were placed in plastic perifusion chambers. Each chamber contained a glass wool plug ~10 mm high on which the cells with Sephadex were layered. They were perifused with the medium through a Rainin miniature peristaltic pump. The flow rate through each chamber was 0.5 ml/min and the KRBGA gassed with 95% O_2-5% CO_2 and kept at 37°C. The effluent from each chamber was collected directly into scintillation vials at different intervals of time. At the end of each experiment, the glass wool plug with cells was expelled from each chamber, and analyzed for the respective radioisotope content. The radioactivity of ^{86}Rb was measured by Cerenkov radiation. ^{86}Rb efflux was expressed either as abolute counts per minute or apparent first-order coefficients calculated from the radioactivity release into the effluent media and the remaining in the cells at the cells at the end of the experiment (11).

Incubations of isolated adrenal cells for steroid production. Two different protocols were used: a dynamic system, similar to that described above, or steroid production was measured under static conditions. In this case, one milliliter of medium containing 1.5-2x10^5 cells were incubated in plastic vials at 37°C under 95% O_2-5% CO_2 in a Dubnoff incubator for 1 h. All experiments were performed with triplicate incubation vials. At the end of the incubation, the cells-free media were decanted into tubes and stored at −20°C for aldoterone determination. The aldosterone content in the incubation medium was determined by direct radioimmunoassay of appropriate aliquots of the medium, as previously described (12) .

Statistics. Statistical comparisons were made with Student's t test for paired data.

RESULTS

Effect of angiotensin II and potassium ions on steroidogenesis and ^{86}Rb fluxes. As shown in Fig. I A, the addition of 100 nM angiotensin II to the perifusate bathing bovine adrenal glomerulosa cells produces an initial transient phase of increased ^{86}Rb efflux followed by a second phase in which a sustained inhibition of the radioisotope efflux was observed. Concomitantly with this inhibition on K permeability, a marked

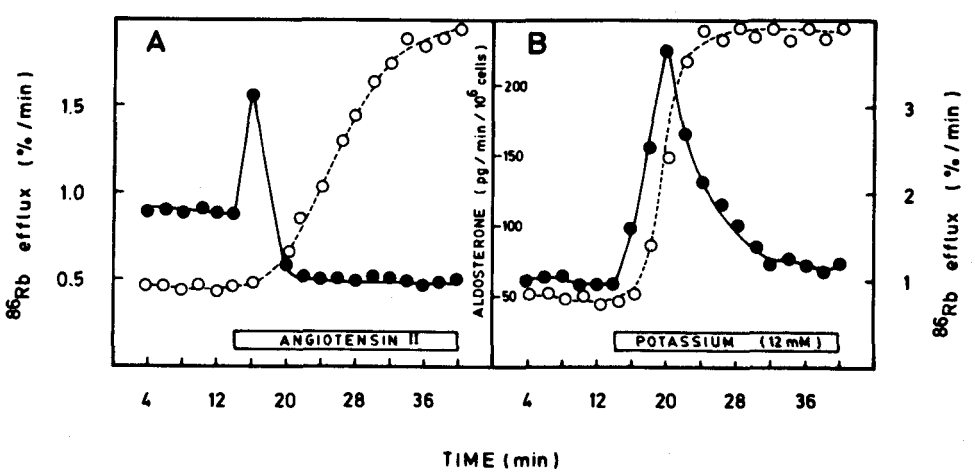

Fig. 1: Time course of the ^{86}Rb release (●) and steroidogenesis (○), from bovine adrenal glomerulosa cells, due to 100 nM angiotensin II (A) or 12 mM K (B). One experiment representative of three is shown. The agonists were present from 14-40 minutes.

increase on steroidogenesis was observed. Figure I B includes the results obtained when external K was increased in the perifusion medium from 4 mM up to 12 mM; under this condition a simultaneous increase on aldosterone production and ^{86}Rb efflux were observed. As in the case of angiotensin II, the effect of 12 mM K on the coefficient rate of ^{86}Rb efflux was transient, but after the peak of ^{86}Rb efflux, the coefficient rates did not return to basal values and remained slightly higher than the initial ones. Typical results from a single representative experiment are shown, and the significance of the results from multiple experiments is indicated in the figure legends or text.

Effect of apamin on the steroidogenic action of angiotensin II and K ions. The effect of apamin, a specific blocker of Ca-mediated potassium permeability, was studied since previous work indicated that apamin complately blocked the initial phase of ^{86}Rb efflux due to angiotensin II without affecting the prolonged reduction of ^{86}Rb efflux causse by the hormone. As shown in Fig. 2, when apamin (10^{-7} M) was added to the perifusate before the addition of 100 nM angiotensin II, the steroidogenic response was even higher than that observed with the hormone alone, added in a parallel column. At the maximal response mean values were: 366±67.1 and 458±76.6 pg aldosterone/min/10^6 cells for angiotensin II alone or plus apamin, respectively (n=4).

The opposite was true when 10^{-7} M apamin was present in the medium containing 12 mM K; apamin had an inhibitory effect on steroidogenesis in each set of parallel columns, but again the inhibitory effect was slightly and was not significant, either under dynamic (mean values± SE were 396±30.5 and 452±30.5 pg aldosterone /min/10^6 cells for K alone or plus apamin) conditions; Fig. 2 B, or with static incubations (Table I).

TABLE 1

Aldosterone production by bovine zona glomerulosa cells in the presence of 12 mM K: effect of apamin

Medium	Aldosterone, ng/10^5 cells	
	Control	Apamin
4 mM K	2.09 ± 0.31	2.55 ± 0.32
6 mM K	2.88 ± 0.81	1.93 ± 0.47
8 mM K	6.32 ± 1.05	5.26 ± 0.97
12 mM K	6.89 ± 1.22	6.26 ± 1.53

Values are means ± SE for aldosterone production under various conditions, 3 to 6 different experiments.

Effect of angiotensin II on the efflux of ^{86}Rb under different conditions. The results shown in Fig. 1 were obtained in cells that were perifused with KRBGA containing 0.6 mM Ca. But when the calcium in the medium was increased to 1.25 mM a different pattern in ^{86}Rb efflux was observed; as shown in Fig. 3 B, 100 nM angiotensin II failed to produce the initial transient phase of ^{86}Rb efflux and was observed only the sustained inhibitory phase on the coefficient

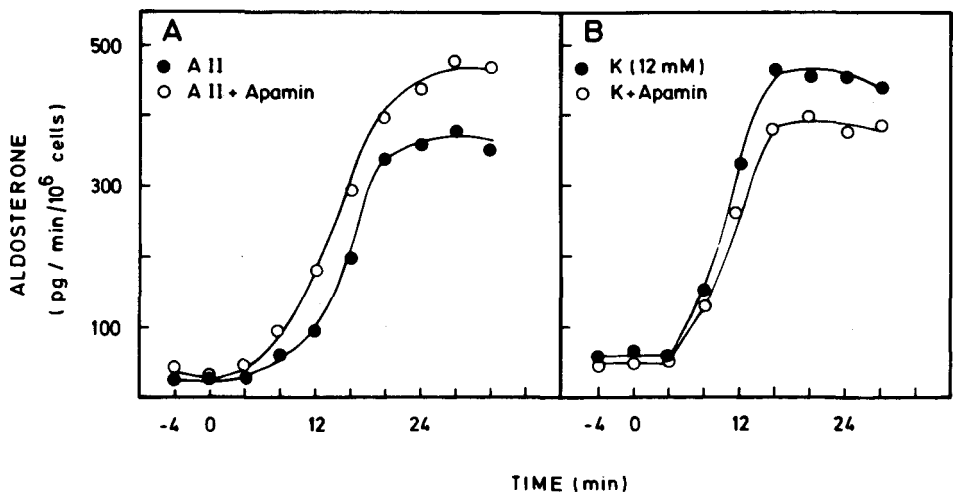

Fig. 2: Effect of 10^{-7} M apamin on the steroidogenic response of 100 nM angiotensin II (A) or 12 mM K (B). Mean values of 4 experiments with the agonist alone or in combination with apamin.

rate of ^{86}Rb efflux. Nevertheless, under these conditions, the steroidogenic response of the glomerulosa tissue is maximal (13) suggesting that depolarization of the plasma membrane is an important step on hormonal action. The same was true when rat glomerulosa slices loaded with ^{86}Rb were subjected to 100 nM angiotensin II in the presence of 0.6 mM Ca, as shown in Fig. 3A, only the inhibition on ^{86}Rb efflux was present. These findings are indicative that the hormone modulates membrane potential by changes in potassium permeability.

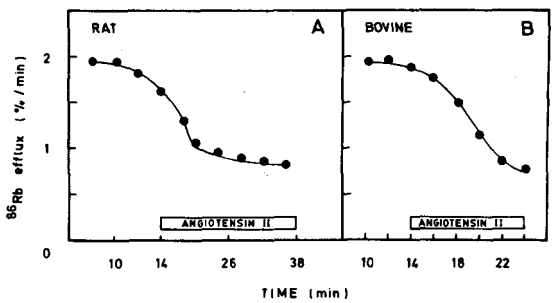

Fig. 3: Time course of the ^{86}Rb release response to 100 nM angiotensin when the hormone was added to rat glomerulosa slices (A) or to bovine tissue that was perifused with 1.25 mM Ca. One experiment representative of 3 is shown, with similar results.

Effect of external ATP on membrane potential and steroidogenesis. Because previous work had shown that external ATP also induces a Ca-mediated K efflux in isolated bovine adrenal cells, the effect of ATP on steroidogenesis was examined. As shown in Fig. 4, when the cells were perifused with a medium containing 10^{-4} M or $5x10^{-4}$ M ATP, a significant increase in aldosterone release was observed ($P<0.05$).

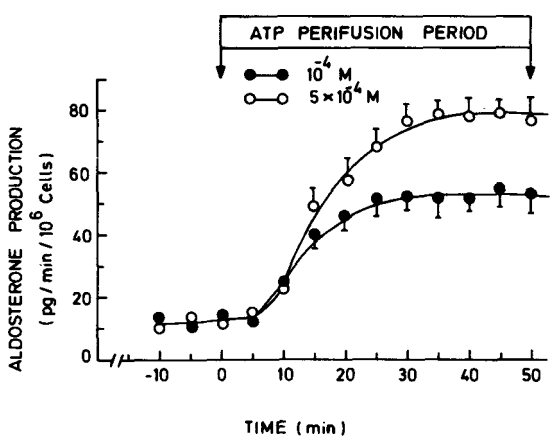

Fig. 4: Effect of external ATP on steroidogenesis from isolated bovine adrenal glomerulosa cells. Mean values ± SE from 3 different experiments.

DISCUSSION

Angiotensin II and K ions are the major hormonal regulators of aldosterone secretion from adrenal glomerulosa cells (7, 8, 14). Recent studies from this laboratory (2) have shown that one of the earliest effects of angiotensin II, together with an enhanced ^{45}Ca efflux, is an increased K permeability. The last effect is apamin sensitive and its role on steroidogenesis was one of the aims of the present study. Also, the effect of the neurotoxic peptide on the potassium-mediated aldosterone production was analyzed, since we have recently shown that an increase in external K also induces an enhanced ^{86}Rb efflux (3) that is partially inhibited by apamin (unpublished observations). Nevertheless, the pattern of ^{86}Rb efflux for both stimuli is different because in the case of angiotensin II a dual responce on K permeability was observed: the transient increase in the rate coefficient ocurring inmediately after the introduction of the polypeptide hormone was followed by a sustained inhibitory effect on ^{86}Rb efflux. It has been postulated that this inhibitory phase could account for the depolarization of the cells and the subsequent opening of the calcium channels.

In fact, as shown in the present results, the inhibition by apamin of the transient increse on ^{86}Rb efflux due to angiotensin II or external K had only minor effects on steroidogenesis. In the case of angiotensin II, and under dynamic conditions, apamin enhanced about 20% the steroidogenic response, whereas the opposite was true with K stimulus. In fact, an small inhibition was observed when external K was increased from 4 to 12 mM; but in any case the effects of apamin were significant. These data would suggest that eventhough the hyperpolarizing effect of Ca-mediated ^{86}Rb efflux may play a role on the control of steroidogenesis, the key step must be related to the depolarizing phase of both stimuli (4, 5). Actually, there are conditions in

which it is possible to see exclusively the inhibitory phase on K permeability, under the action of angiotensin II. As shown in the present results, by increaing external Ca^{2+} to 1.25 mM in the perifusate bathing bovine adrenal cells or rat glomerulosa tissue, angiotensin II has only an inhibitory action on ^{86}Rb efflux. Nevertheless, under these conditions, the steroidogenic effect of angiotensin II is not only preseny but it is more marked that observed at 0.6 mM Ca (13). The lack of activation of a Ca-mediated potassium channels in rat tissue by polypeptide hormones is in agreement with results from other groups working with rat hepatocytes (15).

An additional interesting data was obtained with ATP; we found a significant increase on aldosterone release induce by ATP. Recently, it has been shown that external ATP increase myo-inositol triphosphate (IP_3) in isolated rat hepatocytes (16) and that ATP acting from outside increases Ca uptake (17). However, our results differ from Koritz and Moustafa (18), since these authors found an inhibition of steroidogenesis with ATP in a cell free-system. It is important to denote that the present study was done in a dynamic perifusion system, in which ATP was added to the perifusion medium, but if the cells are incubated with ATP under statics conditions an inhibition of aldosterone production was observed (unpublisned observations). It is interesting to point that the same is true in the case of apamin, as shown previously by us, this drug has rather an inhibitory effect on angiotensin II-mediated steroidogenesis when assayed under static incubations of bovine adrenal glomerulosa cells (2). Further experiments with ATP could be directed to see wether this agent could have an effect on plasma membrane potential, as shown in previous results ATP has a similar pattern on ^{86}Rb efflux to that observed with angiotensin II. Receptor operated Ca-permeable channel activated by ATP was recently described in smooth muscle and mouse macrophage cell line (19, 20).

The present experiments provide evidence that depolarization of adrenal glomerulosa cells is necessary in the sustained steroidogenesic response to different secretagogues. Probably the transient rise in K permeability is a self-regulatory mechanism which protect the cells from excesive Ca entrance mediated by the secretagogues.

REFERENCES

1. PUTNEY, J.W. (1978) Stimulus permeability coupling: role of calcium in the receptor regulation of membrane permeability. *Pharmacol Rev. 30:* 209-245.
2. LOBO, M.V.; MARUSIC, E.T. (1986) Effect of angiotensin II, ATP, and ionophore A23187 on potassium efflux in adrenal glomerulosa cells. *Am. J. Physiol. 250:* E125-E130.
3. LOBO, M.V.; MARUSIC, E.T. (1988) Angiotensin II causes a dual effect on patassium permeability in adrenal glomerulosa cells. *Am. J. Physiol. 254:* E144-E149.
4. FOSTER, R.; LOBO, M.V.; MARUSIC, E.T. (1982) Potassium and angiotensin II induced changes on the membrane potential in adrenal glomerulosa cells. *Arch. Biol. Med. Exp. 15:* R124. (Abstract).
5. QUINN, S.J.; CORNWALL, WILLIAMS, G.H. (1985) Electrophysiological response to angiotensin II by isolated rat adrenal glomerulosa cells. *Biophys. J. 47:* 442a.
6. FOSTER, R.; LOBO, M.V.; MARUSIC, E.T. (1979) Studies of relationship between angiotensin II and potassium ions on aldosterone release. *Am. J. Physiol. 237:* E363-E366.
7. FAKUNDING, J.L.; CHOW, R.; CATT, K.J. (1979) Role of calcium in the stimulation of aldosterone production by adreno corticotropin, angiotensin and potassium in isolated glomerulosa cells. *Endocrinology 105:* 327-333.
8. MULLER, J. (1971) Regulation of aldosterone biosynthesis (Heidelberg, G.; Labhart, A., Ed.), N.Y., Springer-Verlag.
9. LOBO, M.V.; MARUSIC, E.T. (1982) Dissociation of aldosterone response to angiotensin II and potassium in the presence of strontium. *J. Steroid. Biochem. 16:* 629-632.
10. FREDLUND, P.S.; SALTMAN, S.; CATT, K.J. (1975) Aldosterone by isolated adrenal glomerulosa cells: stimulation by physiological concentrations of angiotensin II. *Endocrinology 97:* 1521-1524.
11. PUTNEY, J.W. (1976) Biphasic modulation of potassium release in rat parotid gland by carbachol and phenylephrine. *J. Pharmacol. Exp. Ther. 198:* 375-384.
12. LOBO, M.V.; MARUSIC, E.T.; AGUILERA, G. (1978) Furthers studies on the relationships between potassium and sodium levels and adrenocortical activity. *Endocrinology 102:* 1061-1068.
13. FOSTER, R.; LOBO, M.V.; RASMUSSEN, H.; MARUSIC, E.T. (1981) Calcium: Its role in the mechanism of action of AII and K in aldosterone production. *Endocrinology 109:* 2196-2201.
14. WILLIAMS, B.B.; McDOUGALL, J.G.; TAIT, J.F.; TAIT, S.A.S. (1981) Calcium efflux and steroid output from superfused rat adrenal cells: effect of potassium, adrenocorticotrofic hormone. 5-hydroxy tryptamine, adenosine 3', 5' cyclic monophosphate and angiotensin II and III. *Clin. Sci. 61:* 541-551.

16. HAYLETT, D.G.; JENKINSON, D.H. (1973) Actions of catecholamines on the membrane properties of liver cells in drug receptor, ed by H.P. Rang. University Park Press. Baltimore.
16. CHAREST, R.; PRPIC, V.; EXTON, J.; BLACKMORE, P. (1985) Stimulation of inositol triphosphate formation in hepatocytes by vasopressin adrenaline and angiotensin II and its relationship to changes in cytosolic free Ca^{2+}. *Biochem. J. 227:* 79-90.
17. KRELL, H.; ERMISCH, N.; KASPEREK, S.; PFAFF, E. (1983) On the mechanisms of ATP induced and succinate induced redistribution of cations in isolated rat liver cells. *Eur. J. Biochem. 131:* 247-254.
18. KORITZ, S.; MOUSTAFA, A. (1976) Inhibition of rat adrenal steroidogenesis by adenine containing compounds. *J. Steroid. Biochem. 16:* 629-632.
19. BENHAM, C.; TSIEN, R.W. (1987) A novel receptor operated Ca^{2+} permeable channel activated by ATP in smooth muscle. *Nature 328:* 275-278.
20. STEINBERG, T.H.; SILVERSTEIN, S. (1987) Extracelular ATP^{-4} promotes cation fluxes in the J774 mouse macrophage cell line. *J. Biol. Chem. 262:* 3118-3122.

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Microambiente Hematopoyético: Elementos celulares y de matriz extracelular*

Bone marrow microenvironment

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In bone marrow, cellular stroma together with extracellular matrix (EM) provide an adequate microenvironment for the proliferation and differentiation of hemopoietic progenitor cells. In this article we describe studies on the cell characteristics of a main stromal phenotype, a fibroblast-like cell and its ability to produce in vitro EM components. Comparative studies were performed in fibroblast cultures derived from normal and acute lymphoblastic leukemic (ALL) bone marrow. The grow characteristics of fibroblasts from ALL marrow as well as its capacity to synthetize collagen, fibronectin and GAGs are impaired when compared to fibroblast from normal marrow. Thus, in ALL the impaired production of EM biomolecules by a transient damaged population of stromal cells, may contribute to the development of a defective microenvironment for hemopoiesis.

Estudios tanto en animales de experimentación como en humanos, han señalado que la hematopoyesis ocurre en sitios de la médula ósea en los cuales existe un microambiente (MH) especial, confinado a un tejido intersticial (estroma) y delimitado por una trama de tejido vascular con abundantes elementos reticulares (1).

Las células de estroma junto a factores locales de crecimiento y de regulación y diferentes componentes de matriz extracelular (ME), configuran el microambiente hematopoyético. El MH sirve de "nicho" a la célula troncal hematopoyética modulando su entrada a ciclo desde G^o y su diferenciación (2).

CELULAS DE ESTROMA Y MICROAMBIENTE HEMATOPOYETICO

Entre los fenotipos de estroma hay células "tipo fibroblastos", adipocitos, endoteliales y monocitos/macrófagos. Tanto fibroblastos como adipocitos parecen tener un origen clonal común y con características propias del sistema hematopoyético. Las células endoteliales parecen ser elementos particulares del sistema hematopoyético (3) y no meros constituyentes del sistema vascular asociado. Observaciones recientes, demuestran que en cultivos de médula ósea existen como elementos de estroma linfocitos T maduros, a juzgar por su reactividad con un panel de anticuerpos monoclonales (4).

Los fenotipos antes señalados se corresponden con las células de estroma existentes in vivo en la médula ósea. Estudios histoquímicos y morfológicos practicados in situ en biopsias de médula ósea, revelan una compleja organización celular caracterizada por una definida distribución y relación morfológica de fenotipos de estroma y hematopoyéticos. Entre los primeros se observan células reticulares, endoteliales, macrófagos y adipocitos (5).

Existe poca evidencia acerca del papel particular de cada elemento de estroma en la hemopoyesis; entre éstas, se conoce que:

a) Adipocitos participan en algunas etapas tardías de la granulopoyesis (6) y fibroblastos favorecen la maduración de

* Al doctor Osvaldo Cori, MAESTRO. De sus discípulos, con gratitud.

progenitores tempranos de la eritropoyesis y granulopoyesis a través de interacciones celulares o por la producción de factores de crecimiento (7, 8).

b) Colonias de células de estroma, generadas a partir de médula ósea humana y con características de células endoteliales a juzgar por presencia de cuerpos de Weibel-Palade, son capaces de estimular selectivamente la maduración de progenitores eritroides (BFU-E) (9).

c) La asociación de funciones hematopoyéticas específicas a cada fenotipo de estroma encontraría un elemento "integrador" en el hecho que linfocitos T asociados a monocitos/macrófagos regulan tanto positiva como negativamente la proliferación de progenitores eritroides y granuloides, probablemente vía generación de monoquinas. Estas, a su vez, expresan su efecto hematopoyético vía fibroblastos y células endoteliales (4, 10).

El hecho que la hematopoyesis ocurra en sistemas in vitro, en los cuales no es necesario adicionar factores de crecimiento (Il-3, CSF/GM, CSF/G o EPO), apoya la sugerencia que las células de estroma son capaces de generarlos in situ. Sin embargo, con los bioensayos disponibles, no se ha detectado producción de dichos factores en cultivos de médula ósea.

MATRIZ EXTRACELULAR Y MICROAMBIENTE HEMATOPOYETICO

Las células de estroma de médula ósea, junto a la producción de factores de crecimiento, también producen componentes de la matriz extracelular, entre los que se han detectado colágeno (11), proteoglicanos (12, 13) y fibronectina (14).

La génesis de la ME no parece ser un proceso tipo "todo o nada", sino con adecuadas regulaciones a nivel de las células que la producen y/o degradan. Así, para el caso del sistema hematopoyético, se debería disponer de los tipos de moléculas de matriz adecuadas para satisfacer los requerimientos locales, en el tiempo y en el espacio, relacionados con la migración, proliferación y diferenciación de la célula troncal.

En el sistema murino se sabe que, una vez que el estroma se organiza in vitro y genera ME, recién se inicia la hematopoyesis (15). La inclusión en el cultivo de un análogo de prolina inhibe el depósito de colágeno, con la concomitante alteración en la hematopoyesis (16). Por otro lado, el tratamiento de cultivos de médula ósea con β-xilósidos, que inhiben la síntesis de proteoglicanos, pero no de GAGs, produce un aumento en la producción de células troncales (17). Otra evidencia del papel de ME en la hematopoyesis está dada por la observación que la eliminación selectiva de GAGs de cultivos de células de estroma, bloquea la capacidad de las células troncales de alojarse en ellas con el consiguiente daño en la proliferación y diferenciación hematopoyética (13).

Los datos anteriores indican que la ME no sólo afecta la estructura integral del microambiente hematopoyético, sino que, como ocurre en otros sistemas (18), está influenciando procesos de migración, proliferación y diferenciación celular.

Al ser la hematopoyesis un proceso donde ocurren, en un espacio confinado, eventos de proliferación y diferenciación celular, ambos finamente regulados por la presencia de factores de crecimiento, es evidente que ME y sus componentes participen activamente. Otra característica de la hematopoyesis y que debe estar también relacionada a una ME es el movimiento celular que ocurre y que empieza con el alojamiento de la célula troncal en un espacio preciso y que termina con la salida a la circulación de los linajes hematopoyéticos maduros. La participación de ME en este proceso de reconocimiento y anclaje ha quedado demostrada por el hecho que desde cultivos de largo término de médula ósea sólo es posible recuperar células troncales después de tratar con colagenasa (19) la capa de células adherentes de estroma. Sin embargo, el mecanismo íntimo de este proceso de reconocimiento - anclaje - migración - proliferación y diferenciación no se conoce.

La generación de un microambiente hematocompetente no sólo depende de la síntesis y distribución de los componentes de ME en los espacios peri y extracelular,

sino que también de la posibilidad que tienen las propias células productoras y otras de procesar dichos componentes.

A pesar de que el colágeno intersticial tiene un recambio lento, numerosas actividades colagenolíticas específicas para diferentes tipos de colágeno han sido descritas y adscritas a varios fenotipos celulares similares a los presentes en el estroma de médula ósea. Fibroblastos de piel presentan actividad colagenasa latente o "inactiva" para colágeno I y III (20). Macrófagos liberan una actividad colagenolítica latente que, al ser activada, degrada colágeno V, diferenciándose de otra colagenasa activa que degrada colágeno I (21). Células endoteliales de varios orígenes presentan actividades colagenásicas para colágenos IV y V (22). De gran interés es la existencia de una colagenasa que degrada preferencialmente colágeno I, asociada a granulocitos del tipo de los polimorfonucleares (23).

No se ha determinado aún si las células de estroma de médula ósea producen enzimas degradativas de colágeno, al igual que sus contrapartes celulares en otros tejidos y si estas enzimas juegan un papel en el recambio de colágenos de la ME de la médula ósea.

Existe escasa información sobre presencia de enzimas degradativas de GAGs en fenotipos celulares equivalentes a los encontrados en el estroma de médula ósea. Se sabe, sin embargo, que en cultivos de células epiteliales la presencia de colágeno reduce la degradación enzimática de GAGs (24). Esta observación, asociada al hecho que en fibronectina el sitio de unión a colágeno es igual al sitio de unión a colagenasa, sugiere la existencia de un dinamismo en el establecimiento de la ME. Esto se puede expresar en un cambio en la funcionalidad de la ME para regular formación de membranas basales o para modular procesos de morfogénesis o diferenciación celular.

MICROAMBIENTE HEMATOPOYETICO EN ENFERMEDADES HEMATOLOGICAS

Las características de crecimiento in vitro de células de estroma de médula ósea y su función han sido estudiadas sólo en algunas enfermedades hematológicas.

En anemias aplásticas, cultivos de fibroblastos de médula ósea muestran algunas características comunes con células transformadas (25). Esto sugiere que, en el paciente con anemia aplástica, el agente que ocasiona la enfermedad daña tanto las células troncales hematopoyéticas como las de estroma. La observación que en pacientes aplásticos en remisión después de terapia hormonal, tanto la hematopoyesis como la función del estroma aún sea anormal, apoya la idea de un microambiente dañado profundamente, el cual requiere de un tiempo prolongado para volver a la normalidad.

Al cultivar médula ósea de pacientes con leucemia linfoblástica aguda (LLA) al momento del diagnóstico, se genera una población de células de estroma con características de fibroblastos, en los cuales se observa un crecimiento y función diferente a lo que ocurre en fibroblastos de médula ósea normal. En fibroblastos leucémicos, la proliferación celular no se inhibe por glucocorticoides, como ocurre en fibroblastos normales (26, 27). Este efecto ha sido atribuido a una escasa dotación de receptores para glucocorticoides.

Sin embargo, el efecto de glucocorticoides depende del momento de la terapia anti-LLA en el cual la médula es obtenida y cultivada. Así, se ha visto que médula ósea de pacientes leucémicos, durante quimioterapia, y de pacientes en remisión continua, después de cesasión del tratamiento, generan cultivos de células de estroma, los que se caracterizan por parámetros de crecimiento, de respuesta a glucocorticoides y de dotación de receptores similares a los encontrados en cultivos originados de médula ósea normal (28) (Tabla 1).

Por otro lado, la generación de células grasas o adipocitos, que constituye otro fenotipo del estroma hematopoyético (6), también parece estar alterada en la médula ósea de pacientes con leucemia. Se ha observado que los cambios en la síntesis de triglicéridos por células de estroma de médula ósea (probablemente preadipocitos), proceso mediado por glucocorticoides, se relaciona con su conversión (¿diferen-

TABLA 1

Capacidad proliferativa, respuesta a glucocorticoides (GC), presencia de receptores de glucocorticoides y síntesis de triglicéridos por cultivos de fibroblastos de médula ósea normal y LLA*

Fibroblastos	Capacidad proliferativa (1)	Respuesta a GC (2) %	Receptores GC (3)	Síntesis de triglicéridos (4)
NORMAL	1	50	180	1,6
LLA:				
al diagnóstico	0.7	0	0	1,1
durante terapia	0,7	10	98	
después terapia	1,0	16	78	

* Datos adaptados de referencias N$^{os.}$ 28 y 34.
(1) Valores relativos para la relación de proliferación celular en presencia y ausencia de suero fetal en los cultivos.
(2) Expresada como (%) de inhibición de proliferación celular de cultivos en presencia de GC (10^{-7} M).
(3) Expresado como fmoles de ligando radioactivo/mg prot.
(4) Expresado como razón de incorporación de ^{3}H-palmitato en triglicéridos en presencia y ausencia de glucocorticoides (10^{-7} M).

ciación?) a adipocitos (29). Sin embargo, en células de estroma derivadas de cultivos de médula ósea leucémica al diagnóstico, los glucocorticoides no producen cambios en la síntesis de triglicéridos (Tabla 1). Esta observación, junto a hallazgos de otros autores (30, 31, 32), sugiere que en la condición de leucemia (LLA) existe en la médula ósea una población de células de estroma dañadas en forma reversible, puesto que pueden volver a la normalidad después de una quimioterapia selectiva (28).

Junto a las mencionadas alteraciones en la proliferación celular y en la capacidad de generar adipocitos, la producción de componentes de ME por células de estroma de médula ósea derivada de pacientes con leucemia también está alterada.

Cultivos de fibroblastos de médula ósea humana normal sintetizan y liberan, a lo menos, tres componentes de matriz extracelular: colágeno (11, 33), fibronectina (14) y glicosilaminoglicanos (GAGs) (13, 14).

La síntesis de colágeno, de la misma manera que la exportación con respecto a otras proteínas, es mayor en fibroblastos de médula ósea normal que leucémica. Ambos tipos celulares producen procolágenos I y III; sin embargo, la relación entre ambos es dependiente del origen de las células (normal o leucémica), como también de la densidad celular de los cultivos (33).

La producción de fibronectina (FN) por cultivos de fibroblastos de médula ósea normal y leucémica es también diferente. Aunque ambos fenotipos producen FN, las células normales generan una mayor cantidad que la contraparte leucémica, según evaluación por métodos inmunológicos. Este hallazgo sugiere que en la médula ósea leucémica los fibroblastos presentan un defecto en la síntesis y/o liberación de esta glicoproteína. Esto, a su vez, modificaría en el espacio hematopoyético las características de adhesión y expansión celular de otras células.

Por estudios realizados en este laboratorio, se ha observado una distinta cinética de adhesión y expansión de fibroblastos normales y leucémicos sobre sustratos plásticos en ausencia de FN exógena. Estos resultados apoyan la observación previa, en el sentido de que las células de origen leucémico producen menos fibronectina y/o factor de expansión (spreeding factor).

Finalmente, la producción de glicosilaminoglicanos por cultivos de fibroblastos normales y leucémicos no difiere en cuantía, pero sí en calidad de GAGs sintetizados. El tratamiento selectivo de GAGs liberados al medio de incubación (90 por ciento del total de GAGs sintetizados) con liasas GAGs específicas ha permitido determinar la existencia de cambios importantes en la composición de estas moléculas en ambos tipos de células. De particular importancia es el aumento en el contenido de condroitin sulfato y heparan sulfato en el

medio de incubación de cultivos leucémicos.

En la Tabla 2, se muestra un resumen de los componentes de matriz extracelular producidos por cultivos de fibroblastos de médula ósea normal y leucémica al diagnóstico.

Las células de estroma, que forman el microambiente inductivo, están alteradas en forma transiente en LLA. Esto también implica alteraciones en la síntesis de componentes de la matriz extracelular.

Se ha postulado que el depósito de matriz extracelular (ME) es prerrequisito para el inicio de la hemopoyesis (15), la que empieza con el reconocimiento y anclaje de la célula troncal en el microambiente y luego con su proliferación y diferenciación.

El microambiente, considerado como una estructura dinámica en el espacio y en el tiempo, tiene una organización que permite o induce la proliferación y diferenciación de la célula troncal hematopoyética. La pérdida de esta organización puede ser un factor de génesis de hematopoyesis ineficiente, como parece ser el caso en la médula ósea de pacientes con leucemia linfoblástica aguda.

TABLA 2

Componentes de matriz extracelular producidos por cultivos de fibroblastos de médula ósea normal y LLA al diagnóstico*

Fibroblastos	Colágeno (1)	GAGs (2)			Fibronectina (3)
		HA	CIS	HS	
Normales	100	100	100	100	100
LLA	64	71	200	135	79

* Adaptado de datos en referencias Nos. 14, 33 y 35.
(1) Síntesis de colágeno medida como incorporación de ^3H prolina a material colagenasa-sensible: 100% = 9.000 dpm/10^4 células.
(2) Porcentajes relativos de Ac. Hialurónico (HA), Condroitin Sulfato (CIS) y Heparan Sulfato (HS).
(3) Porcentaje de fibronectina liberada al medio de incubación después de 22 horas de siembra de células en medio libre de suero fetal y determinado por ELISA: 100% = 300 ng/10^5 células.

REFERENCIAS

1. TAVASSOLI, M.; FRIEDENSTEIN, A. (1983) Am. J. Hematol. *15:* 195-203.
2. SCHOFIELD, R. (1983) Biomedicine and Pharmacotherapy *37:* 375-380.
3. IRIE S. and TAVASSOLI, M. (1986) Exp. Hematol. *14:* 912-918.
4. SHIBATA, T. and INOUE, S. (1986) Exp. Hematol. *14:* 234-241.
5. BURGIO, V. L.; MAGRINI, U.; CIARDELLI, L.; PEZZONI, G. (1984) Acta Haematol. *71:* 73-80.
6. DEXTER, T. M; SPOONCER, E.; SCHOFIELD, R.; LORD, B. I.; SIMMONDS, P. (1984) Blood Cells. *10:* 315-339.
7. NAGAO, T.; YAMAUCHI, K.; SHIMIZU, M.; NOGUCHI, K. (1986) Exp. Hematol. *14:* 696-701.
8. TSAI, T.; SIEFF, C. A.; NATHAM, D. G. (1986) Blood. *67:* 1418-1426.
9. CROIZAT, H.; ESKINASI, D.; AXELRED, A. A. (1986) Exp. Hematol. *14:* 324-326.
10. BAGBY, G. C.; McCALL, E.; BERGSTROM, K. A.; BURGE, R. D. (1983) Blood. *62:* 663-668.
11. BENTLEY, S. A. (1982) Br. J. Haematol. *50:* 491-497.
12. WIGHT, T. N.; KINSELLA, M. G.; KEATING, A.; SINGER, J. W. (1986) Blood. *67:* 1333-1343.
13. DEL ROSSO, M.; CAPPELLETTI, R.; DINI, G.; FIBBI, G.; VANUCHI, S.; CHIARUGI, V.; GUAZELLI, C. (1981) B. B. Acta. *676:* 129-136.
14. MINGUELL, J. J.; FERNANDEZ, M.; TETAS, M.; LOPEZ, M. C. (1986) Exp. Hematol. *14:* 581 (Abstr.).
15. ZUCKERMAN, K. S.; WICHA, M. S. (1983) Blood. *61:* 540-547.
16. ZUCKERMAN, K. S.; RHODES, R. K.; GOODRUM, D. D.; PATEL, U. R.; SPARKS, B.; WELLS, J.; WICHA, M. S.; MAYO, L. A. (1985) J. Clin. Invest. *75:* 970-975.
17. SPOONCER, E.; GALLAGHER, J. T.; KRIZA, F.; DEXTER, T. M. (1983) J. Cell Biol. *96:* 510-514.
18. HAY, E. (1981) J. Cell Biol. *91:* 205s-223s.
19. COULOMBEL, L.; EAVES, A. C.; EAVES, C. J. (1983) Blood. *62:* 291-297.
20. WELGUS, H. S.; JEFFREY, J. J.; EISEN, A. Z. (1981) J. Biol. Chem. *256:* 9511-9515.
21. WELGUS, H. G.; CAMPBELL, E. J.; BAR-SHAVIT, Z.; SENIOR, R. M.; TEITELBAUM, S. L. (1985) J. Clin. Invest. *76:* 219-224.

22. KALEBIC, T.; GARBISA, S.; GLASER, B.; LIOTTA, L. A. (1983) Science. *221:* 281-283.
23. HORWITZ, A. L.; HANCE, A. J.; CRYSTAL, R. G. (1979) Proc. Natl. Acad. Sci. *74:* 897-901.
24. DAVID, G.; BERNFIELD, M. R. (1979) Proc. Natl. Acad. Sci. *76:* 786-790.
25. JUNEJA, H,; GARDNER, F. H.; MINGUELL, J. J. and HELMER III, R. E. (1984) Exp. Hematol. *12:* 221-230.
26. MINGUELL, J. J. and MARTINEZ, J. (1983) Exp. Hematol. *11:* 522-526.
27. MINGUELL, J. J.; MARTINEZ, J. and WALTER, T. (1982) Br. J. Haematol. *52:* 307-310.
28. BRUZZONE, M. and MINGUELL, J. J. (1985) Acta Haematol. *73:* 75-79.
29. MARTINEZ, J. and MINGUELL, J. J. (1987) Exp. Hematol. *15:* 221-225.
30. MINGUELL, J. J. (1982) Arch. Biol. Med. Exp. *15:* 457-463.
31. GREENBERG, B. R.; WILSON, F. D.; WOO, L. (1981) Blood *58:* 557-564.
32. BIANCHI-SCARRA, G.; BARRESI, R.; AJMAR, F.; GARRE, C.; RAVAZZOLLO, R.; SESSAREGO, M. (1983) Acta Haematol. *70:* 349-356.
33. FERNANDEZ, M.; MINGUELL, J. J. (1987) Experientia: *43:* 1223-1225.
34. MINGUELL, J. J.; MARTINEZ, J.; BRUZZONE, M. S.; TETAS, M. (1983) Exp. Hematol. *11:* 191 (Abstr.).
35. TETAS, M.; FERNANDEZ, M.; BARAHONA, M. C. (1986) Arch. Biol. Med. Exp. *19:* R258 (Abstract).

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Regulación hormonal heteróloga en glándula mamaria

Heterologous hormonal regulation in mammary gland

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Mammary gland growth and differentiation are largely dependent on a complex and interrelated action of many different hormones which makes the mammary tissue a very suitable one for the study of heterologous hormonal regulation.
This type of control is analyzed by two different approaches: 1. The participation of estradiol in prolactine action during lactation, and 2. The role of glucocorticoids and thyroid hormones in the control of functional activity of rat mammary gland β-adrenergic receptors.

La glándula mamaria, a diferencia de la mayoría de los órganos y tejidos, presenta la mayor parte de su crecimiento después de que el organismo alcanza la madurez, cuando expresa su acción fisiológica, que es la producción de leche. Este proceso que, dependiendo del organismo, requiere una larga preparación (preñez), está controlado por una múltiple interacción hormonal (1, 2). Diversos estudios han señalado que durante la preñez son los esteroides ováricos, estradiol y progesterona, los responsables del crecimiento y diferenciación característicos de este período (3), y la prolactina (PRL), hormona del crecimiento, lactógeno placentario, glucocorticoides y tiroxina, las hormonas responsables del crecimiento y secreción durante el período de lactancia (1, 4). Más recientemente, se ha demostrado también la importante participación del factor de crecimiento epidérmico EGF (5), que actuaría como un estimulador del crecimiento durante la preñez.

Entre los aspectos más interesantes de la glándula mamaria está la posibilidad de estudiar su crecimiento y desarrollo *in vitro*, en cultivo tisular o celular. En este caso, son indispensables en el medio de cultivo las hormonas insulina, PRL y corticosterona (6). El tejido mamario de mediana preñez alcanza luego de 2-4 días de cultivo en presencia de estas hormonas, características de seudolactancia, es decir, producción y secreción de caseína, lactosa y α-lactalbúmina (3).

Nuestro laboratorio ha estado interesado estos últimos años en el estudio de la regulación hormonal heteróloga, que en el tejido mamario es de extraordinaria importancia. Los estudios se han centrado en las hormonas esteroidales, estradiol, glucocorticoides y hormonas tiroideas.

Si bien es cierto que el estradiol presenta su acción a nivel de la preñez, al privar a la glándula de estrógenos durante el período de crecimiento, ésta pierde selectivamente durante la lactancia su respuesta específica a la PRL (síntesis y secreción de caseína y lactosa), manteniendo la respuesta general a dicha hormona así como a las demás (7, 8). Este efecto se estudió en base a la actividad de la enzima γ-glutamil transpeptidasa (γ-GT) de glándula mamaria de rata, la cual es regulada por PRL y es muy activa durante la lactancia (9, 10). Por ello, uno de

A la memoria del profesor Osvaldo Cori Moully, maestro y amigo singular, que supo vivir con plenitud su vocación de humanista y científico curioso de problemas, con los que preparó su mente y la de sus discípulos y colegas para la gran tarea de la investigación y el descubrimiento en nuestro país.

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nuestros objetivos fue estudiar si la γ-GT forma o no parte de la respuesta específica de la glándula a la acción de la PRL en ratas inmaduras ovariectomizadas (OVX)-adrenalectomizadas (ADX) para agotarlas de estrógenos. Se determinó también, como control, la actividad de la lactosa sintetasa.

El otro proceso estudiado, dentro del concepto general de regulación heteróloga, fue la funcionalidad de los receptores β-adrenérgicos en glándula mamaria (16). Nuestro laboratorio describió por primera vez la existencia y propiedades de estos receptores en la glándula, y en el presente trabajo se describirá la regulación de la acción de estos receptores en ratas ADX en presencia y ausencia de hidrocortisona y en ratas hipo e hipertiroideas.

EFECTO DE LA PRIVACION DE ESTROGENOS EN LA RESPUESTA A LA PRL

En las etapas anteriores al ciclo lactogénico, los estrógenos parecen jugar un papel preponderante, ya que, además de participar efectivamente en la normal proliferación de los ductos y alvéolos mamarios, cada vez hay más información acerca de su implicancia en la diferenciación de las células epiteliales. Bolander y col. (8, 11) informaron que células mamarias de ratones hembras vírgenes adultas, privadas de estrógenos, no sintetizan caseína o α-lactalbúmina *in vitro* en respuesta a PRL. Un efecto similar se ha observado en explantes de glándula mamaria de ratas vírgenes adultas privadas de estrógenos desde la pubertad (7); sin embargo, sólo se alteró la acción específica de la PRL relacionada a la síntesis de los componentes de la leche.

La idea de una participación directa del estradiol, en la diferenciación de la glándula mamaria, se ve reforzada por los resultados de la Fig. 1 (12), en que se observan las actividades de la lactosa sintetasa y γ-GT; estos resultados se basan en el siguiente esquema experimental aplicado a ratas inmaduras OVX-ADX y controles (Tabla I).

En la Fig. 1 se observa que los grupos OED y OE.ED, a los que se les administra primero estradiol y luego domperidona, fármaco que actúa a nivel de la hipófisis estimulando la secreción de PRL, se comportan de la misma manera que el grupo SCD, que mantiene sus niveles nor-

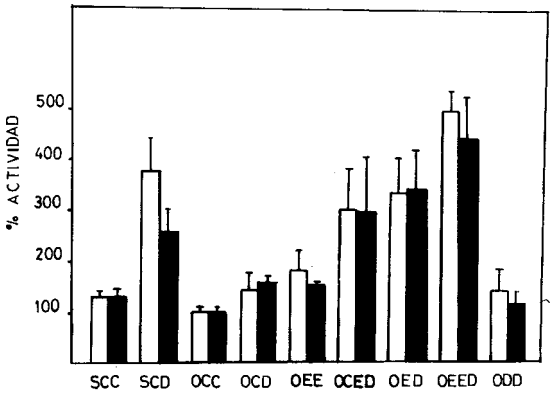

Fig. 1: Efecto *in vivo* de distintos tratamientos con estradiol y domperidona sobre las actividades de la γ-GT (□) y de la lactosa sintetasa (■) de glándula mamaria de ratas vírgenes inmaduras OVX-ADX. Las abreviaturas representan los distintos tratamientos efectuados, descritos en la Tabla I. Los valores corresponden a los $\bar{X} \pm$ E.S. De acuerdo al test "t" de Student, los valores observados para los grupos SCD, OE.ED, OC.ED y OED son significativamente diferentes a los otros grupos estudiados ($P < 0.025$).

TABLA I

Esquema de tratamiento

Grupo	Nº animales	Características	Tratamiento I (4 semanas)	Tratamiento II (1 semana)
SCC	3	Pseudo-operadas	Vehículo	Vehículo
SCD	4	Pseudo-operadas	Vehículo	Domperidona
OCC	3	OVX – ADX	Vehículo	Vehículo
OCD	3	OVX – ADX	Vehículo	Domperidona
OEE	3	OVX – ADX	Estradiol	Estradiol
OE.ED	4	OVX – ADX	Estradiol	Estr. + Domp.
ODD	3	OVX – ADX	Domperidona	Domperidona
OC.ED	3	OVX – ADX	Vehículo	Estr. + Domp.
OED	4	OVX – ADX	Estradiol	Domperidona

Los tratamientos fueron por vía subcutánea. El tratamiento II empezó una vez finalizado el tratamiento I. Estradiol, 1 μg/día; Domperidona 2 mg/día. Vehículo, suero fisiológico - 25% etanol.

males de estrógenos endógenos; todos presentan un marcado aumento de ambas actividades enzimáticas. Sin embargo, no hay respuesta a domperidona cuando las ratas operadas no son previamente tratadas con estradiol.

El estradiol también puede aumentar la secreción de PRL por parte de la hipófisis (13); sin embargo, la manifestación de este efecto no es responsable de los cambios observados en las enzimas estudiadas. Lo anterior se basa en que los grupos tratados únicamente con estradiol o domperidona (OEE y ODD) no presentan cambios importantes en relación a los controles (OCC o SCC).

Los resultados anteriores indican que el estradiol es necesario para que la PRL efectúe su acción sobre la glándula mamaria, aún cuando, como en el caso de la respuesta observada en el grupo OC.ED, no sería necesaria su presencia prolongada. Esto se podría explicar en base a que la acción de los estrógenos sea muy rápida, o bien que actúen en forma sinérgica con la PRL. Se han informado efectos sinérgicos de estradiol y PRL tanto *in vivo* como *in vitro*, indicando una acción directa del estradiol sobre la glándula mamaria (11, 13).

Finalmente, es posible apreciar también que ambas enzimas se comportan de una manera cualitativamente similar, por lo que sería posible seguir la acción de la PRL en la glándula mamaria en base a la determinación de la γ-GT.

REGULACION HORMONAL HETEROLOGA DE RECEPTORES β-ADRENERGICOS

El sistema receptor beta-adrenérgico-adenilato ciclasa ha sido uno de los modelos más utilizados para el estudio de la naturaleza y regulación de los receptores. Básicamente este sistema está conformado por un receptor, una proteína G transductora y por la adenilato ciclasa (15) (Fig. 2).

Dentro de los diversos niveles de regulación molecular, los receptores, entidades macromoleculares capaces de reconocer y discriminar entre las distintas señales del medio externo, constituyen un importante sitio de control, pues al modular su número y/o afinidad por las hormonas circulantes

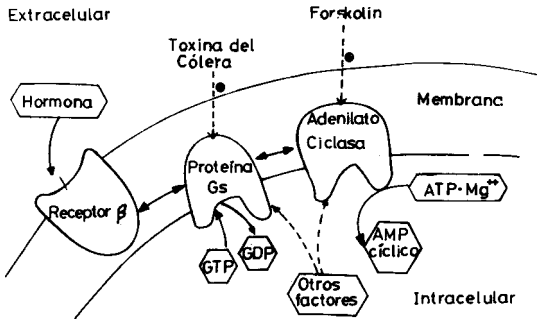

Fig. 2: Sistema receptor β-adrenérgico-adenilato ciclasa.

se está modificando directamente la sensibilidad celular.

Ya que nuestro laboratorio ha descrito recientemente la presencia de receptores β-adrenérgicos en la glándula mamaria (Fig. 3) con características similares a las descritas para otros tejidos (15) y a que ésta es capaz de responder a las catecolaminas, se estudió el efecto regulador heterólogo de los glucocorticoides y de las hormonas tiroideas sobre estos receptores mamarios (16).

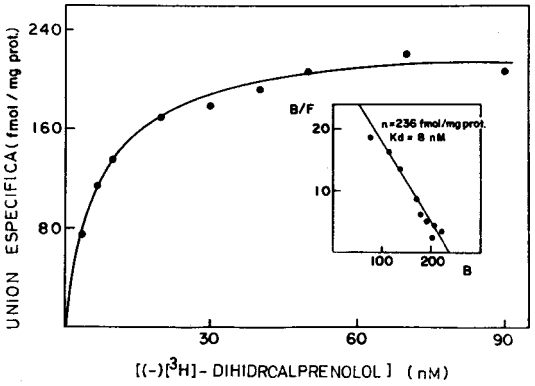

Fig. 3: Curva de saturación y Análisis de Scatchard de la unión de $(-)^3$H-dihidroalprenolol [$(-)^3$H-DHA] a membranas plasmáticas de glándula mamaria (9 días de lactancia). Las membranas se incubaron con concentraciones crecientes de $(-)^3$H-DHA en ausencia (unión total) o presencia (unión inespecífica) de 10 μM de (±) propanolol. Cada valor es el promedio de determinaciones en duplicado. Este es un experimento representativo que se realizó tres veces, en forma independiente. Figura inserta: Graficación de Scatchard de la unión de (–) de ^{3}H-DHA a las membranas de glándula mamaria.

Con este propósito se evaluó el efecto *in vivo* de los glucocorticoides, adrenalectomizando ratas en mediana lactancia y determinando los sitios de unión para el radioligando β-adrenérgico específico, ^{3}H-dihidroalprenolol (^{3}H-DHA), en membranas de glándula mamaria o en células

secretoras aisladas. De los datos de la Tabla II se concluye que los glucocorticoides no regulan el número de receptores β-adrenérgicos, a diferencia de lo que ocurre en otros tejidos controles como el pulmón (17, 18).

La funcionalidad del sistema receptor β-adrenérgico-adenilato ciclasa se determinó en base a la capacidad de producción de AMP cíclico por acción de efectores que actúan sobre los componentes de este sistema: Isoproterenol (a nivel del receptor), toxina del cólera (a nivel de la proteína Gs) o forskolin (a nivel de la adenilato ciclasa). Al ensayar el efecto de los glucocorticoides *in vivo* e *in vitro* se observó un aumento significativo tanto en los niveles basales como en la presencia de los efectores utilizados (Tabla II).

Estos aumentos son independientes del receptor mismo, indicando que el efecto observado puede atribuirse a una regulación en el proceso de acoplamiento entre el receptor y la proteína Gs o entre la proteína Gs y la adenilato ciclasa, o bien a una modulación activadora de la adenilato ciclasa.

El efecto de las hormonas tiroideas sobre el sistema receptor β-adrenérgico-ciclasa mamario se estudió en forma similar al mencionado anteriormente, observándose en ratas hipertiroideas (inducidas experimentalmente por tratamiento por cinco días con tri-yodotironina en una dosis 500 μg/kg peso) un aumento en la constante de disociación. En cambio, en ratas hipotiroideas (inducidas experimentalmente con una dieta deficiente en yodo y la administración simultánea de propiltiouracilo en el agua de bebida) se observó una disminución en la densidad de los receptores beta-adrenérgicos (datos no incluidos).

Ambos estados tiroideos provocaron una disminución en la respuesta a la estimulación beta-adrenérgica determinada en base a la cuantificación de los niveles intracelulares de AMP cíclico.

También trabajos recientes, llevados a cabo en nuestro laboratorio con ratas pregestacionalmente sialoadenectomizadas, parecen indicar que el factor de crecimiento epidérmico (EGF), derivado de la glándula submandibular, juega un importante papel en la expresión de las actividades bioquímico-funcionales y en la regulación heteróloga de los receptores β-adrenérgicos de la glándula mamaria. La sialoadenectomía produjo una disminución en la capacidad de respuesta β-adrenérgica mamaria, medida como AMP cíclico producido, con los efectores isoproterenol (65%), toxina colérica (60%) y forskolin (75%), indicando un compromiso importante a nivel de adenilato ciclasa. También disminuyó la actividad de la γ-glutamiltranspeptidasa y de algunas enzimas lipogénicas durante la lactancia (19).

Es probable, por lo tanto, que el EGF deba ser incluido dentro del grupo de hormonas cuya actividad es esencial para el completo y adecuado desarrollo de la glándula mamaria durante la preñez y que los estudios de regulación heteróloga para las diferentes hormonas y receptores implicados en la diferenciación, crecimiento y función de la glándula mamaria permitan

TABLA II

Efecto de los glucocorticoides (hidrocortisona) *in vivo* sobre el número de receptores beta-adrenérgicos, constante de disociación (Kd) y niveles de cAMP, en glándula mamaria y pulmón de ratas en lactancia

Tejido	Estado	Nº receptores (fmoles/mg prot.)	Kd(nM)	cAMP (pmoles/mg DNA)
Glándula	ADX	134	24	847 ± 33
	ADX + hidrocortisona	140	22	1.464 ± 350
Pulmón	ADX	85	12	246 ± 23
	ADX + hidrocortisona	252	5	641 ± 34

El tratamiento con hidrocortisona (1 mg/kg peso) se hizo por vía subcutánea cada 24 horas, durante 3 días.

dilucidar los complejos mecanismos de control envueltos en el fundamental proceso de la diferenciación y desarrollo celular.

REFERENCIAS

1. TOPPER, J.; FREEMAN, C. (1980) *Physiol. Revs. 60:* 1049-1106.
2. TUCKER, H. (1981) *J. Dairy Sci., 64:* 1403-1430.
3. BANERJEE, M. R. (1976) *Int. Rev. Cytol. 47:* 1-97.
4. HOUDEBINE, L. M. (1985) *Bull. Cancer (Paris) 72:* 121-141.
5. OKAMOTO, S.; OKA, T. (1984) *Proc. Natl. Acad. Sci. 81:* 6059-6063.
6. TOPPER, J.; OKA, T.; VONDERHAAR, B. (1975) in Methods in Enzymology. Vol. 39 (D), (Hardman, J.; O'Malley, B. eds.). *Academic Press, N. York.* pp. 443-454.
7. SANKARAN, L.; QASBA, P.; TOPPER, J. (1984) *Biochem. Biophys. Res. Commun. 125:* 682-689.
8. BOLANDER, F.; TOPPER, J. (1981) *Endocrinology 108:* 1649-1653.
9. PUENTE, J.; VARAS, M. A.: BECKAUS, G.; SAPAG-HAGAR, M. (1979) *FEBS Lett. 99:* 215-218.
10. VILLANUEVA, S.; PUENTE, J.; SAPAG-HAGAR, M. (1987) *Res. Commun. Chem. Pathol. Pharmacol. 57:* 99-105.
11. BOLANDER, F.; TOPPER, J. (1980) *Endocrinology 107:* 1281-1286.
12. RIOS, M. (1986) Tesis de Licenciatura en Bioquímica, U. de Chile.
13. DEIS, R.; DE LOUIS, C. (1983) *J. Steroid. Biochem. 18:* 687-693.
14. LEFKOWITZ, R. J. and STADEL, J. M. (1983) *Ann. Rev. Biochem. 52:* 159-186.
15. LAVANDERO, S.; DONOSO, E.; SAPAG-HAGAR, M. (1985) *Biochem. Pharmacol. 34:* (11) 2036-2040.
16. DEPIX, M. (1987) Tesis de grado para optar al título de Magister en Bioquímica. Fac. de Ciencias Químicas y Farmacéuticas. U. de Chile.
17. FRASER, C. M. and VENTER, C. (1980) *Biochem. Biophys. Res. Commun. 94:* 390-397.
18. DAVIES, A. O. and LEFKOWITZ, R. J. (1984) *Ann. Rev. Physiol. 46:* 119-130.
19. LAVANDERO, S.; PUENTE, J. and SAPAG-HAGAR, M. (1988) *Biochem. Soc. Trans.* (en prensa).

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Identification of insulin binding sites in isolated cells from rat submaxillary gland

Identificación de los sitios de unión de insulina en células aisladas de glándula submaxilar de rata

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Isolated cells from rat submaxillary gland bound ^{125}I-labelled insulin in a time-dependent process that reached a maximum at 30-40 min at 25°C. The radioactivity bound to cells could be dissociated by dilution of the binding site-hormone complex with the incubation buffer.

The presence of unlabelled insulin in the incubation buffer inhibited ^{125}I-labelled insulin degradation according to the amount of hormone added. After 10 min of incubation at 25°C, radioactivity associated to cells was almost exclusively identified as intact ^{125}I-labelled insulin. With increasing times, a greater contribution of final products of degradation in total radioactivity bound to cells was observed; nevertheless, in the presence of unlabelled insulin the radioactivity associated to low molecular weight products markedly decreased.

Equilibrium binding data analysis gave rise to a non-linear Scatchard plot, whose high affinity component showed a dissociation constant of 6.6 ± 0.4 nM.

These observations are consistent with the presence of insulin binding sites in rat submaxillary gland cells which are similar in their characteristics to those identified in other tissues.

INTRODUCTION

It has been postulated that insulin might play a role in the maintenance of the normal structure of the submaxillary glands (growth and histological integrity), being in that way necessary for the glandular function (Liu and Lin, 1969). Anderson and Shapiro (1980) demonstrated that insulin stimulates ^{3}H-leucine incorporation into protein in submaxillary gland slices. This finding, that strongly suggests a direct effect of insulin on the submaxillary gland, was confirmed in our laboratory with the identification of specific binding sites for insulin in rat submaxillary gland microsomes (Scacchi *et al.*, 1983). The properties of these sites are similar to those of insulin receptors already described in other tissues (Cuatrecasas and Hollenberg, 1976). The aim of the present work was to investigate the presence of specific binding sites for insulin in isolated submaxillary gland cells, as a first step in the study of the role of this hormone in the endocrine control of the gland activity.

EXPERIMENTAL

Materials

Na^{125} I (17.4 Ci/mg) was obtained from New England Nuclear, Boston, MA, U.S.A. Insulin from procine pancreas, collagenase from *Clostridium histolyticum* (Type I), hyaluronidase from calf testis (Type III), soybean trypsin inhibitor (Type I-S), bovine serum albumin (Cohn fraction V) and Hepes were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Sephadex G-50 and blue dextran 2.000 were from Pharmacia Fine Chem. AB, Uppsala, Sweden.

All other chemicals were of analytical reagent quality or of the highest purity commercially available.

Animals

Adult male rats of the Long-Evans strain (125-185 g) were used throughout these experiments. They were fed with commercial food and water *ad libitum.*

Iodination and purification of porcine insulin

Porcine insulin was iodinated and purified before use, as already described (Turyn *et al.*, 1985).

Preparation of the Submaxillary cells suspension

The animals were anesthetized with ether and the submaxillary-sublingual complexes were removed

through a ventral midline incision of the neck. The submaxillary glands were separated from the sublingual glands, finely minced with scissors, suspended in the dispersion solution, and incubated in a gyratory water bath shaker (150-200 rpm) at 37°C for 20 min. The dispersion solution consisted of a Ca^{2+} –and Mg^{2+}– free Hanks' balanced salt solution (Hanks and Wallace, 1949) (20 ml/g of tissue) containing 3.5 mg/ml collagenase, 1.0 mg/ml hyaluronidase, 3.5 mg/ml soybean trypsin inhibitor and 15 mM Hepes. The suspension was repeatedly pippeted in order to favor cell dispersion. The suspension was then passed through a nylon cloth (mesh size 77 μm) and cells were collected by centrifugation at 400 rpm for 2 min. The cellular pellet was washed twice with a Ca^{2+} –and Mg^{2+}– and free Hanks' balanced salt solution (15 mH Hepes), and finally resuspended in Hanks' balanced salt solution (15 mH Hepes), pH 8.0, containing 1% (w/v) bovine serum albumin (HBSS) for binding assays.

The cell suspension obtained as described above was kept viable for up to 3 h at 25°C (the viability was always greater than 90%, as measured by exclusion of trypan blue dye). The DNA content of cell samples was determined by the method of Fleck and Munro, 1962.

Measurement of ^{125}I-labelled insulin binding

Binding assays were performed by incubating, for 35 min at 25°C, a tracer amount of ^{125}I-labelled insulin (0.3 - 0.6 ng) with the cell suspension (15-25 μg DNA) in a total volume of 300 μl. Nonspecific binding (^{125}I-labelled insulin bound in the presence of 20 μg of unlabelled insulin) was substracted from the total binding to obtain the specific binding. Polystyrene tubes were used, and the incubation buffer was HBSS, pH 8.0.

Once the incubation was over, ^{125}I-labelled insulin bound to cells was separated from free labelled hormone by centrifugation at 1.400 x g for 10 min at 4°C, after the addition of 4 ml of the ice-cold incubation buffer. The supernatant was discarded, and the tubes kept inverted on a filter paper for at least 12 h at room temperature before counting the radioactivity in a well gamma counter.

Dissociation of the hormone bound to cells

Following the insulin binding assay, cells were collected by centrifugation and resuspended in 20 ml of HBSS buffer. At different times, aliquots of 300 μl of the incubation medium were added to 4 ml of ice-cold HBSS buffer, and the labelled hormone bound to cells was separated from the free labelled ligand, as described above.

Insulin degradation

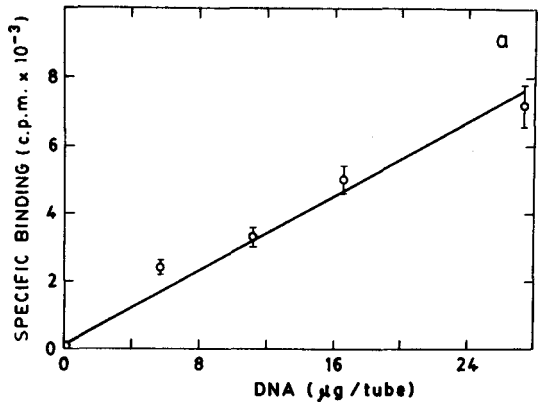
Cells were incubated with ^{125}I-labelled insulin in the presence or absence of unlabelled insulin as previously described. At stated times, ^{125}I-labelled insulin degradation was estimated by two methods: (a) Precipitation with trichloroacetic acid: each sample was centrifuged at 1.400 x g for 10 min at 4°C, and 200 μl of the supernatant were tranferred to another tube, diluted up to 1 ml with ice-cold HBSS buffer and treated with 1 ml of 20% (w/v) ice-cold trichloroacetic acid. The precipitate was separated by centrifugation and the radioactivity present in the supernatant represented degraded ^{125}I-labelled insulin. (b) Gel filtration: each sample was centrifuged twice at 1.400 x g for 10 min at 4°C, after the addition of 4 ml of ice-cold HBSS buffer The resultant pellet was resuspended in 500 μl of a solution of 4 M urea/1 M acetic acid/0.1% (w/v) Triton X-100, and chromatographed on a column (2 x 23 cm) of Sephadex G-50 equilibrated and eluted with the same solution. Fractions of 1 ml were collected. Over 95% of the radioactivity applied to the column was recovered in the eluate.

RESULTS AND DISCUSSION

At 25°C, the specific binding of ^{125}I-labelled insulin to cells showed a linear increase with cell concentration up to 90 μg/ml DNA (Fig. 1a). According to this finding all experiments described below were carried out with 50-80 μg/ml DNA.

Specific insulin binding to cells at 25°C was a time-dependent process that reached its maximum at 30-40 min (Fig. 1b). When the incubation time was higher than 60 min a significant decline in binding was observed, probably due to a binding site and/or hormone inactivation or degradation.

The optimum pH for specific insulin binding to cells was 7.4 - 8.5 (data not shown). ^{125}I-labelled insulin bound to cells was dissociated by diluting the binding site-hormone complex with a 100-fold excess of the incubation medium (Fig. 2). In this condition, approximately a 50% of the bound hormone was dissociated from cells



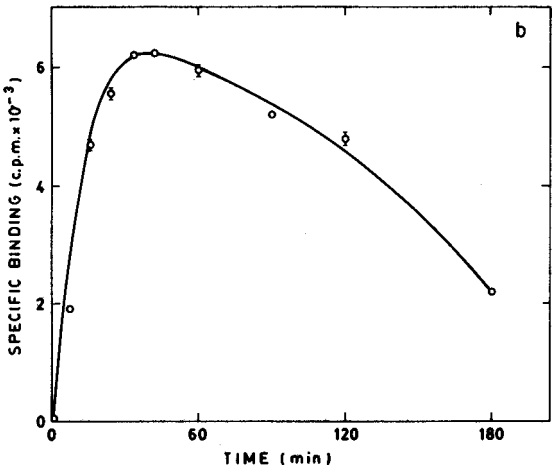


Fig. 1: a) Effect of cellular concentration on specific ^{125}I-labelled insulin binding. ^{125}I-labelled insulin was incubated for 35 min at 25°C with increasing amounts of cells, in the presence or absence of unlabelled insulin. Results are expressed as the mean ± S.E.M. of triplicate experiments. b) Kinetic of association. Cells (23 μg DNA) were incubated for different periods of time at 25°C with ^{125}I-labelled insulin. Specific binding was measured as described. Results are expressed as the mean ± S.E.M. of triplicate determinations.

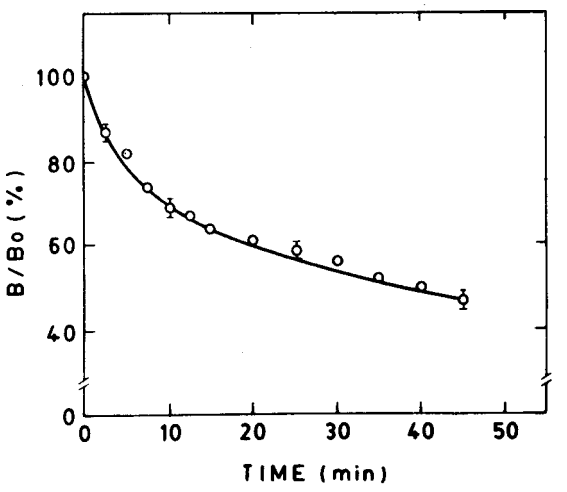


Fig. 2: Kinetic of dissociation. Cells (17 μg DNA) were incubated with ^{125}I-labelled insulin for 35 min at 25°C. Following incubation, cells were collected by centrifugation and resuspended in 20 ml of the incubation buffer. At the stated times, radioactivity associated to cells was determined in aliquots of 300 μl. Bo is the amount of ^{125}I-labelled insulin bound at t=0 min, and B, the amount bound at the indicated time.

in 40 min. The reversibility of the binding reaction suggests that the labelled hormone was bound at specific sites rather than simply adsorbed to the cells. The biphasic character of the dissociation curve suggests that two component can be implicated: a faster one, that could represent hormone associated to the cell surface, and a slower one, probably related to degraded radioactivity released by cells.

Submaxillary gland cells degraded ^{125}I-labelled insulin in a time dependent process (Fig. 3a). The presence of trichloroacetic acid-soluble radioactivity was evident after a 15 min incubation of the cells with the labelled hormone, and progressively increased after this period of time.

Unlabelled insulin added to the incubation medium inhibited ^{125}I-labelled insulin degradation, according to the amount of hormone added (Fig. 3b). The fact that

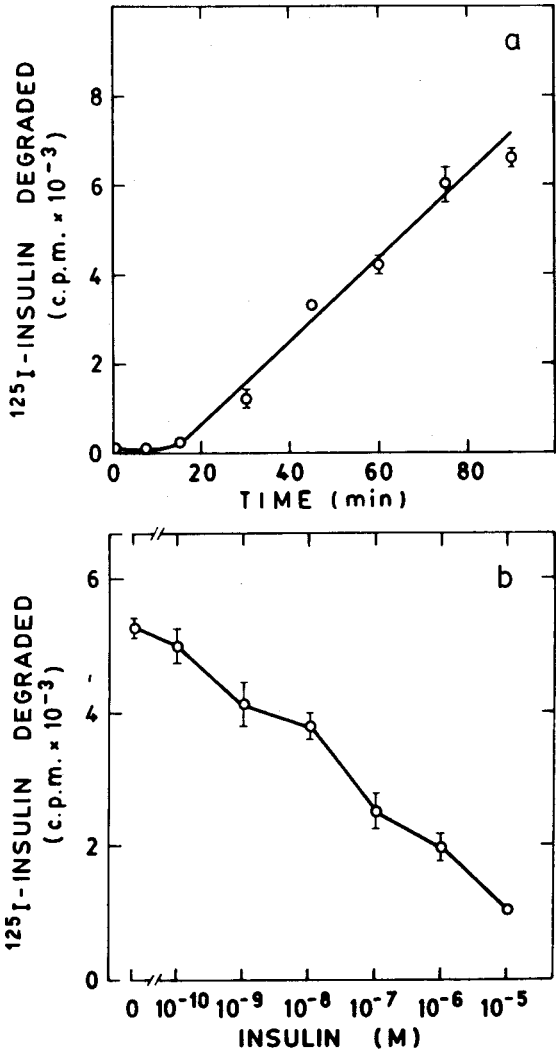


Fig. 3: a) ^{125}I-labelled insulin degradation by rat submaxillary gland cells. Cells (15 μg DNA) were incubated with ^{125}I-labelled insulin at 25°C. At the stated times, the presence of degraded hormone in the incubation buffer was estimated by trichloroacetic acid precipitation. b) Idem a), except that incubation was performed for 60 min in the presence of increasing amounts of unlabelled insulin. Results are expressed as the mean ± S.E.M. of triplicate determinations.

^{125}I-labelled insulin was prevented in the presence of increased amounts of unlabelled insulin is in agreement with previous results, suggesting the existence of a relationship between ligand binding and its intracellular receptor mediated-degradation (Terris and Steiner, 1975; Marshall and Olefsky, 1980; Goldstein and Livingston, 1981). Nevertheless, the possibility of a membrane proteases contribution to ^{125}I-labelled insulin degradation can not be discarded because we have not experimental evidences to assure that hormone degradation is only mediated by the interaction with its specific binding site.

In order to investigate the nature of the radioactivity taken up by the cells, cells were dissolved in a solution of 4 **M** urea/1 **M** acetic acid/0.1% (w/v) Triton X-100 and the resultant solution chromatographed on a column of Sephadex G-50. Figure 4 shows the gel filtration profiles that were resolved in three peaks of radioactivity from left to right: the first one represents material eluting in the void volume, as indicated by the blue dextran 2.000, and can be attributed to aggregates present in ^{125}I-labelled insulin preparations, membranes fragments attached to ^{125}I-labelled insulin or insulin-receptor non dissociable complexes (Katzen and Soderman, 1975). The second peak represents ^{125}I-labelled insulin and the third peak, final degradation products that coeluted with Na^{125}I. The amount of radioactivity associated with the second and the third changed with the time assayed; in the presence of unlabelled insulin the peak of radioactivity in (Vo + Vi) markedly decreased.

^{125}I-labelled insulin binding to cells was inhibited by increasing amount of unlabelled hormone (Fig. 5a). When data were subjected to Scatchard (1949) analysis, a curvilinear plot was obtained (Fig. 5b). This kind of plot is characteristic in insulin-receptor interactions (Kahn, 1976; Baxter and Funder, 1979) and can be ascribed to a variety of causes, the best known being: a) the presence of more than one class of binding sites that have different but fixed affinities or, b) the existence of site-site interactions of the type defined as "negative-cooperativity". By analyzing the Scatchard

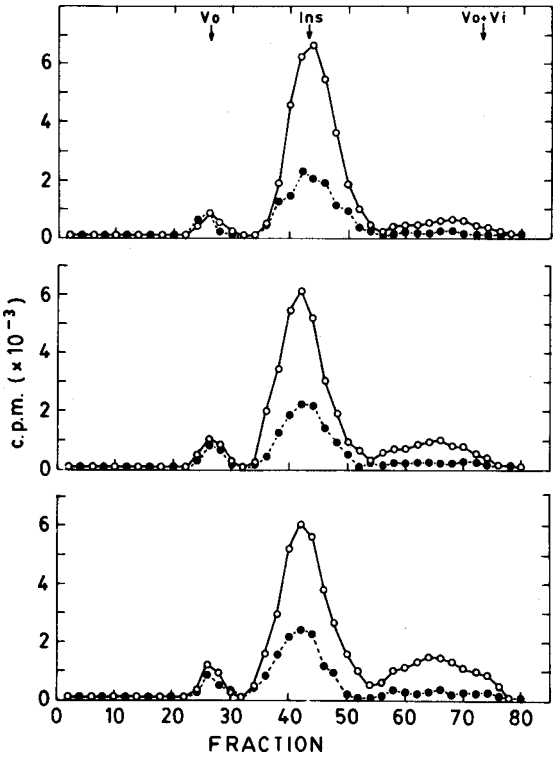


Fig. 4: Nature of the radioactivity bound to cells. Cells (18 μg DNA) were incubated with ^{125}I-labelled insulin in the presence (•) or absence (o) of unlabelled insulin. At different times (10, 20 or 30 min, from upper to lower panel), cells were collected by centrifugation, dissolved in a solution of 4 M urea/1 M acetic acid/1% (w/v) Triton X-100 (UAT), and chromatographed on a column (2 x 23 cm) of Sephadex G-50 equilibrated and eluted with UAT solution. One ml fractions were collected. Arrows indicate the elution volumes of blue dextran 2.000 (Vo), ^{125}I-labelled insulin, recently purified, (Ins) and Na^{125}I (Vo + Vi).

plot according to a two binding sites model, the dissociation constant calculated for the high affinity component was 6.6 ± 0.4 x 10^{-9} **M** (n=3), and is in a good agreement with the values obtained for the high affinity site of the insulin receptor in different cell and membrane preparations (Cuatrecasas and Hollenberg, 1976).

The results presented here indicate the presence of insulin binding sites in rat submaxillary gland cells. This conclusion is consistent with our previous observations in microsomes from rat submaxillary gland (Scacchi *et al.*, 1983) and with the fact that insulin affects the rate of protein synthesis in rat submaxillary gland slices (Anderson and Shapiro, 1980).

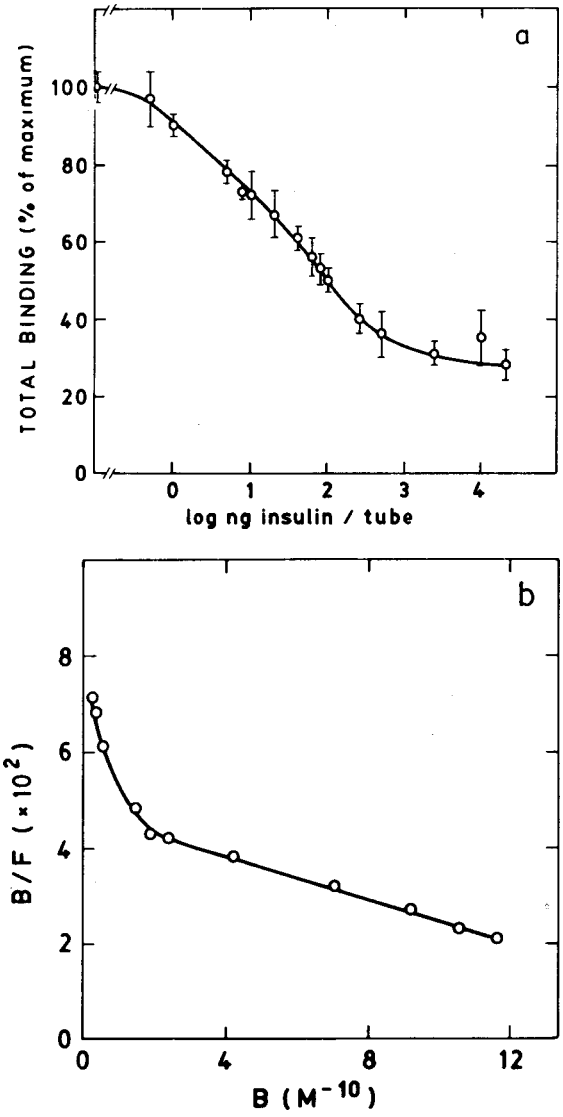


Fig. 5: a) Competition between ^{125}I-labelled insulin and unlabelled insulin for binding to cells. Cells (27 μg DNA) were incubated with ^{125}I-labelled insulin for 35 min at 25°C in the presence of varying amounts of unlabelled insulin. Each point represents the mean ± S.E.M. of triplicate determinations. b) Scatchard plot of the data shown in a). B and F represent bound and free insulin, respectively.

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REFERENCES

ANDERSON, L.C. and SHAPIRO, B.L. (1980) The effect of alloxan diabetes and insulin on the rate of protein synthesis in the rat submaxillary gland. Horm. Metab. Res. *12:* 47-51.

BAXTER, J.D. and FUNDER, J.W. (1979) Hormone receptors. N. Engl. J. Med. *297:* 974-983.

CUATRECASAS, P. and HOLLENBERG, M.D. (1976) Membrane receptors and hormone action. Adv. Protein Chem. *30:* 251-451.

FLECK, A. and MUNRO, H.N. (1962) The precision of ultraviolet absorption measurements in the Schmidt-Thannhauser procedure for nucleic acid estimation. Biochim. Biophys. Acta *55:* 571-583.

GOLDSTEIN, B.J. and LIVINGSTON, J.N. (1981) Insulin degradation by insulin target cells. Metabolism *30:* 825-835.

HANKS, J.H. and WALLACE, R.E. (1949) Relation of oxygen and temperature in the preservation of tissues by refrigeration. Proc. Soc. Exp. Biol. Med. *71:* 196-200.

KAHN, C.R. (1976) Membrane receptors for hormones and neurotransmitters J. Cell Biol. *70:* 261-286.

KATZEN, H.M. and SODERMAN, D.D. (1975) A time-dependent modification of solubilized insulin-receptor complex leading to a loss of reversibility and misinterpreted because of insulin-degrading enzymes. Fed. Proc. *34:* 626.

LIU, F.T.Y. and LIN, H.S. (1969) Role of insulin in body growth and the growth of salivary and endocrine glands in rats. J. Dent. Res. *48:* 559-567.

MARSHALL, S. and OLEFSKY, J.M. (1980) The endocytotic-inte...lization pathway of insulin metabolism: relationship to insulin degradation and activation of glucose transport. Endocrinology *107:* 1937-1945.

SCACCHI, G.E., TURYN, D. and DELLACHA, J.M. (1983) Specific binding sites for ^{125}I-iodo insulin in the rat submandibular gland. *In vivo* and *in vitro* studies. Horm. Metab. Res. *15:* 167-171.

SCATCHARD, G. (1949) The attractions of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. *51:* 660-672.

TERRIS, S. and STEINER, D.F. (1975) Binding and degradation of ^{125}I-insulin by rat hepatocytes. J. Biol. Chem. *250:* 8389-8398.

TURYN, D., SCACCHI, G.E. and DELLACHA, J.M. (1985) Unmasking of insulin receptors in rat submaxillary gland microsomes: effect of high ionic strenght, phospholipase C and S-adenosyl-L-methionine. Biochim. Biophys. Acta *845:* 333-342.

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Mitochondrial DNA and RNA Polymerases. A review

DNA y RNA polimerasas de mitocondria. Una revisión

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INTRODUCTION

All eukaryotic cells are endowed of an extranuclear genetic system confined in two types of organelles: mitochondria in animal and lower eucaryotic cells, mitochondria and chloroplasts in photosynthetic cells. Since the characterization of the "petite mutants" in yeast, it is clear that the integrity of the mitochondrial chromosome is essential for the respiratory function of eucaryote cells (1).

Mitochondria are considered as "semi-autonomous" organelles because the coding capacity of the mitochondrial genome (mt DNA) is very limited; most of the mitochondrial proteins are coded in the nuclear DNA, synthesized in the cytoplasm and imported into the organelles by a well known mechanism which involves the cleavage of a peptide signal at the level of the outer mitochondrial membrane (2). Although the size of the covalently closed circular mt DNA can vary from 15 kb in the case of animal organelles to 2000 kb in the case of some higher plants the number of peptides coded in the mt DNA is fairly constant. About 12-16 hydrophobic proteins are coded in the mitochondrial DNA; most of them are subunits of multimeric enzyme complexes involved in the respiratory function and ATP synthesis (3). The role of the huge excess of mt DNA found in lower eukaryotes and specially in plants, as compared with animal mitochondria, is still unexplained although some of them are due to repeated sequences involved in intramolecular recombination leading to subgenomic molecules which are characteristic of higher plant mitochondria. It is interesting to point out that although an important amount of sequences of chloroplast DNA are found in the mt DNA from higher plants, this plastid DNA is not expressed in the mitochodrial compartment (4).

The mitochondrial genome is replicated, transcribed and translated inside the organelle by enzymes which, as mentioned above, are coded in the nuclear DNA. This fact is clearly exemplified in the case of some yeast petite mutants which posses a mt DNA unable to code for any protein and whose mitochondria contains a normal level of the enzymes involved in the replication and transcription of the mt DNA (5). Other argument in this sense is provided by the fact that the entire sequence of several animal mt DNA have been determined; all the proteins coded in those genomes are known; no enzymes or protein factors involved in mitochondria biogenesis are coded in the sequenced mt DNA (6-8).

Nucleic acids biosynthesis is performed by a universal enzymatic mechanism involving the polymerization of deoxynucleotide triphosphate precursors into DNA by a DNA-dependant DNA polymerase (DNA polymerase) and of ribonucleotide triphosphates precursors into RNA by a DNA-dependant RNA polymerase (RNA polymerase) (9). Both enzymes are absolutely dependant of a single stranded template DNA, the nucleotide incorporated following the Watson-Crick rule (A–T and G–C). An important difference in the

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This article is dedicated to the memory of Profesor Osvaldo Cori from the Universidad de Chile as a humble tribute for his oustanding efforts in the emergence of chilean biochemistry.

mechanism of action of these enzymes is that DNA polymerases cannot initiate DNA synthesis in the absence of a primer oligonucleotide; this primer will provide a free 3' OH end to accept the first nucleotide incorporated by the polymerase; in the case of RNA polymerases no primer is needed, thus, the first nucleotide incorporated by this enzyme will carry a triphosphate group.

Replication and transcription in mitochondria

Very recently it has been shown, thanks mainly to the work of the laboratory of David Clayton, that DNA replication and RNA synthesis are very closely related in the animal mitochondrial compartment. A clear picture with a general scheme of animal mt DNA replication has emerged these last years, first using the electron microscopy approach and more recently using the molecular genetics techniques (10). Figure 1 shows the model proposed by Clayton. The light (L) and heavy (H) strands contain origins of replication located in different regions. DNA replication is initiated by the synthesis of a new strand of the H strand. A mt DNA with a three stranded complex containing a newly synthesized H strand (D-loop for displacement loop) is very abundant in some animal cells and can be easily isolated. When about two thirds of the daugther H strand has been synthesized, the origin of replication of the L strand is unveiled, probably by formation of a hairpin structure, and the synthesis of the L daugther strand can start leading to two circular molecules of mt DNA. Evidences have accumulated that the priming of the daugther H strand and the transcription of the L strand start at the same nucleotide suggesting that the mitochondrial RNA polymerase can act also as the priming enzyme of the H strand (11). What would determine that the newly syntesized RNA from the L strand promoter would become an RNA transcript or a primer of the H strand? The answer seems to be an exciting new endonuclease activity isolated from mouse L-cells mitochondria (12, 13). This activity has been termed RNAase MRP (mitochondrial RNA processing) and contains an endogenous nucleic acid component which is essential for enzyme activity. As expected, the MRP nuclease is coded in the nucleus, and surprisingly enough, the RNA component of this enzyme is also coded in the nuclear DNA; this result opens the question concerning the mechanism of transfer of nucleic acids through the mitochondrial membrane, a phenomenon not described up to now. The study of this process may lead to unexpected advances in the field of organelle genetic transformation. It is also interesting to point out to the possible regulatory role of the nucleus on the mt DNA replication step by controlling the activity of the MRP nuclease. Priming of the L strand seems to follow a completely different mechanism, since the RNA which acts as initiator is probably synthesized by a particular type of RNA polymerase called DNA primase (see below) (14). The detailed mechanism of mt DNA replication in yeast and plants mitochondria is not known, but probably will follow a similar pattern with some differences related to the size genomic discrepancies. Thus multiple replication origins have been described in yeast mt DNA and good evidences exist that, as in the case of animal mt DNA, replication and transcription of the yeast mt genome have the same initiation point (15, 16). Specific sequences involved in the origin of DNA replication have been described in yeast. Sequences similar to the yeast *ars* (for autonomous replication sequences) have been described in yeast and plant organelles but no direct proof has been provided that the *ars* sequences can act as replication origins elsewhere than in yeast.

As described above, mt DNA replication is an assymetrical process, while mt transcription is perfectly symetrical (17). RNA synthesis proceeds from the L and H strands promoter to give raise to a polycistronic transcription product which is further processed at the level of the different tRNAs which act as recognition signals. As mentioned above the transcription product of the L strand can act as

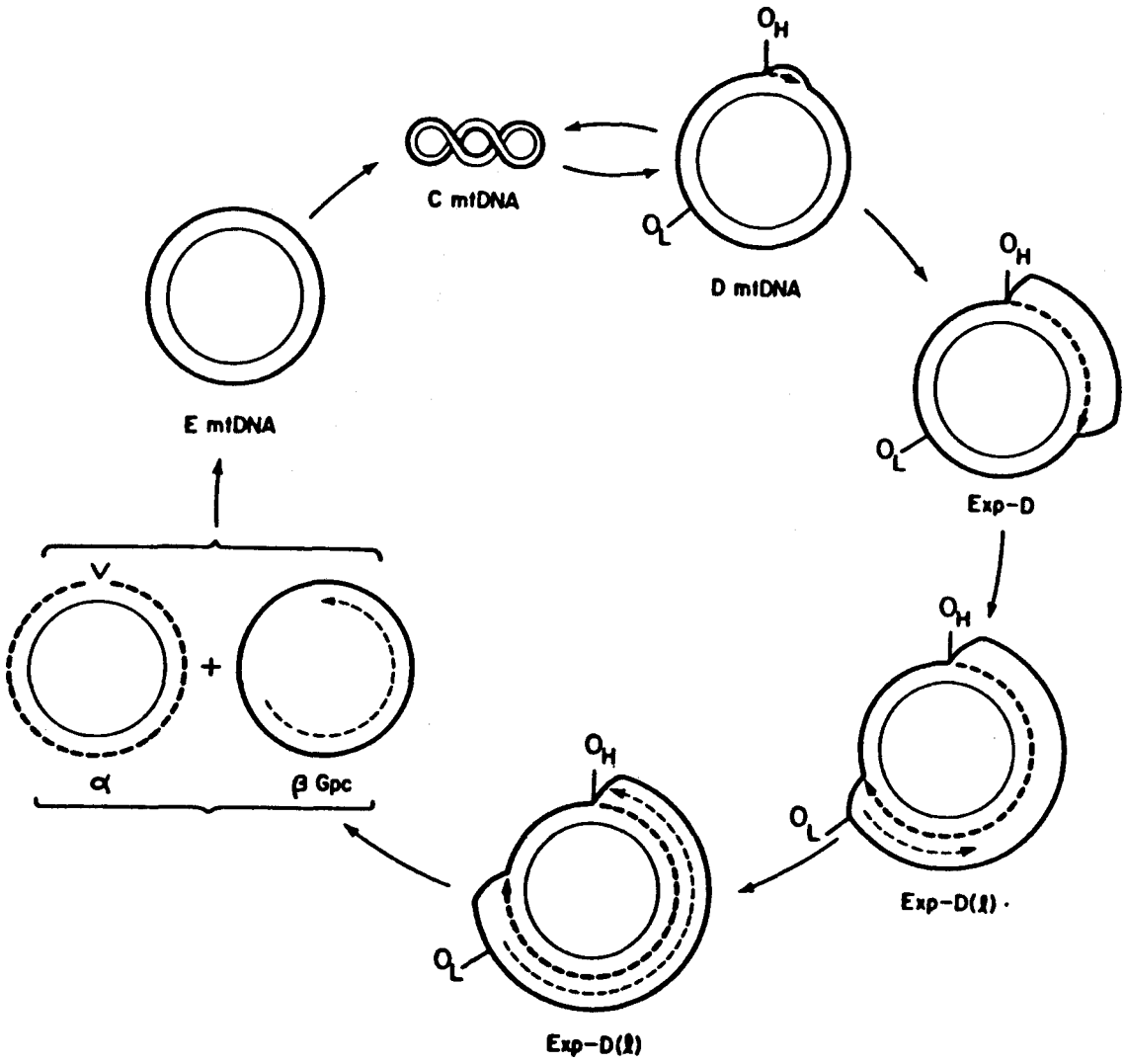

Fig. 1: Replication Model for Animal mt DNA (10). Thick solid lines: parenteral heavy (H) strands. Thin solid lines: parental light (L) strands. Thick dashed lines: daughter H strands. Thin dashed lines: daughter L strands. The order of replication is clockwise starting at D mt DNA. O_H and O_L: origins of H –and L– strand synthesis respectively. The double arrows reflect the metabolic instability of D-loop strands and consequent equilibrium between D mt DNA and C mt DNA. Expanded D-loop replicative intermediates are termed Exp-D prior to initiation of L-strand biosynthesis and Exp-D(ℓ) after initiation of L-strand synthesis. β Gpc: gapped circular daughter molecule. Both α and β daughter molecules are then converted to closed circles (E mt DNA) with few if any superhelical turns. Approximately 100 negative superhelical turns are then introduced into these E mt DNA molecules resulting in the formation of C mt DNA. This serves as the template for the formation of D mt DNA (D-loop mt DNA) which completes the cycle.

mRNA, as well as primer for DNA synthesis, while the only known function of the RNA product of the H strand is like mRNA.

Mitochondrial DNA polymerase

A unique DNA polymerase has been isolated and characterized from animal mitochondria. In the nomenclature used for the multiple DNA polymerases found in animal cells the mitochondrial enzyme is called DNA polymerase gamma. DNA polymerases alpha and beta are located in the nucleus and they are involved respectively in the replication and repair of the nuclear genome (18). In some specific tissues a fourth DNA polymerase called delta has been described, the latter polymerase is very similar in its properties to DNA polymerase alpha but an exonuclease activity is found in the same subunit carrying the polymerase activity (19). Very recently it has been proposed that

DNA polymerase delta is identical to a dissociated form of DNA polymerase alpha since a cryptic exonuclease activity is revealed when the multimeric DNA polymerase alpha complex is dissociated (20).

When DNA polymerase gamma was first described in 1972 (21), the property of recognizing very efficiently some synthetic polyribonucleotides as templates prompted the comparison with the retroviral RNA-dependant DNA polymerase (reverse transcriptase) which had been described two years before (22). Although the ability to use preferentially poly rA-oligo dT or poly rC-oligo dC is a general property of all animal mitochondrial DNA polymerases described up to now, it is clear that reverse transcriptase and DNA polymerase gamma are unrelated proteins, as shown using antibodies directed against both enzymes, as well as by the fact that the mitochondrial polymerase is not able to use natural RNAs as templates (23). DNA polymerase gamma is not only found in the mitochondrial compartment but it has been also characterized in the soluble cytoplasm and in the nuclear fraction (24). Whether the presence of this enzyme in the nucleus is a contamination from mitochondrial leaking, or it is nuclear and plays a specific function in this compartment remains to be established. For some time it was believed that polymerase gamma found in the nucleus was involved in the replication of the adenovirus genome, but recently it was shown that this virus is replicated by its own DNA polymerase which shares many properties with DNA polymerase gamma (25). A role of polymerase gamma in the replication of parvovirus DNA has been suggested (26). Parvovirus must be replicated by a host cell DNA polymerase, since the genome of these viruses is too small to code for its own polymerase.

The mitochondrial DNA polymerases from HeLa (27), EMT-6 (28) and synaptosomal cell (29), as well as from Xexopus oocytes (30), drosophila (31) and chicken embryos (32, 33) have been studied in some detail. Only the latter two enzymes have been purified to apparent homogeneity; thus, it has been proposed that chicken embryo mt DNA polymerase is a tetramer formed of four identical subunits of 45 Kd, while the drosophila mitochondrial DNA polymerase seems to be an heterodimer with subunits of 125 and 35 Kd molecular weight. Under non denaturing conditions the chicken and drosophila polymerases have similar sizes (160-180 Kd).

The use of inhibitors affecting nucleic acids biosynthesis is a very useful tool for the characterization of DNA polymerases. Aphidicolin, which is a very specific inhibitor of DNA polymerase alpha, does not affect the activity of polymerase gamma. The use of this inhibitor showed that DNA polymerase alpha was not involved in mt DNA replication (34). The deoxynucleoside triphosphate analog, dideoxy TTP (ddTTP), is a very strong inhibitor of mitochondrial DNA polymerases under conditions where polymerase alpha is not affected. It has been shown that ddTTP inhibits DNA polymerase gamma by acting as a chain terminator, since this enzyme can incorporate the analog as efficiently as dTTP (35). The DNA intercalating agent ethidium bromide also inhibits mitochondrial DNA polymerase more strongly than polymerases beta and alpha. We have shown that the effect of this type of inhibitor cannot be explained only on the basis of its interaction with the template, since in the presence of the same DNA template, mitochondrial DNA polymerase from mice cells was more inhibited than polymerases alpha and beta from the same source (28).

Mitochondrial DNA polymerases other than those purified from animal cells have some properties that make it difficult to classify them as typical gamma DNA polymerases. Thus mitochondrial DNA polymerase from yeast and plant cells (36-39) recognize very poorly a poly rA-oligo dT template. However, it is important to point out that the ability of a DNA polymerase to use a specific template may be very dependant on the assay conditions (temperature, cation,

ionic strength, etc.). The answer to different inhibitors showed a similar pattern for animal, yeast or wheat mitochondrial DNA polymerases. All these polymerases are strongly inhibited by ddTTP and ethidium bromide and are resistant to aphidicolin. The size of the native yeast DNA polymerase determined by sucrose gradient centrifugation is 120 to 130 Kd while a unique band of 60 Kd was observed by SDS-polyacrylamide electrophoresis. The native wheat mitochondrial enzyme has an apparent size of 180 kd (38).

The mitochondrial membrane seems to play an important role in the replication of the organelle genome. Thus, we have found that part of wheat mitochondrial DNA polymerase activity is found associated to a very high molecular weight complex. Electron microscopy observations, as well as the protein composition of this complex, indicate a possible membrane localisation. Associated with this complex we have also found the mitochondrial RNA polymerase, suggesting that this enzyme may play an important role in mt DNA replication, and a topoisomerase activity, as well as mitochondrial DNA (our unpublished results). Similar evidences for a role of the mitochondrial membrane have been described in lower eukaryotes and animal cells (40, 41).

Mitochondrial DNA dependant RNA polymerases

Specific mechanism regulate the order and level of expression during the transcription of nuclear genes. Transcription (RNA synthesis) is performed essentially by a DNA dependant RNA polymerase. Regulation of the activity of this enzyme is acomplished by several protein factors associated to it. The basic enzymatic mechanism of all RNA polymerases described up to now is the same: recognition and binding to a promoter region of DNA, initiation and elongation of an RNA strand by complementary copying of the template DNA, termination and release of the newly synthesized RNA strand.

Three distinct RNA polymerases are involved in nuclear DNA transcription. RNA polymerase I or A synthesizes the precursors of ribosomal RNAs 28S and 18S; this enzyme is localized in the nucleolus. RNA polymerase II of B is involved in the synthesis of messenger RNA. RNA polymerase III or C transcribes the tRNAs and 5S genes (42). In the case of mitochondria the organelle genome is transcribed by only one RNA polymerase. The three nuclear RNA polymerases are characterized by their large size, between 500 and 650 Kd, as well as by the number of subunits, between 10 and 15.

The inhibitor alpha amanitin has been extremely useful in characterizing the nuclear activities, since this drug inhibits RNA polymerase II at very low concentrations, while the activity of RNA polymerase III is about 100 times less affected and RNA polymerase I is completely resistant to alpha-amanitin. The promoter sequence recognized by RNA polymerase III is found inside genes coding for tRNAs and 5S RNA, while the promoter of RNA polymerase II is the heptanucleotide TATAAAT (TATA box) located at about 12-32 nucleotides upstream of the transcription start (17). As mentioned above animal mitochondrial RNA is synthesized as a unique transcript, thus only one promoter should be found in each strand. The huge size of yeast and plant mitochondrial genomes suggest the existence of several transcripts. Sequence analysis of mitochondrial genes from these organisms has allowed the finding of a consensus promoter sequence (43, 44).

Mitochondrial RNA polymerases are coded in the nuclear genome. As in the case of mt DNA polymerases they are synthesized in the cytoplasm and imported into the organelles. The extensive purification of human (45) and yeast (46) mitochondrial RNA polymerase shows striking differences with the nuclear enzymes. Human mitochondrial RNA polymerase sediments at approximately 8S which is in the range of the sedimentation values of 6-7 obtained for the RNA polymerases from *N. crassa* (47), *Xenopus laevis* (48, 49) and yeast (46). Data from these groups

indicate that the mt RNA polymerases are probably multimers of single polypeptide chains of 45-64 Kd. Although no specific polypeptide has been associated with human mt RNA polymerase, a polypeptide of about 60 Kd is the most abundant in the highly purified enzyme preparation. However, it has been recently reported a yeast mt RNA polymerase which after purification to near homogeneity showed a 145 Kd polypeptide band by SDS-electrophoresis (50). It is important to consider, concerning the latter finding, that the 145 Kd enzyme was purified from a total yeast extract, ommiting a previous mitochondria purification step. Moreover, the assay used by these authors to follow the mt RNA polymerase activity is typical of a DNA primase (see below). Whether the 45 Kd yeast mt RNA polymerase, described before, is a proteolytic product of the 145 Kd enzyme, or if the latter enzyme coresponds to a nuclear or a mt DNA primase activity remains to be established. Using an antisera directed against the purified 145 Kd RNA polymerase a library of yeast genomic inserts constructed in the expression vector lambda gt11 has been immunoscreened. A 4 Kb yeast DNA fragment appears to contain most or all of the gene encoding the 145 Kd catalytic subunit (51). In higher plants the mt RNA polymerase has an hydrophobic nature; in the presence of the nonionic detergent Triton X-100 a 50 Kd active enzyme form was obtained by sucrose gradient centrifugation or gel filtration (52).

The effect of KCl concentration on the activity also distinguishes animal, yeast and plant mt RNA polymerases from the nuclear RNA polymerases. The activity of the mitochondrial enzymes is progressively inhibited by increasing salt concentrations. The mt RNA polymerases require the presence of Mg^{+2} for optimal activity while Mn^{+2} inhibits their activity. A notable feature of the human mt RNA polymerase is the requirement for ATP at a much higher concentration (15-20 fold) than that for the others NTPs (53). This optimum cannot be explained by an increased degradation of the nucleotide. At the present time it is not possible to determine if the high requirement of ATP corresponds to the initiation or elongation step of RNA synthesis, although some preliminary experiments indicate that high ATP requirement may be related to a high Km for the process of initiation. As the ATP concentration in HeLa cell mitochondria is about 0.8 mM, i.e. close to the optimum ATP concentration for *in vitro* transcription obtained in that system, it is appealing to think that the mt RNA polymerase has evolved to function in a high ATP environment.

No specific inhibitor of mt RNA polymerases has been described. These enzymes are resistant to alpha-amanitin and rifampicin, a specific inhibitor of the single RNA polymerase found in procaryotic organisms, although a derivative of rifampicin: 3-formylrifamycin SV 0-n-octyloxime (AF/013), has proven to inhibit animal mt RNA polymerase (48). However, this derivative has no effect on yeast or plant mt RNA polymerases (our own unpublished results).

Partially or highly purified RNA polymerases are able to copy efficiently several type of DNA templates but generally they show a lack of specificity concerning promoter recognition. In the case of procaryotic RNA polymerases a whole set of protein factors (sigma factors) confer a high degree of specificity to the polymerase for the transcription of a given gene (54). An analogous system may operate in eukaryotic cells since a multicomponent mt RNA polymerase has been isolated from a whole extract of bakers' yeast (55). Under *in vitro* incubation conditions this activity is able to recognize the consensus mitochondrial promoter sequence ATATAAGTA. Using promoter-containing templates these authors have been able to chromatographically separate the mt specific RNA polymerase from the nuclear RNA polymerases.

DNA primase

As mentioned in the first part of this review a peculiar RNA polymerase activity,

called DNA primase, is involved in the synthesis of short primers necessary for the initiation of DNA synthesis. Nuclear DNA primases have been characterized in several organisms but very few data is available on mt enzymes (18). Animal primases have been characterized from human cells (56) and rat liver mitochondria (57). Although they have not been extensively purified they can be distinguished from the classical mt RNA polymerase by the size of the transcription products, as well as the effect of KCl which inhibits more strongly the mt RNA polymerase than primase. The latter enzyme can be separated from RNA polymerase and DNA polymerase gamma by sucrose gradient centrifugation at relatively high ionic strength. The easy separation of DNA polymerase gamma and the mt DNA primase points out to an important difference with the situation in animal nuclei, since the association of DNA polymerase alpha and the nuclear primase is extremely strong and a chromatographic separation can be achieved only under drastic conditions (58). A primase-like activity has also been found in wheat mitochondria but the enzyme has not been further characterized (39).

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REFERENCES

1. TZAGOLOF, A. (1982) In Mitochondria. Plenum Press.
2. HURT, E.C., and VAN LOON, A.P.G.M. (1986) *Trends in Biochem. Sci. 11:* 204-207.
3. TZAGOLOFF, A. and MYERS, A.M. (1985) *Ann. Rev. Biochem. 55:* 249-285.
4. STERN, D.B. and LONDSDALE, D.M. (1982) *Nature 299:* 698-702.
5. EPHRUSSI, B.; HOTTINGER, H. and CHIMENES, A.M. (1949) *Ann. Inst. Pasteur 76:* 351-368.
6. BERNARDI, G. (1979) *Trends in Biochem. Sci. 4:* 197-201.
7. BIBB, M.J., VAN ETTEN, R.A.; WRIGHT, C.T.; WALBERG, M.W. and CLAYTON, D.A. (1981) *Cell 26:* 167-180.
8. ANDERSON, S., BANKIER, A.T., BARRELL, B.G. DE BRUIJN, M.H.L., COULSON, A.R.; SANGER, F. *et al.* (1981) *Nature 290:* 457-465.
9. KORNBERG, A. In DNA Replication. Freeman. 1980 and 1982 Supplement.
10. CLAYTON, D.A. (1982) *Cell 28:* 693-705.
11. CHANG, D.D.; HAUSWIRTH, W.M. and CLAYTON, D.A. (1985) *EMBO J. 4:* 1559-1567.
12. CHANG, D.D. and CLAYTON, S.A. (1987) *EMBO J. 6:* 409-417.
13. CHANG, D.D. and CLAYTON, D.A. (1987) *Science 235:* 1178-1184.
14. WONG, T.W. and CLAYTON, D.A. (1985) *Cell 42:* 951-958.
15. BALDACCI, G. and BERNARDI, G. (1982) *EMBO J. 1:* 987-994.
16. BALDACCI, G., CHERIF-ZAHAR, B. and BERNARDI, G. (1984) *EMBO J. 3:* 2115-2120.
17. CLAYTON, D.A. (1984) *Ann. Revs. Biochem. 53:* 573-594.
18. FRY, M. and LOEB, L.A. (1986) In Animal Cell DNA Polymerases. CRC Press.
19. LEE, M.; TAN, C.; DOWNEY, K. and SO, A. (1981) Progress in Nucleic Acid. *Res. and Mol. Biol. 26:* 83-86.
20. COTTERILL, S.M.; REYLAND, M.E.; LOEB, L.A. and LEHMAN, I.R. (1987) *Proc. Natl. Acad. Sci. USA 84:* 5635-5639.
21. FRIDLENDER, B.; FRY, M.; BOLDEN, A. and WEISSBACH, A. (1972) *Proc. Natl. Acad. Sci. USA 69:* 452-455.
22. TEMIN, H. and BALTIMORE, D. (1972) *Adv. Virus Res. 17:* 129-186.
23. LEWIS, B.J.; ABRELL, J.W.; SMITH, R.G. and GALLO, R.C. (1974) *Science 183:* 867-870.
24. SPADARI, S. and WEISSBACH, A. (1974) *J. Biol. Chem. 249:* 5809-5815.
25. FIELD, J.; GRONOSTAJSKI, R.M. and JURWITZ, J. (1984) *J. Biol. Chem. 259:* 9487-9495.
26. KOLLEK, R. and GOULIAN, M. (1981) *Proc. Natl. Acad. Sci. USA 78:* 6206-6210.
27. TIBBETS, C.J.B. and VINOGRAD, J. (1973) *J. Biol. Chem. 248:* 3367-3379.
28. TARRAGO-LITVAK, L.; VIRATELLE, O.; DARRIET, D.; DALIBART, R.; GRAVES, P.V. and LITVAK, S. (1978) *Nucleic Acids Res. 5:* 2197-2210.
29. HUBSCHER, U.; KUENZLE, C.C. and SPADARI, S. (1977) *European J. Biochem. 81:* 249-258.
30. DUNON-BLUTEAU, D.; CORDONNIER, A. and BRUN, G. (1987) *J. Mol. Biol. 197:* 175-185.
31. WERNETTE, C.M. and KAGUNI, L. (1986) *J. Biol. Chem. 261:* 14764-14770.
32. BERTAZZONI, U.; SCOVASSI, A.I. and BRUN, G. (1977) *European J. Biochem. 81:* 237-248.
33. YAMAGUCHI, M.; MATSUKAGE, A. and TAKAHASHI, T. (1980) *J. Biol. Chem. 255:* 7002-7009.
34. ZIMMERMAN, W.; CHEN, S.M.; BOLDEN, A. and WEISSBACH, A. (1980) *J. Biol. Chem. 255:* 11847-11852.
35. ONO, K.; OGASAWA, M. and MATSUKAGE, A. (1979) *Biochem. Biophys. Res. Comm. 88:* 1255-1262.
36. WINTERSBERGER, U. and WINTERSBERGER, E. (1970) *Eur. J. Biochem. 13:* 20-27.
37. WINTERSBERGER, U. and BLUTSCH, H. (1976) *Eur. J. Biochem. 68:* 199-207.
38. CHRISTOPHE, L.; TARRAGO-LITVAK, L.; CASTROVIEJO, M. and LITVAK, S. (1981) *Plant Science Letters 21:* 181-192.
39. RICARD, B.; ECHEVERRIA, M.; CHRISTOPHE, L. and LITVAK, S. (1983) *Plant Mol. Biol. 2:* 167-175.
40. KAWANO, S. and KUROIWA, T. (1985). *Exptl. Cell Res. 161:* 460-472.

41. VAN TUYLE, G.C. and PARCO, P.A. (1981) *J. Biol. Chem. 256:* 12772-12779.
42. LEWIS, M.K. and BURGESS, R.B. (1982) In *The Enzymes XV (Part B):* 110-150.
43. CHRISTIANSON, T. and RABINOWITZ, M. (1983) *J. Biol. Chem. 258:* 14025-14033.
44. YOUNG, E.G.; HANSON, M.R. and DIERKS, P. M. (1986) *Nucleic Acids Res. 14:* 7995-8006.
45. WALBERG, M.W. and CLAYTON, D.A. (1983) *J. Biol. Chem. 258:* 1268-1275.
46. LEVENS, D.; LUSTIG, A. and RABINOWITZ, M. (1981) *J. Biol. Chem. 256:* 1474-1481.
47. KUNTZEL, H. and SHAFER, K.P. (1971) *Nature New Biology 231:* 265-269.
48. WU, G.J. and DAWID, I.B. (1972) *Biochemistry 11:* 3589-3595.
49. BOGENHAGEN, D.F. and YOZA, B.K. (1986) *Mol. Cell. Biol. 6:* 2543-2550.
50. KELLY, J.L. and LEHMAN, I.R. (1986) *J. Biol. Chem. 261:* 10340-10347.
51. KELLY, J.L.; GREENLEAF, A.L. and LEHMAN, I.R. (1986) *J. Biol. Chem. 261:* 10348-10351.
52. MARTIN, M.T.; ECHEVERRIA, M.; LITVAK, S. and ARAYA, A. (1987) *Plant Science 49:* 199-207.
53. SHUEY, D.J. and ATTARDI, G. (1985) *J. Biol. Chem. 260:* 1952-1958.
54. McCLURE, W.R. (1985) *Ann. Revs. Biochem. 54:* 171-204.
55. WINKLEY, C.S.; KELLER, M.J. and JAEHNING, J.A. (1985) *J. Biol. Chem. 260:* 14214-14223.
56. WONG, T.W. and CLAYTON, D.A. (1985) *J. Biol. Chem. 260:* 11530-11535.
57. LEDWITH, B.J.; MANAM, S. and VAN TUYLE, G.C. (1986) *J. Biol. Chem. 261:* 6571-6577.
58. SUZUKI, M.; ENOMOTO, T.; HANAOKA, F. and YAMADA, M. (1985) *J. Biochem. 98:* 581-584.

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Molecular Action of Erythropoietin on RNA Synthesis: 30 Years of Study*

Acción molecular de la eritropoyetina en la síntesis de RNA: 30 años de estudio

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Studies on erythropoietin (Epo) are briefly reviewed in their historical perspective from mid 19th century until its identification as a product of genetic recombination and its clinical trials in 1987.

Our own work throughout three decades deals mainly with Epo action on RNA biosynthesis in rat bone marrow. Results show that Epo participation as a fundamental compound in the erythropoietic process, inducing the biosynthesis of a giant RNA probably with hnRNA characteristics, which is processed to functional RNA with the participation of testosterone (Te).

Despite the so many experimental data available at present, there is still no conclusive evidence allowing to define the specific molecular mechanisms of action of both hormones at transcriptional level.

A model is postulated to explain the effects of Epo and Te on RNA biosynthesis.

Finally, the implications of present knowledge for the conceptual approach and design of future work are discussed.

INTRODUCTION

Brief Story of Erythropoietin

Glycoproteic hormonal erythropoietin (Epo) is the central factor initiating and regulating erythropoiesis. It may, thus, control the synthesis of the hemoglobin.

In mid 19th century Denis Jourdaret, a French physician observed that the blood of surgical patients living in the highlands of Mexico, was thick and viscous and contained an increased number of red corpuscles. He concluded that the low atmospheric pressure in the mountains contributed to augment the number of red cells (1). En 1906, Carnot and Deflandre (2) postulated that erythropoiesis is controlled by a humoral factor secreted into the blood stream and directly stimulating red blood cell formation in the bone marrow. They originally named this factor hemopoietin and then, once its hormone structure was recognized it was more appropiatelly named erythropoietin (3).

The search for the humoral factor started and continued for almost 50 years with confusing and unconvincing results. In 1953, Erslev (4) demonstrated that it was present in the plasma of anemic rabbits, while Hodgson and Tohá (5) were able to show its presence in the urine for repeatedly bled rabbits.

The attempts to investigate the site of Epo production have been the subject of numerous investigations. In 1957 Jacobson *et al.* (6) claimed that Epo was produced by the kidney, an assertion that is being questioned for a long time. At the present time, however, it is accepted that the kidney is the main organ for Epo synthesis, although about 10% to 15% of the total

* En homenaje a Osvaldo Cori, que, como maestro y amigo, supo ser el conductor y guía de tantas generaciones. En este trabajo resumo treinta años de esfuerzo y dedicación para desarrollar esta línea. Si bien Osvaldo no participó en ella, siempre su espíritu y su estímulo estuvieron presentes. En su evolución participaron muchos de los que fueron sus alumnos, por lo que parte de este trabajo también es de él.

synthesis occurs in the liver. The extrarenal production of Epo is the consequence of the low rate of red cell production in patients affected by severe kidney failure.

Purification and physicochemical characterization of Epo have involved a long and difficult task, because the minute quantities of the availables hormone and the lack of a sensitive bioassay have slowed down the experimental search for the factor structure. The most important advances have been those of Goldwasser *et al.* (7, 8) and Espada and Gutnisky (9). Human urinary Epo, the only purified form of the hormone obtained so far is a 166 aminoacid protein with an apparent molecular weight of 39,000 Da and 30% of carbohydrate content (galactose, mannose, glucosamine and glucose of which 40% is sialic acid).

An impressive contribution was the development of a sensitive radioimmunoassay (10) to detect small amounts of circulating Epo. This technique made it possible to monitor the isolation and fractionation procedures needed to determine its amino acid sequence. With a highly purified Epo and using fractions of amino sequences, a cDNA was prepared. K. Jacobs *et al.* (11) described the cloning of human erythropoietin gene and the expression of an Epo cDNA clone in a transient mammalian expression system to yield a secreted product with biological activity. Recently Sytekowsky and Fisher (12) isolated and characterized an anti-peptide monoclonal antibody to human Epo, while Lin *et al.* (13), McDonald *et al.* (14) and Shoemaker *et al.* (15) have reported results related to the cloning and expression of human and mouse Epo genes.

The advent of recombinant DNA techniques has allowed to obtain enough amounts of Epo for use in clinical trials. Eschbach *et al.* (16) have administred recombinant human Epo to patients with endstage renal disease in order to reverse the concurrent anemia. The treatment was successful in that it made transfusions unnecessary, reducing thereby the risks of immunologic sensitization, infection and iron overload. The hematocrit revested to normal levels.

The availability of recombinant human Epo may facilitate the study of its molecular mechanism of action in which the initial events are still only partially understood. A considerable amount of comprehensible general and specific information on various aspects of erythropoietin structure and function has been published in numerous books and reviews (3, 17, 18, 19, 20, 21, 22).

J.L. Spivak has presented a recent review dealing with the biochemistry of the hormone (23).

PROLOGUE

The biochemical events in wich Epo participates are still undefined. Even though some molecular aspects have been studied specially those related with the action of Epo at DNA level in the transcription stages and although numerous papers have been published, the molecular reactions triggered by Epo are still an unsettled issue. In this paper I am not attempting to write a review on the subject and will only discuss the participation of Epo and testosterone (Te) in the regulation of RNA synthesis in the rat bone marrow basically referred to our works, with emphasis on marrow cells because the erythropoietic process occurs principally in the marrow of normal adult animals. The RNA synthesis seems to represent the most early response to the Epo action triggering the erythropoietic process for the production of hemoglobin, which from the cellular point of view determines the red cells production.

For aspects related to the physiology and biochemistry of erythropoiesis, hemopoietic growth factors, hemapoietic stem cells, receptors, clinical erythropoiesis, etc., the readers may consult the references 17, 18, 19, 20, 21, 22, 23, 24 and 25.

Erythropoiesis

Erythropoiesis is the process of differentiation and proliferation of committed stem cells to erythrocytes. From the molecular point of view this cellular mechanism is directed to the synthesis of hemoglobin, the protein of erythrocytes that will ac-

count for oxygen transport, fundamental molecule that characterizes the event. The role of Epo as an inducer of the process has been extensively demonstrated and well documented. The molecular action of Te on the phenomenom has being studied only recently and some results will be commented further.

Erythropoietin as a molecular signal

Epo initiates erythropoiesis by acting on the erythropoietin-sensitive cells (ESC) derived by differentiation from the stem cells. Perhaps, ESC can be considered as cells bearing specific high-affinity receptors to recognize Epo. It is not clear which kind of erythroid progenitor cell has the ability to synthesize the receptor capable of interacting with Epo. In general, the Epo receptor has not been completely characterized and it has been proposed that it is located in the external surface of the ESC (26, 27, 28). The complex Epo-receptor may activate a cytoplasmic factor as a second messenger. The possible role of cyclic nucleotides in erythropoiesis has been studied (29, 30) the results being so far incomplete and controversial. We have shown (29) that cAMP does not stimulate *in vitro* RNA synthesis in isolated rat bone marrow nuclei as to mimic the Epo action, even in the presence of a cytoplasmic fraction that alone increases the synthesis of nuclear RNA. Preliminary results (29) demonstrated that 10^{-4} M dibutyryl ciclic GMP (db cGMP) stimulated RNA synthesis in whole rat bone marrow after 2 hours incubation, while no effect was observed with db cAMP at various concentrations. The phosphodiesterase inhibitor 3-isobutyl-1-methyl-xantine (MIX) abolished the effect of Epo when whole rat bone marrow cells are incubated with Epo and MIX (29). The possible role of second messengers in the Epo action remains to be elucidated.

Erythropoietin effect on RNA synthesis

A) 1960 decade

If cell differentiation and proliferation are interpreted as the synthesis of specific proteins which characterize particular types of cells, it could be expected that RNA synthesis occurs as an initial molecular event after the impact of a growth factor or a hormone at DNA level. To explain and discuss the results obtained at transcription levels it is assumed that the Epo message reaches the bone marrow chromatin where an effect on the genome is produced. When Jacob and Monod postulated (31) that information necessary for protein synthesis was carried from DNA to the ribosomal site through messenger RNA, appeared the idea to find out what effect this factor might have on nuclei acid biosynthesis. The first attempt to study the effect of Epo on RNA synthesis was reported by Perretta and Thomson (32) when they showed a small increase in ^{14}C-formate incorporation into RNA and DNA of normal rabbit bone marrow *in vitro*, after the action of a crude preparation of Epo obtained from the urine of rabbits made anemic with phenylhydrazine. However, though other investigators failed to confirm these experiments (33) the finding was the beginning of several strategies to stablish the experimental methodology to demonstrated the effect of Epo on RNA synthesis. In 1965 our group (34) was able to demonstrate that Epo stimulated the synthesis of RNA earlier than the synthesis of the hemoglobin in a similar system *in vitro*. While RNA activity was increased after 4 h incubation, hemoglobin synthesis measured by ^{59}Fe incorporation into the heme began to rise at the 15 h of incubation to reach a peak at the 29th.

Despite existed substantial data supporting an early increase in marrow or spleen cell RNA synthesis following the action of erythropoietin the evidence was not sufficient to know the exact nature and function of this RNA. The action of Epo on RNA from rat bone marrow *in vitro* can be demonstrated within 15 min (35), while the effect on nuclear RNA from spleen of polycythemic mouse *in vivo* was observed within 30 min of hormone action (36). At that time the operational difficulties due to the lack of specific methods for identifying several RNA species, including messenger RNA began to be recognized. We perform-

ed some experiments in order to show that the synthesis of more than one class of RNA may be affected by Epo in the bone marrow and spleen of erythrocyte-transfused mice (37, 38). Epo increased the synthesis of nuclear and cytoplasmic RNA between one and two hours after injection of the hormone.

At the end of the decade Gross and Goldwasser (39) found that other types of RNA were also affected by Epo. They described increased radioactivity in rapidly labeled RNAs with sedimentation coefficients of 4, 6, 9, 45, 55, 65 and 150 S. The rate of synthesis of 150 S RNA was sharply increased in the 0-15 min period after the hormone stimulus, decreased at 2-4 h and disappeared after 10 h. The synthesis of other RNA types gradually increased to a maximum at about 6 h and then quickly diminished after that time.

Many workers have observed that actinomycin D markedly inhibited the bone marrow response to the hormone. The effect of Epo on hemoglobin synthesis by rat bone marrow cells *in vitro,* was completely abolished if actinomycin D was added before or shortly after the hormone (40).

To summarize: during the decade it was demonstrated that Epo participates in the molecular mechanism of regulation of erythropoiesis. It was suggested that the hormone probably acts at the transcription step and that under its influence several types of RNA are synthesized. Our group and Goldwasser *et al.* made valuable contributions to this idea.

The principal finding was that several RNA types are activated by the Epo. This latter may correspond fundamental molecules in the process by which the biochemical machinery for the synthesis of hemoglobin is being prepared.

B) *1970 decade*

That period had various characteristics the principal being, the knowledge of gene expression in eukaryotes and the variety of possible molecular mechanisms to explain it. The attention of several investigators was focused on studying the synthesis of primary pre messenger RNA (pre m RNA) and its processing (maturation) to secondary pre m RNAs by a splicing reactions, giving rise stepwise to functional mRNA. During this process chemical modifications as methylation, 5'-terminal capping and 3'-terminal polyadenylation take place. Processing is a regulated process, involving many of the possible phases and mechanisms of post-transcriptional regulation. Confirmation of the existence of the pre m RNA (41) and of messenger ribonucleoprotein on informosomes (42) constituted the positive challenge to face with some expectation of success to elucidate the molecular mechanism of Epo action at this level.

The synthesis of RNA constitutes the main event of the transcription step. During this decade our efforts were dedicated to the study of the effect of Epo and Te on the activity of bone marrow RNA polymerase and the analysis of the characteristics of the several species of RNA synthesized under the effect of the hormones. We have reported (43) that the activity of RNA polymerase from rat bone marrow is increased under the action of Epo *in vivo,* while *in vitro* the response to the hormone was negliglible. The different response of the enzyme seemed to indicate that Epo might act in a molecular reaction that heads the action of RNA polymerase. This hypothesis was never proved.

In 1971 (44) we published a paper in which we reported a discriminatory effect of Epo on RNA synthesis. Using the hot phenol technique to extract RNA at 10°C, 40°C and 60°C we were able to show that an RNA of fraction-60°C isolated in MAK columns exhibited an increased ability to stimulate the incorporation of ^{14}C-valine into the proteins of a cell-free system obtained from rabbit reticulocytes. The hormone effect was abolished by high doses of actinomycin D. Epo augmented the synthesis of bone marrow RNA fractions extracted at 10°C, 40°C and 60°C, but only the latter had a prominent effect on protein synthesis *in vitro.* These results were confirmed and extended later on by us in another paper (45).

The RNAs obtained at 60°C might correspond to DNA-like RNA and mRNA

strongly activated by Epo (46). The fraction was very heterogenous, with sedimentation coefficients of 4, 10, 18-23, 45-50 and 85 S. The rate of synthesis of 85 S was sharply increased by Epo, being these results very similar to those obtained by Gross and Goldwasser (39) which detected the appearance of a 150 S RNA at 15 min of hormone action.

The activity of Epo was selective because it activated an RNA species of high molecular weight RNA precursor, whose processing gave rise to different RNA with intermediate sedimentation coefficients up to 9 S RNA which corresponds to the globin mRNA.

The activation of ribosomal and RNA (or their precursors) could reflect and indirect effect of the hormone or the action of other effectors.

The study of the high molecular weight precursor of functional RNA that participates in protein synthesis had become an increasingly important issue in the understanding of eukaryotic cells metabolism. The precursor RNAs have some common properties such as high molecular weight, short half-life, heterogenous base composition and they are confined exclusively to the cell nucleus. They had been denominated under a variety of names: heterogenous nuclear RNA (hnRNA) giant nascent RNA, nuclear nascent RNA and giant D-RNA. We will refer to them as hn RNAs (47, 48).

It is believed that when hnRNAs are processed by specific enzymes, they can originate functional RNA and it was postulated that hormones may be involved in this mechanism (46).

The presence of hnRNA in bone marrow has been reported by several authors. Scherrer (47) has described a giant RNA of 5-10 x 10^6 MW in duck erythroblasts. Gross and Goldwasser (39) have reported the synthesis of a 150 S RNA after the action of Epo in rat bone marrow cells *in vitro*, while Perretta *et al.* have shown the appearance of a precursor RNA of size with informational properties under the effect of Epo in rat bone marrow cells *in vivo* (44, 45, 46).

In 1978 we published a paper (49) in which we presented experimental evidence of the existence and the relationships between hnRNA with poly adenylic sequences and the formation of 9 S RNA in rat bone marrow cells. We showed the presence of 85 S, 72 S, 32 S, 17 S and 9 S RNA, all containing poly A residues in their molecules, a finding suggesting a role for these RNA molecules in the maturation hnRNA process to functional 9 S RNA. Experiments with this approach to investigate whether the different stages on the maturation of hnRNA were under Epo control unfortunatelly were never carried out.

Testosterone entered in scene

The metabolic activity of androgens in erythropoiesis was reviewed by Minguell and Sierralta (50) and some aspects of their molecular mechanism of action were discussed. Minguel *et al.* (51) have demonstrated that testosterone may act on polychromatophilic erythroblasts, stimulating the synthesis of ribosomal RNA or its precursor. The steroid enhanced the nuclear ribonuclease activity, an effect that could represent a control mechanism for the processing of hnRNA (30, 46, 51, 52).

Since 1976 we are trying to demonstrate our working hypothesis that Epo and Te act synergically to create the biochemical machinery for hemoglobin synthesis, the macromolecule that characterizes the erythropoietic process.

In 1979 we studied the effect of Epo on the synthesis of rat bone marrow RNA at different times using the technique of sucrose-gradient analysis. We found at 30 min; Epo induced the synthesis of high molecular weight RNA of 93 S and 85 S, which dissappeared at 45 min; at 45 to 60 min after Epo, RNA in the range 18 to 4 S appeared (63). From these results, the main possible conclusion to be established was that Epo selectively stimulated the transcription of high molecular weight RNA probably a hnRNA type while Te specifically activated the synthesis of ribosomal RNA species.

To assess the possibility that Epo and Te stimulate the synthesis of different RNA types, the activity of RNA polymerases was tested. The presence of several RNA polymerases in eukaryotic cells is well established and has been reviewed by Chambon (54). Eukaryotic cells contain at least three classes of RNA polymerases whose enzymatic characteristics can be aproximatelly identified and measured under appropiate conditions of ionic strength, different concentrations of α-amanitin and presence of specific divalent cations.

We have reported (43) that Epo increase RNA polymerase activities in rat bone marrow *in vivo.* The ability of Epo and Te to increase the activity of multiple forms of RNA polymerase in isolated nuclei from rat bone marrow cells confirmed that the molecular action of both hormones is exerted at some level in the transcription stage. The selective effect of Te on the activity of the nucleolar Mg^{++} dependent RNA polymerase I and the discriminatory action of Epo on the activation of the nucleoplasm Mn^{++} dependent RNA polymerase II, indicated that both hormones might act at different sites in the genome.

Piantadosi *et al.* (55) described a sequential activation of splenic nuclear RNA polymerases by Epo while Valladares *et al.* (56) were able to show that the incorporation of ^{3}H-UTP into RNA by isolated rat bone marrow nuclei is stimulated by Te. Using α-amanitin and different ionic strength conditions they found that Te enhanced preferentially RNA polymerase I activity.

The main conclusion to be drawn for this period was that transcription in rat bone marrow cells was under hormone regulation in which Epo induced the synthesis of a hnRNA while Te stimulated the synthesis of rRNA species. Both hormones might participate in molecular steps of processing mechanisms.

C) *1980 period*

In the years 1980 to 1982 we have published some papers (57, 58, 59) in which RNA polymerase activities were measured in isolated rat bone marrow nuclei obtained from normal polycythemic, anemic and castrated animals. In the same experiments, nuclear RNAs were characterized by polyacrylamide-gel electrophoresis obtaining different kinds of RNA profiles whose sedimentation coefficients were calculated. The animals were injected with Epo and Te for 3 h.

Epo stimulated RNA polymerase II which cathalizes the synthesis of RNA species 30, 22, 16-15,9 and 6 S when the nuclei are incubated at high strength (HIS) conditions, while at low ionic strength (LIS) conditions no activation was observed, with the exception of a 4 S RNA. Te exhibited great efficiency to activate RNA polymerase I which synthesizes RNA species of 26, 18 and 4 S RNA. Epo alone and plus Te at LIS showed only a light increase in 9 and 4 S RNA (see Fig. 1).

The 30, 22 and 16-15 S RNA may correspond to intermediate messengers precursors for functional hemoglobin 9 S mRNA. The action of Epo plus Te at HIS resembled the RNA profile obtained from anemic rats in which the blood Epo level is markedly augmented. Bastos and Aviv (60) demonstrated in cultures of dimethyl sulfoxide-treated Friend erythroleukemic cells, the existence of three RNA species containing base sequences common to globin mRNA and with sedimentation coefficients of 27, 15 and 10 S.

Scherrer and Marcaud (61) found in duck erythroblast ribosomes, RNA species consisting of 9 S RNA and a specie of polydisperse RNA with sedimentation coefficients whitin the range of 6 to 28 S.

The characterization of the different classes of RNA synthesized in the presence of Epo found by us and the RNA species found in erythropoietic cells demonstrated by other authors may represent the pattern of RNA maturation in rat bone marrow in which Epo is implicated in the induction of the pre-mRNA. Table I summarizes the different RNA types of erythropoietic cells under the effect or not of Epo.

We extended these observations (58, 59) and clearly demonstrated that Epo stimulated synthesis of 30 S RNA in

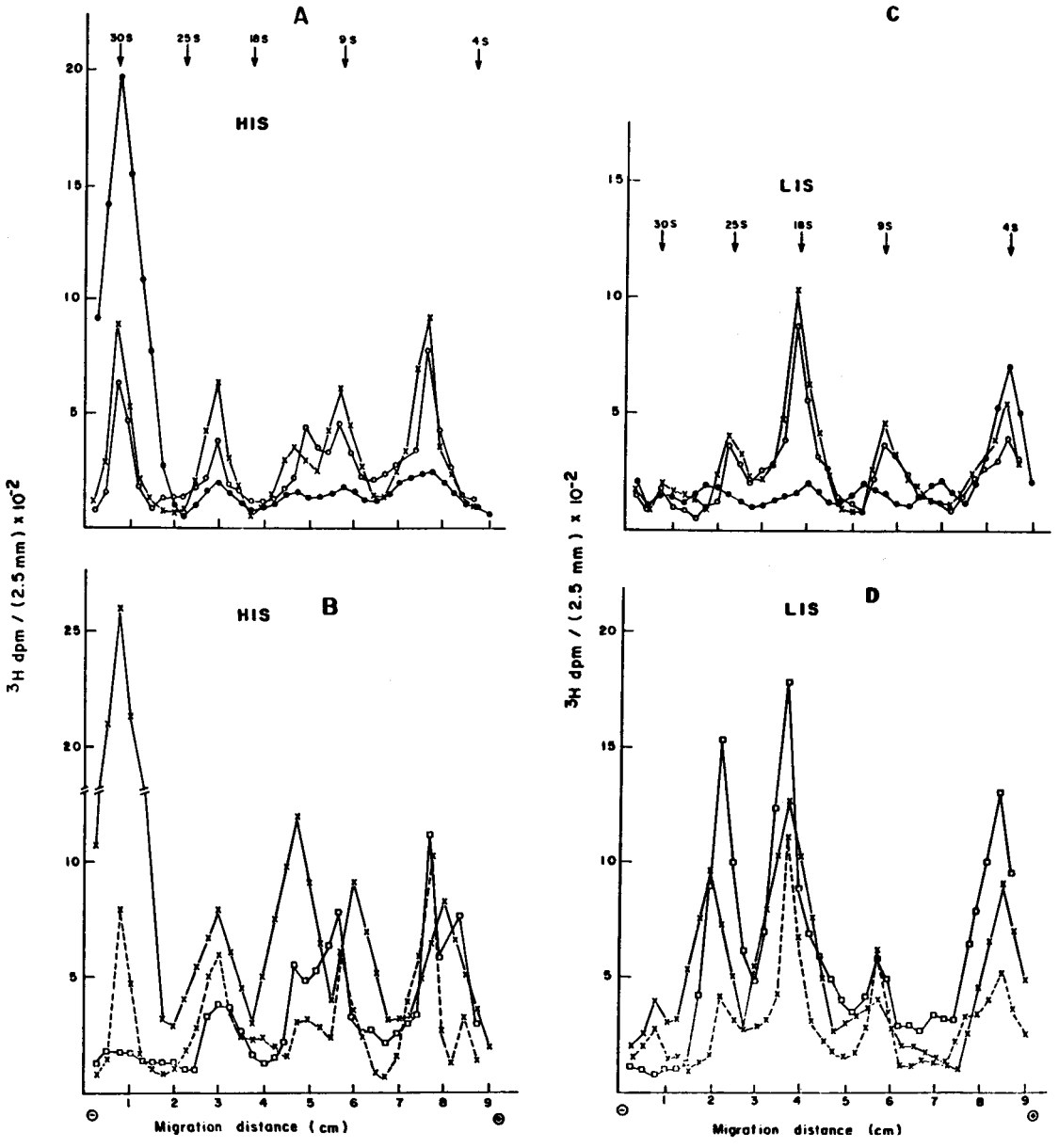

Fig. 1: In vitro experiments. Polyacrylamide-gel electrophoretic profiles of *in vitro* synthesized RNA. Effect of erythropoietin (Epo) and testosterone (Te).

Patterns of nuclear RNA obtained from isolated normal (N) and castrated polycythemic (CP) rats bone marrow nuclei. RNA was synthesized under HIS and LIS conditions, isolated and processed as indicated elsewhere (58, 59). The rats were injected with 5 UI of Epo and 250 ug/100 g rat weight of Te for 3 h.
The specific activities (dmp/ug RNA) of total nuclear RNA applied were:

HIS conditions

Control	: 2504	○——○	Panel A
N + Epo	: 3140	×——×	Panel A
CP + Epo	: 4468	●——●	Panel A
N + Te	: 2270	□——□	Panel B
N + Epo-Ete	: 3240	×– –×	Panel B
CP + Epo-Te	: 4800	×——×	Panel B

LIS conditions

N Control	: 1020	○——○	Panel C
N + Epo	: 1070	×——×	Panel C
CP + Epo	: 1070	●——●	Panel C
N + Te	: 1510	□——□	Panel D
N + Epo-Te	: 1412	×– –×	Panel D
CP + Epo-Te	: 896	×——×	Panel D

Arrows show calculated sedimentation coefficients (77).

TABLE I

Different RNA species described in erythropoietic cells

Years	Conditions Tecnique	RNA species	Cells	Affected by Epo[1]	(References)
1961	*In vitro*	Whole RNA: isolated bases	rat bone marrow	Yes	(34, 79)
1965	*In vitro*	Whole RNA: isolated bases	rat bone marrow	Yes	(34, 79)
1965	*In vivo*	Nuclear and cytoplasmic RNA	rat bone marrow, mouse spleen	Yes	(36, 37)
1968	*In vitro*	28 to 6 S; 9 S	duck erythroblast	N D[2]	(61)
1969	*In vitro*	150, 65, 55, 45, 9 and 4 S	rat bone marrow	Yes	(39)
1973-73	*In vivo*	60°C RNA fraction MAK column	rat bone marrow	Yes	(44, 45)
1976	*In vivo*	85, 50-45, 23-18, 10 and 4 S	rat bone marrow	Yes	(46)
1977	*In vitro*	27, 15 and 10 S	erythroleukemic	No	(60)
1979	*In vivo*	85, 72, 32, 17 and 9 S	rat bone marrow	N D[2]	(49)
1979	*In vivo*	93, 85, 18 and 4 S	rat bone marrow	Yes	(53)
1980-82	*In vitro*	30, 22, 16-15, 9 and 6 S	rat bone marrow	Yes	(57, 58, 59, 68)
1984-86	*In vivo*	85, 79-70, 45, 13, 9 and 4 S	rat bone marrow	Yes	(69, 70,72)
1985	*In vitro*	RNA from RNP[3]	rat bone marrow	Yes	(71)

[1] Erythropoietin.
[2] Not done.
[3] Ribonucleoprotein.

castrated-polycythemic rats while Te increased the formation of 28, 18, 6-5 and 4 S RNA. The combined action of both hormones influenced the 9 S RNA formation and 22 and 15 S RNA, that may represent intermediary RNA in 9 S RNA induction.

The stimulation of 6-5 S RNA synthesis by Te at HIS conditions and the joint action of Epo plus Te may represent the main finding of these works and suggested the idea that these kinds of RNA may correspond to low molecular weight nuclear RNA species designated U2 to U6, which in combination with specific proteins form ribonucleo-protein particles (RNP) that participate in the pre mRNA processing by splicing mechanisms (63, 64, 65, 66).

As it had been shown by our group (52, 67) that Te activated nuclear endonucleases, the participation of Te at this stage seems possible.

In summary, Epo induced the synthesis of 30S pre-mRNA, which is processed to 9 S RNA by RNP, and these, in turn, were stimulated by Te.

The increased synthesis of different species of RNA by the *in vitro* action of Epo and the activation of RNA polymerases may provide preliminary arguments to explain the molecular activity of the hormone (43, 44, 45, 46, 53, 57, 58, 59, 62). The hypothesis is still completely insufficient to explain a mechanism of the molecular action of the factor.

Many reasons have arisen to justify why so many biochemical steps of the erythropoietic process are still unsolved. Concerning nucleic acid the principal reason may be the operational difficulties to analyze and to isolate RNA species in eukaryotic organisms. These problems have led several investigator to apply different techniques; this approach allowed to get a large variety of results, that conform a highly complicated and cumbersome picture of the origen and maturation of pre RNA species.

Being the analysis of RNA synthesis strictly dependent on the methodology applied, the action of Epo and Te was analyzed applying and comparing different methodological strategies.

We have performed some experiments according to these concepts obtaining some results that will be published soon.

In Table I are summarized the different RNA types isolated from erythropoietic cells using several methodologies. It can be observed that RNAs appear in the wide range of 150 to 4 S, many of them activated by Epo. Two main problems have to be solved in order to obtain comparable results. One of them is the selection of an appropiate method to isolate the several

RNA species. The other involves the choice of the biological model to be used. It is necessary to consider that the cellular composition of rat bone marrow is markedly heterogenous with a small content of Epo target cells. This hinders the study of the process in this kind of cells.

The practical consequences were that we had to try a variety of methods using whole rat bone marrow or erythroblasts enriched fractions. Even though the results obtained were not satisfactories most of them were comparable proving to be being very useful for the construction of models to explain the molecular action of Epo and Te.

In an effort to support the general evidences demonstrated in the various works cited in this paper I will now briefly discuss the preliminary results obtained following three experimental strategies:

1. *In vitro Experiments:* Determination of the RNA polymerases activity and the profile of RNA types in isolated rat bone nuclei under the effect of Epo and Te.

2. *In vivo Experiments:* Estimation of the rat bone marrow RNA under the effect of Epo and Te, extracting the marrow RNA inmediately after the animal being sacrificed and

3. *Ribonucleoprotein (RNA) Experiments:* Analysis of the RNA under the *in vitro* effect of Epo isolating the marrow ribonucleoprotein (RNP).

In Table II are depicted the results obtained when rats under different physiological conditions were submitted to the action of Epo and/or Te. Nuclei isolated from normal, polycythemic, castrated and castrated-polycythemic rats 3 h after exposure to the hormones, were used to measure the activity of RNA polymerase. It is observed that Epo enhances the activity of RNA polymerase II at HIS conditions in the four groups of rats while under LIS conditions no effect is produced. By contrast, Te significantly increases enzyme activity under LIS and HIS conditions, except in normal rats at HIS. The combined action of both hormones results in a 39% to 83% increase respectively in the activity of the enzyme, when measured under HIS and LIS conditions.

In *in vitro* experiments, RNA species synthetized by normal and castrated-polycythemic bone marrow nuclei the effect of Epo and Te alone or in combination were resolved and their sedimentation coefficients appeared characterized by gel-electrophoresis as it is shown in Fig. 1.

TABLE II

In vitro experiments.
Effect of erythroproietin (Epo) and testosterone (Te) on the RNA polymerases activity of isolated bone marrow nuclei obtained from normal, polycythemic, castrated and castrated-polycythemic rats

	Ionic Strength							
	HIGH (HIS): RNA Polymerase II				LOW (LIS): RNA Polymerase I			
	Control	Epo	Te	Epo-Te	Control	Epo	Te	Epo-Te
Normal	100± 5	126 ± 7[a]	109 ± 6	139± 11[a]	100± 6	114± 4	163 ± 5[b]	178 ± 8[b]
Polycythemic	100± 4	135 ± 8[a]	137 ± 9	156± 8	100± 7	113± 6	114 ± 5[b]	163 ± 12[b]
Castrated	100± 2	120 ± 4	146 ± 14[a]	164± 4[b]	100± 8	123± 5	152 ± 8[b]	161 ± 13[b]
Castrated-Polycythemic	100± 4	154 ± 10[b]	163 ± 6[b]	183± 6[b]	100 ± 4	114± 5	130 ± 8[a]	141 ± 6[b]

The rats were injected intravenously *in vivo* for 3 h with 5 IU of erythropoietin and with 200 ug of testosterone per 100 g of body weight. The bone marrow nuclei were isolated and purified by a method already described (52, 58, 59).

The experiments were carried out *in vitro* at high (HIS) and low (LIS) ionic strength. The nuclei were incubated for 15 min at 30°C with 3H- UTP and a proper reaction mixture and the radioactive was measured in acid insoluble material. Experimental conditions were indicated elsewhere (58, 59).

The results are expressed in percentages (means of 20 samples ± SE) of the RNA specific radioactivities in comparison with controls taken as 100%. The RNA radioactivities were in the ranges of 813-1440 dpm/ug of DNA at HIS conditions and of 119-232 dpm/ug of DNA at LIS conditions.

a) and b) significantly different from their respective controls at $P < 0.05$ and $P < 0.001$ by the t test, respectively.

It is observed in panel A that Epo stimulated solely the synthesis of a 30 S RNA, and Te solely that of 6-5 S RNA (panel B). The combination of Epo and Te provoked the appearance of RNA species of 30, 22, 15, 9 and 6-5 S. At LIS conditions, the joint effect of both hormones stimulated the synthesis of 28, 18 and 4 S RNA.

On the basis of these results that we have already reported and discussed (57, 58, 59, 60) we have concluded that Epo induces the synthesis of a pre mRNA, that is then processed to 9 S RNA.

The results of *in vivo* experiments to determine the RNA profiles obtained with the thermic method of Dabeva *et al.* (69, 70) are shown in Fig. 2. The four RNA fractions correspond to: 4°C to nucleoplasmic RNAs, 50°C to nucleolar RNAs, 80°C to heterogenous nuclear RNAs and cytoplasmic RNA.

In panel A the presence of 85, 79-70, 45 and 4 S RNA is the most remarkable characteristic of the profile. The high molecular weight RNAs seem to be processed by the joint action of Epo and Te (panel D) to pre mRNA 13 S and 9 S,

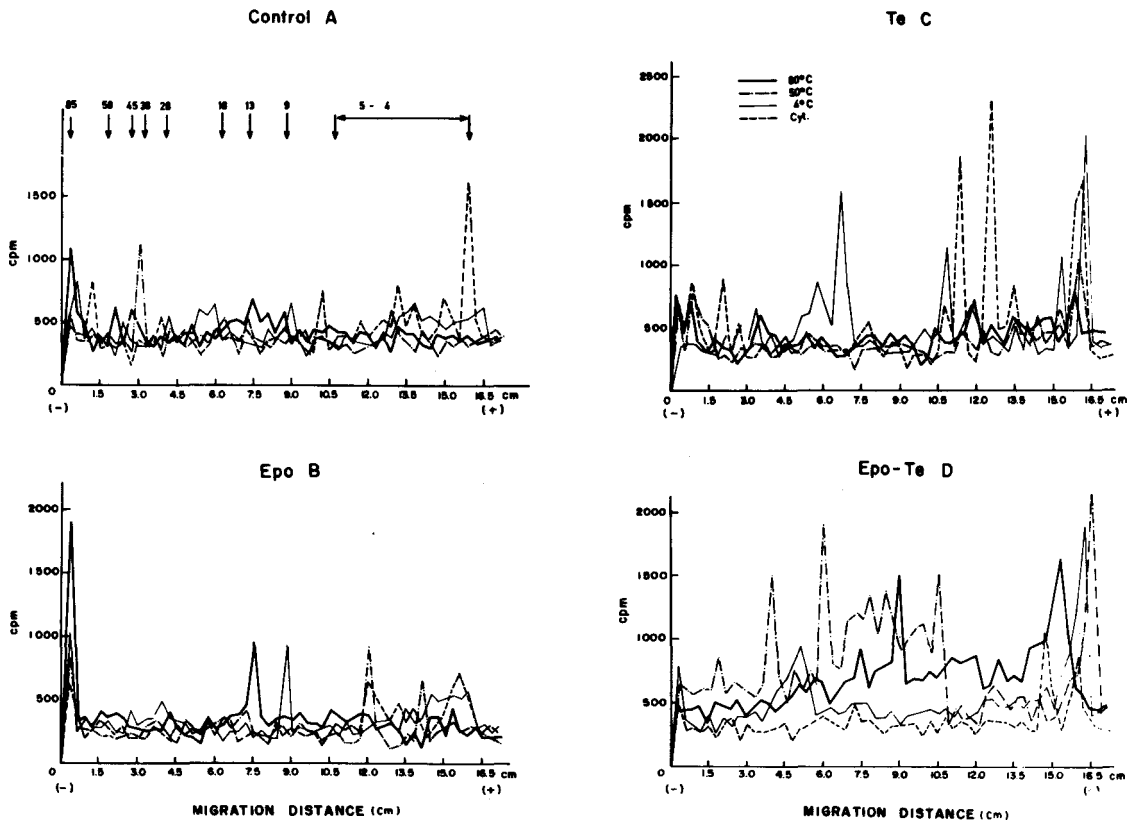

Fig. 2: In vivo experiments. Polyacrylamide-gel electrophoretic profiles of *in vivo* synthesized RNA. Effect of erythropoietin (Epo) and testosterone (Te).

Patterns of RNA fractions isolated by the thermic procedure of Dabeva (60, 70, 72, 73) at 4°C, 50°C, 80°C and cytoplasmic RNA. The RNA profiles were obtained by gel electrophoresis: polyacrylamide 2% and agarose 0.5%. The rats were injected intravenously with 6 UI of Epo/200 g rat weight and with 125 uCi of 5,6-3H-uridine and intraperitoneal with 600 ug/200 g rat weight of Te in propilenglycol solution. After 3 h the animals were sacrificed and the marrow removed. 400 ug of RNA of each fractions were applied on the gel.

RNA fractions

80°C ——————
50°C —·—·—·—·
4°C ——————
cyt. — — — — — —

Arrows show calculated sedimentation coefficients (77).

while the r 45 S are processed to r 18 and r 28 S.

These results are in agreement with those already obtained in our laboratory in 1979 (53).

We have carried out some preliminary experiments (71) estimating the synthesis of RNA under the effect of Epo in marrow cells *in vitro* measuring the RNA activity in ribonucleoprotein (RNP). In Table III it can be seen that the RNA of the RNP augmented by 19% while the activity of the total cellular RNA shows an increase of only 11%.

TABLE III

Ribonucleoproteins (RNP) experiment. Effect of erythropoietin (Epo) on synthesis of a RNP-RNA by rat bone marrow cells *in vitro*

	CPM/Total cells		
RNA specie	Control	+ Epo	% of increase
Whole RNA	494468	542801	11
RNA from RNP	15648	18564	19

Model experiment from a series of 8 similar.
The figures indicate the main of 5 samples.
10^6 rat bone marrow cells per ml were incubated at 37°C in a MEM-Eagle medium at pH 7.2 during 60 min with 0.15 IU of Epo and 2 uCi of 5,6-^{3}H-uridine. Then the whole RNA and RNP were isolated and processed as described elsewhere (71, 74, 75, 76).

To summarize: the molecular mechanism for the early increase in transcriptional activity following exposure to Epo and Te was not established, but our experimental results, as described have made a contribution towards it elucidation.

The main finding seems to be that Epo induces the synthesis of a giant RNA probably with the characteristics of a hnRNP which in turn is processed under the form of RNP to the functional 9 S mRNA, in complex reactions with the participation of Epo and Te.

GENERAL CONCLUSIONS

Despite so many experimental data available at present, there is still no conclusive evidence of the specific molecular mechanisms at transcription levels and consequently, it is not yet possible to understand the general principles and overall organization of mRNA formation and its regulation in eukaryotic cells.

In relationships with the regulatory effect of erythropoietin and testosterone on erythropoiesis, several facts are conclusive.

A) *General*

1. The differentiation and proliferation of committed erythroid progenitor cells are regulated by erythropoietin.

2. Erythropoietin provokes an early increase in transcriptional activity triggering the erythropoietic process.

3. Testosterone may act once erythropoietin triggers the phenomenom.

B) *Specific*

1. Erythropoietin induces the synthesis of a giant pre RNA in erythroid progenitor cells.

2. The giant RNA is processed to functional RNA with the participation of erythropoietin and testosterone.

3. In the presence of actinomycin D the synthesis of RNA by erythropoietin is abolished and by consequence the erythropoiesis as well.

4. On the basis of our present day data, it would be tempting to draw a general model of Epo and Te at transcriptional level, that is presented in Fig. 3. The model supports some ideas reported by Perretta in 1969 (78).

C) *Some facts to be demonstrated*

1. Biochemical and cellular biological events governing erythropoiesis and in general hemopoiesis:
- Role of erythropoietic (hemopoietic) growth factors *in vivo.*
- Existence of receptors for erythropoietin and other growth factors.
- Role of erythropoietin as a hormone, a growth factor or a mitogen?

2. Molecular aspects:
- Identification of second messengers mediating the erythropoietin action.

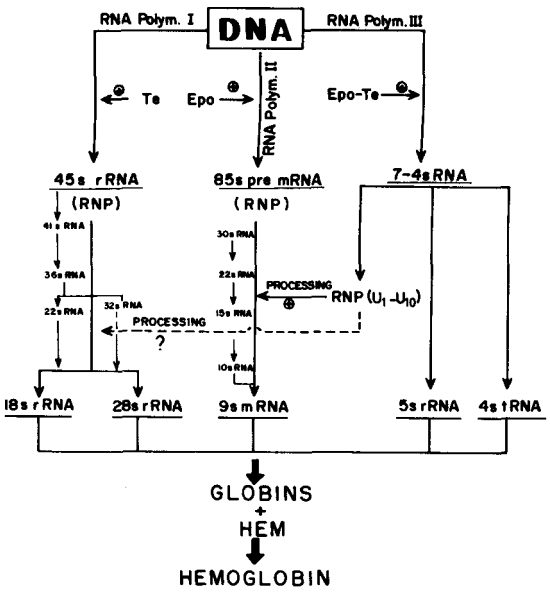

Fig. 3: Proposed mechanisms for the molecular action of erythropoietin and testosterone on erythropoietic process at transcriptional and post-transcriptional level.

- Molecular impact of Epo and Te at molecular level on the characterization of RNA types induced and stimulated by erythropoietin and testosterone.
- Are the RNAs involved in the synthesis of hemoglobin in the form of RNP?
- Characterization of RNP induced by the hormone.
- Are the proteins of RNP involved in the processing of giant RNA?

Future Development

Much work will have to be carried out in order to comprehend the complex reactions of gene expression and its regulation.

Erythropoiesis is an important biological process not only because of its clinical implication but also because it represents an excellent biological model to study the regulation mechanism involved in cell proliferation and differentiation in which participate hormones, as well as, growth factors.

We have at present the advantage of available modern experimental tools such as purified human erythropoietin in adequate quantity and sensitive radioimmunoassay techniques to measure it; new methods to separate cells; monoclonal antibody to human Epo; the recombinant DNA techniques, etc. Because of all of this it may possible to work out more accurate experimental designs in order to obtain more consistent results than when only crude erythropoietin preparations were used.

It will possible to elucidate how growth factors control the survival, proliferation and development of normal erythropoietic cells and to determine which molecular events are disturbed in the control mechanisms that operated in the transition of healthy to diseased marrow. This could allow more rapid advances in the understanding of Epo and Te regulation of red cell production to be expected.

From the molecular point of view the life-span of primary and secondary pre-mRNA molecules will be elucidated. The study of the many variants of processing hnRNA would be possible because there is no unique mechanism to produce mRNA. The analysis can be done in marrow cells in spite of their complexity.

Being the erythropoiesis especially in bone marrow cells, the suitable biological model, to study the molecular basis of cytodifferentiation and proliferation, we intend to pursue the demonstration that the hormonal regulatory mechanisms that control the process may follow straight metabolic pathways inside the cell, through a supramolecular compartmentation, that includes the cytoskeletal organization.

It should be fundamental to show that the initial molecular reaction in which Epo interacts with the membrane receptor follows a straight supramolecular pathway by which the chemical signal reaches the nuclear chromatin. At this nuclear level would be generated the pre RNAs, which in turn under the form of RNPs would start another straight pathway in which RNA processing occurs, to be completed outside the nucleus with the synthesis of the globins in the endoplasmic reticulum.

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REFERENCES

1. ERSLEV, A. (1987) Erythropoietin coming of Age. *The New Eng. J. Med. 316:* 101-103.
2. CARNOT, P. and DEFLANDRE, C. (1906) Sur l'activité hemapoietique des differents organes au cours de la regeneration du sang. *C.R. Acad. Scienc. 143:* 432.
3. FISHER, J. (1972) Erythropoietin: Pharmacology, Biogenesis and Control of Production. *Pharmacol. Rev. 24:* 459-508.
4. ERSLEV, A. (1953) Humoral regulation of red cell production. *Blood, 8:* 349-357.
5. HODGSON, G. and TOHA, J. (1954) The erythropoietic effect of urine and plasma of repeatedly bled rabbits. *Blood, 9:* 299-304.
6. JACOBSON, L.; GOLDWASSER, E.; FRIEND, W. and PLZAC, L. (1957) Role of the kidney in erythropoiesis. *Nature, 179:* 633-634.
7. GOLDWASSER, E. and KUNG, C.K.H. (1971) Purification of erythropoietin. *Proc. Nat. Acad. Sci. 68:* 697-701.
8. MIYAKE, T.; KUNG, C.K.H. and GOLDWASSER, E. (1977) Purification of human erythropoietin. *J. Biol. Chem. 252:* 5558-5564.
9. ESPADA, J. and GUTNISKY, A. (1970) Purification of human urinary erythropoietin. *Acta Physiol. Latinoamer. 20:* 122-129.
10. SHERWOOD, J.B. and GOLDWASSER, E. (1979) A radioimmunoassay for erythropoietin. *Blood 54:* 885-893.
11. JACOBS, K. *et al.* (1985) Isolation and characterization of genomic and c DNA clones of human erythropoietin. *Nature, 313:* 806-810.
12. SYTKOWSKY, A.J.; FISHER, I.W. (1985) Isolation and characterization of an antipeptide monoclonal antibody to human erythropoietin. *J. Biol. Chem. 260:* 14727-14731.
13. LIN, F.K. *et al.* (1985) Cloning and expression of the human erythropoietin gene. *Proc. Natl. Acad. Sci. USA, 82:* 7580-7584.
14. McDONALD, J.D.; LIN, F.K. and GOLDWASSER, E. (1986) Cloning sequencing and evolutionary analysis of the mouse erythropoietin gene. *Mol. Cell. Biol. 6:* 842-848.
15. SHOEMAKER, C.B. and MITSOCK, L.D. (1986) Murine erythropoietine gene: cloning expression and human gene homology. *Mol. Cell. Biol. 6:* 849-858.
16. ESCHBACH, I.W. *et al.* (1987) Correction of the anemia of endstage renal disease with recombinant human erythropoietin. *New Eng. J. Med. 316:* 73-78.
17. JACOBSON, L.O. and DOYLE, M. (1962) Erythropoiesis. Grune and Stratton, New York.
18. GORDON, A.S. (1970) Regulation of Hematopoiesis. Vol. I and II. Appleton-Century-Crofts. New York.
19. KRANTZ, S.B. and JACOBSON, L.O. (1970) Erythropoietin and regulation of erythropoiesis. Chicago. The University of Chicago Press.
20. SPIVAK, J.L. and SEIBER, F. (1983) Erythropoietin in hormones. In: Fotherby K., Pat, S.B. eds. Normal and abnormal human tissues. New York: Walter de Gruyter and Co. Vol. III: 63-96.
21. SHERWOOD, J.B. (1984) The chemistry and physiology of erythropoietin. *Vitamins and Hormones, 41:* 161-211.
22. POPOVIC, W.J. and ADAMSON, J.N. (1978) Erythropoietin assay: present status of methods pitfalls and results in polycythemic disorders. *CRC Critical Rev. Clin. Lab. Sci. 10:* 57-87.
23. SPIVAK, J.L. (1986) The mechanism of action of erythropoietin. *Int. J. Cell Cloning 4:* 139-166.
24. WHATTON, A.D. and DEXTER, T.M. (1986) Haematopoietic growth factors. *Trends Biochem. Sci. 11:* 207-211.
25. MINGUELL, J. (1982) Bone marrow stroma and disease. *Arch. Biol. Med. Exp. 15:* 457-463.
26. CHANG, S.C-S; SIKKEMA, D. and GOLDWASSER, E. (1974) Evidence for an erythropoietin receptor protein on rat bone marrow cells. *Biochem. Biophys. Res. Commun. 57:* 399-405.
27. KRANTZ, S.B. and GOLDWASSER, E. (1984) Specific binding to erythropoietin to spleen cells infected with the anemic strain of Friend virus. *Proc. Nat. Acad. Sci. USA, 81:* 7574-7578.
28. WEISS, T.L.; KUNG, C.K.H. and GOLDWASSER, E. (1985) The frequency of bone marrow cells that bind erythropoietin. *J. Cell. Biochem. 27:* 57-65.
29. GARRIDO, A. (1983) Acción *in vitro* de eritropoyetina y nucleótidos cíclicos sobre la síntesis de RNA en fracciones celulares de médula ósea de rata. Tesis. Instituto de Nutrición y Tecnología de los Alimentos, Universidad de Chile.
30. MINGUELL, J.; VALLADARES, L.; VALENZUELA, D.; CAÑAS, P.; GARRIDO, F. y PERRETTA, M. (1976) Regulación hormonal de la eritropoyesis. *Rev. Méd. Chile, 104:* 925-933.
31. JACOB, F. and MONOD, J. (1961) On the regulation on gene activity. *Cold Spring Harbor Symp. Quant. Biol. 26:* 193-204.
32. PERRETTA, M.A. and THOMSON, R.Y. (1961) Effect of erythropoietin on incorporation of formate labelled with Carbon-14 into the nucleic acids of normal rabbit tissues *in vitro. Nature, 190:* 912.
33. DUKES, P.P. and GOLDWASSER, E. (1962) Lack of effect of plasma erythropoietin on formate incorporation into nucleic acids *in vitro. Nature, 195:* 1222-1223.
34. PIEBER-PERRETTA, M.; RUDOLPH, W.; PERRETTA, M. and HODGSON, G. (1965) Effect of erythropoietin on ^{14}C –formate incorporation into rat bone marrow RNA. *Biochim. Biophys. Acta, 95:* 360-362.
35. KRANTZ, S.B. and GOLDWASSER, E. (1965) On the mechanism of erythropoietin-induced differentiation II. The effect on RNA synthesis. *Biochim. Biophys. Acta, 103:* 325-331.
36. PERRETTA, M.; RUDOLPH, W. and PIEBER-PERRETTA, M. (1966) Acción de la eritropoyetina sobre la síntesis de los ácidos nucleicos de bazo y médula ósea. *Arch. Biol. Med. Exp. 3:* 93-99.
37. RUDOLPH, W. and PERRETTA, M. (1967) Effects of erythropoietin on ^{14}C –formate uptake by spleen and bone marrow nucleic acids of erythrocyte-transfused mice. *Proc. Soc. Exp. Biol. Med. 124:* 1041-1045.
38. PERRETTA, M. (1967) Different 14C-formate and 6-^{14}C orotic acid incorporation patterns in the

nucleic acids of spleen from normal a polycythemic mice treated with erythropoietin. *Biochim. Biophys. Acta, 142:* 548-551.

39. GROSS, M. and GOLDWASSER, E. (1969) On the mechanism of erythropoietin-induced differentiation. V. Characterization of ribonucleic acid as a result of erythropoietin action. *Biochemistry, 8:* 1795-1805.
40. GALLIEN-LARTIGUE, O. and GOLDWASSER, E. (1965) On the mechanism of erythropoietin-induced differentiation. I) The effect of specific inhibitors on hemoglobin synthesis. *Biochim. Biophys. Acta. 103:* 319-326.
41. SCHERRER, K.; IMAIZUMI-SCHERRER, M.; TEREZA, REYNAUD, CLAUDE-AGUS and THERWATH, A. (1979) On pre-messenger RNA and transcription. A review. *Molec. Biol. Rep. 5:* 5-28.
42. SPIRIN, A. (1979) Messenger ribonucleoproteins (informosomes) and RNA-binding proteins. *Mol. Biol. Rep. 5:* 53-57.
43. PERRETTA, M. and BOSCO, C. (1970) Effect of erythropoietin on the activity of RNA polymerase. *Experientia, 26:* 594-596.
44. PERRETTA, M.; VALENZUELA, A.; SAGE, N. and OYANGUREN, C. (1971) Discriminatory effect of erythropoietin on the RNA metabolism from rat bone marrow. *Arch. Biol. Med. Exp. 8:* 30-38.
45. PERRETTA, M.; VALENZUELA, A. and VALLADARES, L. (1973) mRNA synthesis of DNA-like RNA. In: Gene Expression and Its Regulation. Edited by Francis T. Kenney, Barbara A. Hamkalo, Gabriel Favelukes and J. Thomas August. Plenum Publishing Corporation, New York, pp. 137-148.
46. PERRETTA, M.; VALLADARES, L.; ROMERO, C.; SIERRALTA, W.; VALENZUELA, A.; SPENCER, E.; CAÑAS, P. and MINGUELL, J. (1976) Hormone Action on the cell nucleus. Effect of erythropoietin and testosterone on bone marrow. *Arch. Biol. Med. Exp. 10:* 35-40.
47. SCHERRER, K. (1973) Messenger RNA in eukaryotic cells: The life history of duck globin messenger RNA. In: Protein synthesis in reproductive tissue. E. Diczfalusy (ed) Karolinska Institutet. Stockhölm, pp. 95-129.
48. SAMARINA, O.P.; LUKANIDIN, E.M. and GEORGIEV, G.P. (1973) Ribonucleoprotein particles containing mRNA and pre- mRNA. In Protein synthesis in reproductive tissue. E. Diczfalusy (ed) Karolinska Institutet. Stockhölm, pp. 130-167.
49. VALENZUELA, A.; SPENCER, E. and PERRETTA, M. (1978) Polyadenylic acid-rich RNA in rat bone marrow cels. *Arch. Biol. Med. Exp. 11:* 97-103.
50. MINGUELL, J. and SIERRALTA, W. (1975) Molecular mechanism of action of the male sex hormones. *J. Endocr. 65:* 287-315.
51. MINGUELL, J.; CARAVAGNO, A. and YAÑEZ, J. (1971) Effect of methyltestosterone on the nucleic acid metabolism of rat bone marrow cells. *Proc. Soc. Exp. Biol. Med. 138:* 438-445.
52. SIERRALTA, W.; GONZALEZ, M. and MINGUELL, J. (1974) The effect of testosterone on rat bone marrow nuclear RNA metabolism. *J. Steroid Biochem. 5:* 645-649.
53. PERRETTA, M.; VALLADARES, L.; GARRIDO, F.; VALENZUELA, D. and LUDWIG, U. (1979) Hormonal control of gene expression: differential activation of rat bone marrow RNA polymerases by erythropoietin and testosterone. *Arch. Biol. Med. Exp. 12:* 309-318.
54. CHAMBON, P. (1975) Eukaryotic nuclear RNA polymerases. *Annu. Rev. Biochem. 44:* 613-638.
55. PIANTADOSI, C.A.; DICKERMAN, H.W. and SPIVAK, L.J. (1976) Sequential activation of splenic nuclear RNA polymerases by erythropoietin. *J. Clin. Inv. 57:* 20-26.
56. VALLADARES, L.; CAÑAS, P. and MINGUELL, J. (1976) Bone marrow ribonucleic acid polymerase. Effect of testosterone on nucleotide incorporation into nuclear RNA. *Nucleic Acid. Res. 3:* 3077-3086.
57. PERRETTA, M.; WAISSBLUTH, L.; LUDWIG, U. and GARRIDO, F. (1980) Hormonal control of RNA polymerases in rat bone marrow nuclei. The action of erythropoietin and testosterone. *Arch. Biol. Med. Exp. 13:* 247-257.
58. PERRETTA, M.; WAISSBLUTH, L.; LUDWIG, U.; GARRIDO, F.; GARRIDO, A. and RONCO, A.M. (1981) Different RNA species stimulated by testosterone and erythropoietin in isolated bone marrow nuclei obtained from polycythemic and anemic rats. *J. Steroid Biochem. 14:* 537-543.
59. PERRETTA, M.; JOHNSON, C.; CHEVESICH, J.; GARRIDO, A.; GARRIDO, F. and RONCO, A.M. (1982) Effect of erythropoietin and testosterone on RNA synthesis of isolated bone marrow nuclei from castrated-polycythemic rats. *Arch. Biol. Med. Exp. 15:* 371-379.
60. BUSTOS, R.N. and AVIV, H. (1977) Globin precursors molecules. Biosynthesis and processing in erythroid cells. *Cell, 11:* 641-650.
61. SCHERRER, K. and MARCAUD, L. (1968) Messenger RNA in avian erythroblast at the transcriptional and translational levels and the problems of regulation in animal cells. *J. Cell. Physiol. 72:* 181-212.
62. MANIATIS, G.M.; RIFKIND, R.A.; BANK, A. and MARKS, P.A. (1973) Early stimulation of RNA synthesis by erythropoietin in cultures of erythroid precursor cells. *Proc. Natl. Acad. Sci. USA, 70:* 3189-3194.
63. KNOWLER, J.T. and WILKS, A.F. (1980) Ribonucleoprotein particles and the maturation of eukaryote RNA. *Trends Biochem. Sci. 5:* 268-271.
64. WILKS, A.F. and KNOWLER, J.T. (1981) The phosphorylation of the proteins of rat liver heterogenous ribonucleoprotein particles by an endogenous kinase activity. *Biochim. Biophys. Acta 652:* 228-233.
65. KNOWLER, J.T. (1982) The processing of eukaryote RNA. *Biochem. Education 10:* 130-137.
66. KNOWLER, J.T. (1983) An assesment of the evidence for the role of ribonucleoprotein particles in the maturation of eukaryote RNA. *Intern. Rev. Cytol. 84:* 103-153.
67. MINGUELL, J. and PERRETTA, M. (1967) Ribonuclease activity in rat bone marrow. *Biochim. Biophys. Acta 142:* 545-548.
68. CHEVESICH, J. (1981) Análisis de diferentes especies de RNA inducidos por eritropoyetina y testosterona en médula ósea de rata. Tesis. Facultad de Ciencias Básicas y Farmacéuticas, Universidad de Chile.
69. CONTRERAS, M. (1984) Efecto *in vivo* de eritropoyetina y testosterona sobre el metabolismo de RNAs en médula ósea de ratas. Tesis. Facultad de Ciencias Básicas y Farmacéuticas. Universidad de Chile.
70. CARRASCO, G. (1986) Acción *in vivo* de eritropoyetina y testosterona sobre la síntesis y proce-

samiento de pre RNAs en médula ósea de rata. Tesis. Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile.

71. TIJMES, M. (1985) Efecto de eritropoyetina sobre la síntesis de ribonucleoproteínas en médula ósea de rata. Tesis. Universidad Metropolitana de Ciencias de la Educación.
72. DABEVA, M.; DUDOV, K.; HADJIOLOV, A.; EMANUILOV, J. and TODOROV, B. (1976) Intranuclear maturation pathways of rat liver ribosomal ribonucleic acids. *Biochem. J. 160:* 495-503.
73. DABEVA, M. and DUDOV, K. (1982) Quantitative alterations in the nucleolar and nucleoplasmic ribosomal ribonucleic acids in regenerating rat liver. *Biochem. J. 204:* 179-183.
74. SAMARINA, O.P.; LUKANIDIN, E.M.; HOLMAR, E.M. and GEORGIEV, G.A. (1968) Structural organization of nuclear complexes containing DNA-like RNA. *J. Mol. Biol. 33:* 251-263.
75. PEDERSON, T. (1974) Proteins associated with heterogenous nuclear RNA in eukaryotic cells. *J. Mol. Biol. 83:* 163-183.
76. PEDERSON, T. and DAVIS, N.C. (1980) Messenger RNA processing and nuclear structure: isolation of nuclear ribonucleoprotein particles containing B-globin messenger RNA precursors. *J. Cell Biol. 87:* 47-54.
77. LEWICK, P. and SINSKEY, A. (1969) Precision of RNA separation by polyacrylamide gel electrophoresis. *Anal. Biochem. 33:* 273-278.
78. PERRETTA, M. (1969) Mecanismos bioquímicos de acción hormonal. *Arch. Biol. Med. Exp. 6:* 89-105.
79. THOMSON, R.; PERRETTA, M. and RICCERI, G. (1960) The nucleic acid metabolism of animal cells *in vitro* III). Factors influencing nucleotide biosynthesis. *Biochim. Biophys. Acta, 45:* 87-102.

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Methylation of proteins from the translational apparatus: an overview*

Metilación de proteínas del aparato de traducción: una visión

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Several of the translational apparatus proteins are methylated in all kinds of organisms. Although most of the modified proteins play key roles during protein biosynthesis, the biological function of these chemical modifications still remains elusive. Our recent data indicate a highly conserved pattern of ribosomal protein methylation in eubacteria, with methylated proteins being both structurally and functionally homologous in several microorganisms. Chloroplast ribosomes also appear to have a rather eubacterial pattern of ribosomal protein methylation. On the other hand, there is an apparently ubiquitous methylation of some of the translational factors in several organisms. These findings suggest an important, albeit unknown role for the post-synthetic methylation of the translational machinery. The analysis of the sequences of known methylation target sites and the search of similar sites in other proteins of known sequence, allows to predict those ribosomal proteins or translational factors that may be subjected to post-translational modifications with one or more methyl groups. Although a definitive answer with respect to the biological role of these N-methylations is still missing, a direct correlation between the methylation of some proteins and their biological activity is just beggining to emerge.

INTRODUCTION

Several of the translational apparatus proteins are modified post-synthetically (1-12). Methylations, acetylations and phosphorylations are the most studied of these chemical modifications (1-13). We have been specifically interested in the study of the N-methylations that take place at the protein synthesis machinery (6, 7, 9, 14).

Although the biological significance of these methylations is not understood, it has been suggested that methylation of the ribosomal components may be important for the assembly of the particle (4, 15). On the other hand, the pattern of ribosomal protein methylations appeared to be highly conserved in eubacteria (6, 7), suggesting an important, albeit unknown, role for these chemical alterations. Some of the methylated proteins are known to have important functions for protein synthesis in *Escherichia coli.* Ribosomal proteins L7/L12 are essential for translation and translational accuracy in protein biosynthesis (16-18). The methylated proteins L11 and L16 have both been implicated at or near the peptidyltransferase center of the ribosome (15, 19). The elongation factor EF-Tu and its equivalents, which are also methylated in several organisms (8-12) also play a key role during the translation step in protein synthesis (20, 21). Nevertheless, in spite of the effort of several researchers, no biological function for most of these methylations has yet been established.

However, as very recently pointed out by Stock *et al.* (22), a comparison of the methylation site sequences of the modified proteins from several organisms may be useful not only in establishing the specificity and the possible types of

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methylating enzymes involved, but may eventually allow to predict proteins that may be subjected to post-translational modifications. In addition, comparative studies may allow interesting evolutionary speculations with respect to some of these post-synthetic modifications.

In this minireview, we concentrate our analysis on the available information on most of the N-methylations that take place at the translational machinery, trying to find some common features with respect to the methylation sites and possible specificities of the modifying enzymes, which may be interesting and that may eventually help to find the rather elusive role for these protein modifications.

The methylated proteins from the translational apparatus

Methylation of ribosomal proteins has been studied in several organisms as shown in Table I. Some *E. coli* ribosomal proteins and the elongation factor IF3 are known to be methylated at their amino terminal amino acids. The sites of these methylations in *E. coli* are the N-terminal Me-Met* in IF3, L16 and some of the unprocessed L33 molecules (see below) (5, 24); Me-Ala in mature L33 and S11 (24, 26, 27); Me2-Ala and Me3-Ala in L11 (23, 37) and possibly Me3-Ala in BSL11 from *Bacillus subtilis* (7). The *E. coli* L11 protein not only has three methyl groups at the amino-terminal alanine, but is in addition trimethylated at lysines No 3 and 39 (37). On the other hand, some ribosomal proteins such as L7/L12 or its homologous are methylated only at a single residue, such as Lys No 81 in *E. coli* (25, 28). In addition to the above mentioned methylated amino acids, other such as Me-Lys, Me2-Lys, Me3-Lys, N^G, N^G-Me2-Arg and methyl glutamine are also found in the proteins from the translational machinery (1, 2, 22).

* The abbreviations used for the methylated amino acids are: Me-Lys, ϵ-N-monomethyllysine; Me2-Lys, ϵ-N-dimethyllysine; Me3-Lys, ϵ-N-trimethyllysine; Me-Met, N-methylmethionine; Me-Ala, N-monomethylalanine; Me2-Ala, N-dimethylalanine; Me3-Ala, N-trimethylalanine; N^G, N^G-Me2-Arg, N^G, N^G-dimethylarginine.

Several of the ribosomal proteins methylated in eubacteria are structurally and functionally homologous, indicating a conservation of the methylation on equivalent proteins in different species (6, 7).

As noted by Stock *et al.* (22), many methylated proteins appear to function by interacting with other proteins, often as part of macromolecular complexes. This is specially evident in the case of the translational apparatus, since it can be considered as a supramolecular structure including both, methylated ribosomal proteins and translational factors. Within the ribosome, the pentameric complex formed by two L7/L12 dimers and protein L10 is a relatively independent region of the 50S subunit (17) and all of its constituents are methylated both, in *E. coli* and *B. stearothermophilus* (1, 7). Additionally, the monomethylated lysine-81 of L7/L12 is a residue with a large-side chain mobility (40) and appears to be at the interface of the two L12 molecules which form each of the two dimers in the pentameric complex (41). Nevertheless, whether the methylation of Lys-81 affects the dimer formation or not, remains to be seen. This macromolecular complex is not only important as a site of interaction with the elongation factors (38), but also appears to exert an autogenous control, regulating the translation of both constitutive proteins by interaction with the leader sequence of the L10 operon mRNA (39).

Many other ribosomal proteins have been reported methylated, including those from both cytoplasmic and chloroplastic ribosomes from *Euglena gracilis* (Sanhueza and Jerez, unpublished results), (Table I). However, the sites of methylation are presently unknown.

Two of the elongation factors from *E. coli* are known to be methylated. The IF3 being monomethylated at its amino terminal methionine (5) and EF-Tu, with a single Me-Lys-56 (42) (Table I), which is apparently located in an exposed region of the molecule near the aa-tRNA binding site (43).

TABLE I

Methylated proteins from the translational apparatus in several organisms

Source	Methylated protein		
	Ribosomal	*Factor*	*Reference*
I. EUBACTERIA			
Escherichia coli	S11, L1, L3[a] L5[b], L7/L12[c], L8+9, L10[d], L11[e,*], L16[f], L18, L33		(1, 2, 4, 23, 27)
		EF-Tu	(8, 9, 28)
		IF3	(5)
Salmonella typhimurium		EF-Tu	(8)
Bacillus subtilis	BSL9[c], BSL10, BSL11[e,*], BSL16, BSL18, BSL20		(6, 7)
Bacillus stearothermophilus	BTL5[b], BTL6[a], BTL8[d], BTL11[e,*], BTL13[c], BTL16, BTL20b[f]		(7)
Bacillus megaterium	spots N[o] 1, 3, 9, 11[e,*], 12, 17		(29)
Moderate halophile NRCC 41227	HXA[c]		(30)
Alteromonas espejiana	AL2, AL5, AL10*, AL12, AL13, AL14, AL15		(7)
II. ARCHAEBACTERIA			
Halobacterium cutirubrum	HL3, HL8, HL10*, HL11, HL14		(7)
III. CHLOROPLASTS			
Euglena gracilis	spots A, E, 4, 5b, 7, 14, 18*, 22, 23, 25		(+)
IV. EUKARYOTES			
HeLa cells	spots 3, 20, 28, 50, 51, 52, 55		(31)
	spot 2, 15, 31L S1, S18*, S38, L30, L42		(32, 33)
Saccharomyces cerevisiae	S4, S10, S15, S25, L3, L15[e,*], L18a, L42		(34, 35)

(Table I continued)

Source	Methylated protein		
	Ribosomal	*Factor*	*Reference*
Saccharomyces carlsbergensis	S31, S32, L15*, L41		(36)
Euglena gracilis	spots 39, 41, 47, 49, 51*, 56		(+)
Mucor racemosus	–	EF1-α	(10)
Artemia salina	–	EF1-α	(11)
Mouse 3T3 B cells	–	EF1-α	(12)

[a-f] Methylated proteins that are structurally and functionally homologous to each other (eg.: L3 with BTL6, L5 with BTL5, etc.).
(+) Sanhueza and Jerez, unpublished results.
* The most highly methylated of the ribosomal proteins in each organism.

As Table I also shows, EF-1α, the EF-Tu equivalent in eukaryotes, has been reported as extensively methylated in *A. salina* (11), *M. racemosus* (10) and transformed 3T3 cells (12).

Methylating enzymes and target sites

In general, most of the N-methyltransferases known so far are specific for their natural sustrates since they apparently recognize both, the length and sequence of amino acids and the species of protein (2). As pointed out by Kim and Paik (2), the length of the amino acid sequence near the methylation site necessary for recognition by the methyltransferase might not be long. De Lange *et al.* (44) suggested earlier the general amino acid sequence of X-Lys-Lys-Y, in which X and Y may vary, for the ε-NH2 methylation of the lysine residues of histones and cytochrome c. However, only two *in vitro* methylating activities involved with the translational apparatus proteins have been studied but without too much detail (9, 45). Nevertheless, Stock *et al.* (22) have speculated defining for all proteins, three possible types of putative N-terminal methyltransferases, according to the sequence they would recognize around the N-terminal methylated amino acid. Thus, a QP transferase would monomethylate the N-terminal methionine, recognizing the sequence M L *Q P* K———— in L16 (24) and other proteins non-related to the ribosomal function (22). On the other hand, IF3 having Me-Met (5) and L33 with Me-Met or Me-Ala (5, 26, 27, 46) at their N-terminal amino acid, would be methylated by a hypothetical MAK methyltransferase which would recognize the sequence *M K* G G K R V for IF3 and *(M) A K* G I R G K for L33. The same type of enzyme could recognize the *A K* A P I R A present in the ribosomal protein S11 (22, 24). However, in some cases not only the target sequence of amino acids is important for the recognition by the methyltransferase, but also the tertiary structure of the entire protein being methylated (2). In *E. coli* K strains, almost 25% of L33 is methylated at the initial methionine and the remainder is methylated at the Ala residue exposed by the expected cleavage of the methionine preceding this alanine, as reviewed by Stock *et al.* (22). By using an *in vitro* DNA-dependent system, in which L11 was synthesized and methylated de novo, we found in addition to Me3-Lys, some trace of a neutral amino acid which may correspond to Me3-Ala, suggesting that the *in vitro* system employed would be capable of removing part of the formylmethionine from the N-terminal end of L11 (14). A system like this would be very useful to study *in vitro* the posible control of the methylation of the

N-terminus of proteins such as L11 or L33. In BSL11 from *B. subtilis*, which is structurally and functionally homologous to L11 (47), being both the most methylated proteins of their respective ribosomes, we also found the presence of Me3-Ala in addition of Me3-Lys (6, 7).

The N-terminus of L11 (A K K V Q A Y) (37) could be recognized in part by the proposed MAK enzyme, since it has the N-methyl-Ala-Lys characteristic of this type of enzyme, except that the alanine is trimethylated, as in some eukaryotic N-methylated proteins (22). L15 from *Saccharomyces cerevisiae* is known to be structurally and functionally equivalent to L11 from *E. coli* (48), and both are the most methylated proteins in the large ribosomal subunit (1, 34) (see Table I). Unfortunately, the information on the primary structure of L15 is limited to a 20 amino acids long N-terminal sequence (49) that shows no homology with any region of the L11 sequence (37). This suggests that if L15 is methylated at its amino-terminal end, a different type of enzyme would be responsible. Alternatively, the ribosomal protein L15 can be methylated at internal positions only. On the other hand, a partially purified enzyme or complex, which methylates mainly L11 has been described, although the sites of methylation were not defined (45).

Several of the methylated amino acids can exist in the form of more than one derivative, depending on the number of methyl groups they contain. Some evidence indicates that at least in certain systems a single enzyme rather than different activities would be responsible for a stepwise addition of each methyl group (2).

The sequences containing internal methylated amino acids are known in only a few of the proteins from the translational apparatus. For example, Table II shows the sequences around the *E. coli* monomethyl-lysine-81, which is present in the acidic ribosomal proteins L7/L12

TABLE II

Comparison of actual or putative methylation sites in the acidic ribosomal proteins (L7/L12 type) from several sources

Source	Sequence	Methylation and Reference
	81	
E. coli	R G A T G L G L *K* E A K D L V E S	+ (1, 25, 50)
NRCC 41227	R E I T G L G L *K* E A K A A V D G	+ (30)
M. lysodeikticus I	R E I T G L G L K E A K E V V D N	
B. stearothermophilus	R E I T G L G L K E A K D L V D N	+ (7)
B. subtilis	R E I T G L G L K E A K E L V D N	+ (7)
M. lysodeikticus III	R A L T S L G L K E A K D L V D G	
S. griseus	R E L T S L G L K E A K D L V D G	
D. vulgaris	R A L T G L G L K E A K D K V D G	
R. spheroides	R A I T G L G L K E A K D L V E –	
Chloroplasts	R A L T S L G L K E A K E L I E G	n. d. (+)
E. coli rp1L-518	R (D) A T G L G L K E A K D L V E S	n. d. (51)
E. coli rp1L-564	R G A T G L G L K (K) A K D L V E S	n. d. (51)

The sequences were from *Escherichia coli* (25), NRCC 41227 (30), *Bacillus subtilis* (55), *Bacillus stearothermophilus* (56), *Micrococcus lysodeikticus* I and II (57) and as referred to by Leijonmarck and Liljas (41), *Streptomyces griseus* (58), *Rhodopseudomonas spheroides* (59), spinach chloroplasts (60), *Desulfovibrio vulgaris* (61), *E. coli* rp1L-518 and rp1L-564 (51). Methylation of the protein is indicated by +. The known methylated amino acid in these proteins is indicated by (*). The *E. coli* mutants changes are indicated by parenthesis. (+), Sanhueza and Jerez, unpublished results.

(L12 is identical to L7, except that the last one is acetylated at its N-terminus). This is an evolutionarily conserved region, which could be directly or indirectly involved in interactions with translational factors. In eukaryotic acidic ribosomal proteins however, this region would not be conserved. The Lys-81 has been identified as methylated in the two bacterial species indicated (1, 25, 30). Also, the equivalent acidic ribosomal proteins have been found methylated in two other gram (+) microorganisms (7) although the sites of these methylations are presently unknown.

Since the sequences shown in Table II are highly conserved in eubacteria, it is likely that the same Lys-81 could be recognized and by the same type of methylating enzyme. This enzyme however, would probably be a different entity compared with the N-terminal methyltransferases already mentioned, since their respective target sites differ.

If the putative L7L12 methyltransferase is present in each of the organisms listed in Table II, one could possibly predict the methylation of all the eubacterial type L7/L12 ribosomal proteins. However, we found that the acidic ribosomal proteins from *Alteromonas espejiana,* a moderate halophile (7) and the ones isolated from chloroplasts from *E. gracilis* (Sanhueza and Jerez, unpublished results) were apparently not methylated under the conditions employed. This lack of modification may be due to the fact that the degree of modification may vary depending on the growth conditions. In this regard, the methylation of L7/L12 in *E. coli* greatly varies with growth temperature (50). Alternatively, the L7/L12 methyltransferase could not be universally distributed.

The acidic ribosomal proteins from the *E. coli* mutants rplL-518 (Gly-74 replaced by Asp) and rplL-564 (Glu-82 replaced by Lys) seen in Table II did not show Me-Lys at position 81 (51). As the authors speculated, it could be that the methylating enzyme is inactive in the strains which lack the methylation or more likely, that the structure of the mutationally altered L7/L12 protein is such that the methyltransferase does not recognize its target sequence (51). These data also suggest that the methyltransferase involved is highly specific for the conserved sequence present in L7/L12.

Although the eukaryotic acidic protein calmodulin is completely unrelated to the translational apparatus, it is a methylated protein having Me3-Lys-115 in several species (52, 53). The sequence around this modified residue (H V M T N L G E K* L T D E E V D E) is strikingly similar to the methylated sequence of L7/L12, possesing at least seven identical residues (for example compared with the *B. subtilis* sequence in Table II) and eleven homolog residues out of the 17 shown if one considers the following Dayhoff conservative categories (54): C; S, T, P, A, G; N, D, E, Q; H, R, K; M, I, L, V; F, Y, W.

Table III A shows the sequence of EF-Tu from *E. coli,* which is apparently involved in tRNA-dependent GTP hydrolysis and is the target site for the methylation of Lys-56, and the homolog sequences of the elongation factors from several organisms. However, no information on the methylation of the factors from the last organisms is presently available. Analysing these sequences, one could predict the methylation of the EF-Tu factor from *E. gracilis* chloroplast, since its possible methylating target site is almost identical to that of *E. coli.* To confirm this prediction, we are currently studying the possible *in vivo* methylation of the organellar EF-Tu, assuming the existence of a methylating enzyme in the chloroplast. In this connection, it is known that some protein methyltransferases exist within the organelle (67).

The elongation factors from the other species shown in Table III A also posses a high degree of conservation of the methylating site, except that the target Lys-56 present in *E. coli* is replaced in all of them by an arginine. Whether these proteins are methylated in these arginines remains to be seen. Me-Lys is slightly more basic than Lys and therefore is more similar to arginine. On the other hand, Me2-Lys would apparently decrease the basicity of the ϵ-amino group of lysine (2). Both of

TABLE III

Comparison of *in vivo* methylation sites with similar sequences in elongation factors EF-Tu and EF1-α from several organisms

A.																		
											*							
E. coli	48- 64	:			Q	I	D	N	A	P	E	E	K	A	R	G	I	T	I	N	T
E. gracilis chloroplast	48- 64	:			D	I	D	S	A	P	E	E	K	A	R	G	I	T	I	N	T
Saccharomyces mitochondria	85-101	:			A	I	D	K	A	P	E	E	R	A	R	G	I	T	I	S	T
Methanococcus	59- 75	:			V	M	D	G	L	K	E	E	R	E	R	G	V	T	I	D	V
Saccharomyces	59- 75	:			V	L	D	K	L	K	A	E	R	E	R	G	I	T	I	D	I
Artemia salina	59- 75	:			V	L	D	K	L	K	A	E	R	E	R	G	I	T	I	D	I
B.																					
													***								
Artemia salina	69- 87	:	G	I	T	I	D	I	A	L	W	K	F	E	T	A	S	Y	Y	V	T
Saccharomyces	70- 88	:	G	I	T	I	D	I	A	L	W	K	F	E	T	P	K	Y	Q	V	T
													***								
Artemia salina	209-227	:	W	Y	K	G	W	N	I	E	R	K	E	G	K	A	D	G	K	T	L
Saccharomyces	208-226	:	W	Y	K	G	W	E	K	E	T	K	A	G	V	V	K	G	K	T	L
													***								
Artemia salina	308-326	:	G	F	N	V	K	N	V	S	V	K	E	L	R	R	G	Y	V	A	S
Saccharomyces	307-325	:	G	F	N	V	K	N	V	S	V	K	E	I	R	R	G	N	V	C	G

The sequences indicated were from *E. coli* (43), *Euglena gracilis* chloroplasts (62), *Saccharomyces cerevisiae* mitochondria (63), *Methanococcus vannielii* (64), *Saccharomyces cerevisiae* EF1-α (65) and *Artemia salina* (66). The presence of mono or dimethyllysine is indicated by (*) and that of trimethyllysine by (***).

these modified amino acids have been found in EF-Tu (8, 9, 28), the amount of Me2-Lys increasing towards the late phase of *E. coli* growth (28). It is possible that being both Me-Lys and Arg similar in charge properties and H bonding potential, the replacement of the first amino acid by arginine would maintain the overall chemical properties of the protein. On the other hand, methylation of arginines has also been observed in several proteins, but mainly from eukaryotes (2). Nevertheless, the possible effect of monomethylation of a side chain of an amino acid is much less pronounced than that of a trimethylation. This is a particularly clear in the trimethylation of the N-terminus of the proteins already mentioned. In the case of several calmodulins which contain a Me3-Lys in position 115, it has been shown that Arg and not the unmodified Lys can replace this modified residue in its function (53).

When the known methylated sequences of *A. salina* EF1-α (Table III B) are compared to the sequences shown in A, it is clear that they are completely different. However, comparing the sequences from *A. salina* and *Saccharomyces cerevisiae* shown in B, one could predict that the EF1-α protein from the last organism could have at least two methylated regions: 70-88 and 307-325. In this regard, Fonzi *et al.* (68) anticipated that in addition of the methylation of the EF1-α from *Mucor racemosus* (10), the equivalent factors from all organisms and cell types would be found to contain methylated amino acids. This assumption was based on their own unpublished findings with EF1-α from *Neurospora crassa, Saccharomyces cerevisiae* and HeLa cells (68).

As pointed out by Kim (2), a general rule which can be applied to the *in vivo* methylation sites of several proteins, is that the methylated lysine precedes or is followed by a basic amino acid: X-Lys-Lys-Y or X-Arg-Lys-Y-. However, the *in vivo* methylation sites shown in Tables II and III and most of those discussed by

Stock *et al.* (22) do not apparently follow this rule, suggesting the involvement of different methylating enzymes in many cases.

The possible biological function of methylation

One could think that in general, the methylations of the translational apparatus "just happen", specially in slowly growing cells were both ribosomes and elongation factors, being long-lived would be more exposed to these modifications. However, in many cases there are indications that the methylation takes place while the protein is being newly synthesized (2). Furthermore, as already mentioned, most of the methylations of proteins from the translational machinery take place in components that have important biological roles. Additionally, the apparently high conservation of the methylation patterns for the eubacterial ribosomal proteins (7) and the ubiquitous methylation of some of the elongation factors (5, 8-12, 28, 66, 68) suggest an important albeit unknown role for these modifications.

One line of evidence suggests a role for the methylation of proteins in the protection of the exposed methylated N-terminal sequences to attack by aminopeptidases within the cell (2, 22). It is known that in general, there is an increase of post-translational modifications in cells growing under limiting growth conditions (2). If during these conditions there is a concomitant increase of proteases, the modification of some proteins may serve as a protective device. However, some controversial results have been obtained in this area. Paik and Kim found no significant differences in the rate of tryptic hydrolysis of native versus incompletely methylated proteins (2). On the other hand, methylation of the Lys-115 from calmodulin apparently protects this site from trypsin digestion (69). Finally, a clear evidence indicating that Me2-Lys residues in peptides are not bound by the substrate-binding site of trypsin has recently been shown (70). Nevertheless, dimethylation of Lys-56 in EF-Tu apparently does not protect the protein from proteolytic attack (28).

There have been several attempts to demonstrate the role of N-methylations in proteins in general (2, 4, 15). However, the results obtained so far have not been very clarifying. For example, mutations in the prmA gene of *E. coli* were found to result in defective methylation of the ribosomal protein L11. However, no readily observable phenotype was obtained (71). Furthermore, the methyl groups of the ribosomal protein L11 are not related to the synthesis of ppGpp (72). On the other hand, a possible effect of methylation on the assembly of the ribosomal particle has also been suggested (4, 15).

The methylation of the elongation factor EF1-α from *Mucor racemosus* has been implicated on the acquisition of activity by the protein, since both methylation and the specific activity of the factor increased during the course of spore germination (10, 68). The authors speculated that the apparent ubiquity of EF1-α methylation may imply an important role of the methylated residues in peptide formation. Since methylation increases the basicity of lysine residues, it is not difficult to imagine that the N-methylation of EF1-α could play a role in the ability of the factor to complex with the acidic aminoacyl-tRNA, ribosomes and mRNA and thereby facilitate the hydrolysis of GTP (68).

In the case of the *E. coli* EF-Tu methylation, it was early speculated that the function of methylation, if any, being Lys-56 near the aa-tRNA binding site, could be to improve the binding of aa-tRNA. To study the EF-Tu methylation, we developped some *in vitro* methylating assays in which artificially submethylated EF-Tu or a de novo synthesized EF-Tu were employed as substrates (9). However, one problem that makes these studies difficult, is the separation of the methylated from the unmodified form of the factor, which is usually present in higher amounts. Very recently, Van Noort *et al.* (28) isolated a low methylated EF-Tu (containing about 25% of Me-Lys) from mid log-

arithmic phase cells and the methylated one from late stationary phase grown *E. coli* cells and have compared some properties of both preparations (28). They reported for the first time that both the GTPase activity of the protein and the reactivity of Cys-81 are significantly less stimulated by the tRNA when EF-Tu is methylated. We are currently testing artificially submethylated EF-Tu preparations to confirm these interesting results.

This slowing down of the GTPase activity of EF-Tu by the methylation of the protein may result, as the authors speculated, in a more accurate translation process although it would probably proceed more slowly (28).

Recent techniques, such as site-specific mutagenesis could be elegantly employed to show the participation of the methylation of the side chains of amino acids. One example of this application, which could be applied to other methylated proteins, was very recently described for the methylation of Lys-115 in calmodulin (53). The authors clearly demonstrated that methylation of Lys-115 activated an NAD kinase to a maximal level compared with the unmodified protein (53).

These new results are highly encouraging, and no doubt we soon may have a better description of the up to now mysterious role for these post-translational modifications.

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REFERENCES

1. CHANG, C.N. and CHANG, F.N. (1975) Methylation of the ribosomal proteins in *Escherichia coli.* Nature and stoichiometry of the methylated amino acids in 50 S ribosomal proteins. *Biochem. 14*: 468-477.
2. PAIK, W.K. and KIM, S. (1980) Protein Methylation, pp. 142-166. In A. Meister (ed.), Biochemistry. A series of monographs. John Wiley & Sons, Inc., New York.
3. WOOL, J.G. (1979) The structure and function of eukaryotic ribosomes. *Annu. Rev. Biochem. 48:* 719-754.
4. ALIX, J.H.; HAYES, D. and NIERHAUS, K.H. (1979) Properties of ribosomes and RNA synthesized by *Escherichia coli* grown in the presence of ethionine. V. Methylation dependence of the assembly of *E. coli* 50 S ribosomal subunits. *J. Mol. Biol. 127:* 375-395.
5. BRAUER, D. and WITTMANN-LIEBOLD, B. (1977) The primary structure of the initiation factor IF3 from *Escherichia coli. FEBS Lett. 79:* 269-275.
6. MARDONES, E.; AMARO, A.M. and JEREZ, C.A. (1980) Methylation of ribosomal proteins in *Bacillus subtilis. J. Bacteriol. 142:* 355-358.
7. AMARO, A.M. and JEREZ, C.A. (1984) Methylation of ribosomal proteins in bacteria: evidence of conserved modifications of the eubacterial 50 S subunit. *J. Bacteriol. 158:* 84-93.
8. AMES, G.F.L. and NIAKIDO, K. (1979) *In vivo* methylation of prokaryotic elongation factor Tu. *J. Biol. Chem. 254:* 9947-9950.
9. TOLEDO, H. and JEREZ, C.A. (1985) *In vitro* methylation of the elongation factor EF-Tu from *Escherichia coli. FEBS Lett. 193:* 17-21.
10. HIATT, W.R.; GARCIA, R.; MERRICK, W.C. and SYPHERD, P.S. (1982) Methylation of elongation factor 1α from the fungus *Mucor. Proc. Natl. Acad. Sci. USA 79:* 3433-3437.
11. AMONS, R.; PLUIJMS, W.; ROOBOL, K. and MÖLLER, W. (1983) Sequence homology between EF1-α, the a-chain of elongation factor 1 from *Artemia salina* and elongation factor EF-Tu from *Escherichia coli. FEBS Lett. 153:* 37-42.
12. COPPARD, N.J.; CLARK, B.F.C. and CRAMER, F. (1983) Methylation of elongation factor 1α in mouse 3T3 B and 3T3 B/ SV40 cells. *FEBS Lett. 164:* 330-334.
13. ALIX, J.H. and HAYES, D. (1983) Why are macromolecules modified post-synthetically? *Biol. Cell. 47:* 139-159.
14. JEREZ, C. and WEISSBACH, H. (1980) Methylation of newly synthesized ribosomal protein L11 in a DNA-directed *in vitro* system. *J. Biol. Chem. 255:* 8706-8710.
15. NIERHAUS, K.H. (1979) Analysis of the assembly and function of the 50 S subunit from *Escherichia coli* ribosomes by reconstitution. In Chambliss, G.; Craven, G.R.; Davies, J.; Davis, K.; Kahan, L. and Nomura, M. (ed.), Ribosomes: structure, function and genetics, University Park Press, Baltimore, pp. 267-294.
16. MÖLLER, W. (1974) The ribosomal components involved in EF-G and EF-Tu-dependent GTP hydrolysis. In Nomura, M.; Tissieres, A. and Lengyel, P. (ed.), Ribosomes. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., pp. 711-731.
17. MÖLLER, W.; SCHRIER, P.I.; MAASSEN, J.A.; ZANTEMA, A.; SCHOP, E.; REINALDA, H.; CREMERS, A.F.M. and MELLEMA, J.E. (1983) Ribosomal proteins L7/L12 of *Escherichia coli.* Localization and possible molecular mechanism in translation. *J. Mol. Biol. 163:* 553-573.
18. KIRSEBOM, L.A. and ISAKSSON, L.A. (1985) Involvement of ribosomal protein L7/L12 in control of translational accuracy. *Proc. Natl. Acad. Sci.* USA *82:* 717-721.
19. NIERHAUS, K.H. (1982) Structure, assembly and function of ribosomes. *Curr. Top. Microbiol. Immunol. 97:* 82-155.
20. MILLER, D.L. and WEISSBACH, H. (1977) Factors involved in the transfer of aminoacyl-tRNA to the ribosome. In Molecular mechanisms of protein biosynthesis (Weissbach, H. and Pestka, S., eds.) pp. 323-373, N.Y. Academic Press.

21. KAZIRO, Y. (1978) The role of guanosine 5'-triphosphate in polypeptide chain elongation. *Biochim. Biophys. Acta 505:* 95-127.
22. STOCK, A.; CLARKE, S.; CLARKE, C. and STOCK, J. (1987) N-terminal methylation of proteins: structure, function and specificity. *FEBS Lett: 220:* 8-14.
23. LEDERER, F.; ALIX, J.H. and HAYES, D. (1977) N-trimethylalanine, a novel blocking group, found in *E. coli* ribosomal protein L11. *Biochem. Biophys. Res. Commun. 77:* 470-480.
24. CHEN, R.; BROSIUS, J. and WITTMANN-LIEBOLD, B. (1977) Occurrence of methylated amino acids as N-termini of proteins from *Escherichia coli* ribosomes. *J. Mol. Biol. 111:* 173-181.
25. TERHORST, C.; MÖLLER, W.; LAURSEN, R. and WITTMANN-LIEBOLD, B. (1972) Amino acid sequence of a 50 S ribosomal protein involved in both EFG and EFT dependent GTP-hydrolysis. *FEBS Lett. 28:* 325-328.
26. CHANG, C.N.; SCHWARTZ, M. and CHANG, F.N. (1976) Identification and characterization of a new methylated amino acid in ribosomal protein L33 of *Escherichia coli. Biochem. Biophys. Res. Commun. 73:* 233-239.
27. CHANG, F.N. and BUDZILOWICZ, C. (1977) Characterization of methylated neutral amino acids from *Escherichia coli* ribosomes. *J. Bacteriol. 131:* 105-110.
28. VAN NOORT, J.M.; KRAAL, B.; SINJORGO, K.M.C.; PERSOON, N.L.M.; JOHANNS, E.S.D. and BOSCH, L. (1986) Methylation *in vivo* of elongation factor EF-Tu at lysine-56 decreases the rate of tRNA-dependent GTP hydrolysis. *Eur. J. Biochem. 160:* 557-561.
29. CANNON, M. and CUNDLIFFE, E. (1979) Methylation of basic proteins in ribosomes from wild-type and thiostrepton-resistant strains of *Bacillus megaterium* and their electrophoretic analysis. *Eur. J. Biochem. 97:* 541-545.
30. MATHESON, A.T.; MÖLLER, W.; AMONS, R. and YAGUCHI, M. (1979) Comparative studies on the structure of ribosomal proteins, with emphasis on the alanine-rich, acidic ribosomal, "A" protein. In Chambliss, G.; Craven, G.R.; Davies, J.; Davis, K.; Kahan, L. and Nomura, M. (ed.). Ribosomes: structure, function and genetics. University Park Press, Baltimore, pp. 297-332.
31. CHANG, F.N.; NAVICKAS, I.J.; AU, C. and BUDZILOWICZ, C. (1978) Identification of the methylated ribosomal proteins in HeLa cells and the fluctuation of methylation during the cell cycle. *Biochim. Biophys. Acta. 518:* 89-94.
32. VANDREY, J.P.; GOLDENBERG, C.J. and ELICEIRI, G.L. (1976) *In vivo* isotope incorporation patterns into HeLa ribosomal proteins. *Biochim. Biophys. Acta. 432:* 104-112.
33. GOLDENBERG, C. and ELICEIRI, G.L. (1977) Methylation of ribosomal proteins in HeLa cells. *Biochim. Biophys. Acta. 479:* 220-234.
34. CANNON, M.; SCHINDLER, D. and DAVIES, J. (1977) Methylation of proteins in 60 S ribosomal subunits from *Saccharomyces cerevisiae. FEBS Lett. 75:* 187-191.
35. HERNANDEZ, F. and CANNON, M. (1978) Methylation of proteins in 40 S ribosomal subunits from *Saccharomyces cerevisiae. FEBS Lett. 89:* 271-274.
36. KRUISWIJK, T.; KUNST, A.; PLANTA, R.J. and MAGER, W.H. (1978) Modification of yeast ribosomal proteins. Methylation. *Biochem. J. 175:* 221-225.
37. DOGNIN, M.J. and WITTMANN-LIEBOLD, B. (1977) The primary structure of L11, the most heavily methylated protein from *Escherichia coli* ribosomes. *FEBS Lett. 84:* 342-346.
38. LAKE, J.A. (1985) Evolving ribosome structure: domains in archaebacteria, eubacteria, eocytes and eukaryotes. *Annu. Rev. Biochem. 54:* 507-530.
39. JOHNSEN, M.; CHRISTENSEN, T.; DENNIS, P.P. and FIIL, N.P. (1982) Autogenous control: ribosomal protein L10-L12 complex binds to the leader sequence of its mRNA. *EMBO J. 1:* 999-1004.
40. AQVIST, J.; VAN GUNSTEREN, W.F.; LEIJONMARCK, M. and TAPIA, O. (1985) A molecular dynamics study of the C-terminal fragment of the L7/L12 ribosomal protein. Secondary structure motion in a 150 picosecond trajectory. *J. Mol. Biol. 183:* 461-477.
41. LEIJONMARCK, M. and LILJAS, A. (1987) Structure of the C-terminal domain of the ribosomal protein L7/L12 from *Escherichia coli* at 1.7 Å. *J. Mol. Biol. 195:* 555-580.
42. L'ITALIEN, J.J. and LAURSEN, R.A. (1979) Location of the site of methylation in elongation factor Tu. *FEBS Lett. 107:* 359-362.
43. LAURSEN, R.A.; L'ITALIEN, J.J.; NAGARKATTI, S. and MILLER, D.L. (1981) The amino acid sequence of elongation factor Tu of *Escherichia coli.* The complete sequence. *J. Biol. Chem. 256:* 8102-8109.
44. DE LANGE, R.J.; GLAZER, A.N. and SMITH, E.L. (1969) Presence and location of an unusual amino acid, ϵ-N-trimethyllysine, in cytochrome c of wheat germ and Neurospora. *J. Biol. Chem. 244:* 1385-1388.
45. CHANG, F.N.; COHEN, L.B.; NAVICKAS, I.J.; and CHANG, C.N. (1975) Purification and properties of a ribosomal protein methylase from *Escherichia coli* Q13. *Biochem. 14:* 4994-4998.
46. WITTMANN-LIEBOLD, B. and PANNENBECKER, R. (1976) Primary structure of protein L33 from the large subunit of the *Escherichia coli* ribosome. *FEBS Lett. 68:* 115-118.
47. SMITH, I.; PARESS, P. and PESTKA, S. (1978) Thiostrepton-resistant mutants exhibit relaxed synthesis of RNA. *Proc. Natl. Acad. Sci. USA 75:* 5993-5997.
48. EL-BARADI, T.T.A.L.; DE REGT, V.C.H.F.; EINERHAND, S.W.C.; TEIXIDO, J.; PLANTA, R.J.; BALLESTA, J.P.G. and RAUE, H.A. (1987) Ribosomal proteins EL11 from *Escherichia coli* and L15 from *Saccharomyces cerevisiae* bind to the same site in both yeast 26 S and mouse 28 S rRNA. *J. Mol. Biol. 195:* 909-917.
49. OTAKA, E.; HIGO, K. and ITOH, T. (1984) Yeast ribosomal proteins. VIII. Isolation of two proteins and sequence characterization of twenty-four proteins from cytoplasmic ribosomes. *Mol. Gen. Genet. 195:* 544-546.
50. CHANG, F.N. (1978) Temperature-dependent variation in the extent of methylation of ribosomal proteins L7 and L12 in *Eschericha coli. J. Bacteriol. 135:* 1165-1166.
51. KIRSEBOM, L.A.; AMONS, R. and ISAKSSON, L.A. (1986) Primary structures of mutationally altered ribosomal protein L7/L12 and their effects on cellular growth and translational accuracy. *Eur. J. Biochem. 156:* 669-675.

52. DIETER, P. (1984) Calmodulin and calmodulin-mediated processes in plants. *Plant, Cell Env. 7:* 371-380.
53. ROBERTS, D.M.; ROWE, P.M.; SIEGEL, F.L.; LUKAS, T.J. and WATTERSON, D.M. (1986) Trimethyllysine and protein function. Effect of methylation and mutagenesis of lysine 115 of calmodulin on NAD kinase activation. *J. Biol. Chem. 261:* 1491-1494.
54. DAYHOFF, M.O., ed. (1978) Atlas of protein sequence and structure (National Biomedical Research Foundation, Washington, D.C.), pp. 345-352.
55. ITOH, T. and WITTMANN-LIEBOLD, B. (1980) The primary structure of *Bacillus subtilis* acidic ribosomal protein B-L9. *J. Biochem.* (Tokyo) *87:* 1185-1201.
56. GARLAND, W.G.; LOVIE, K.A.; MATHESON, A.T. and LILJAS, A. (1987) The complete amino acid sequence of the ribosomal "A" protein (L12) from *Bacillus stearothermophilus. FEBS Lett. 220:* 43-46.
57. ITOH, T. (1981) Primary structure of an acidic ribosomal protein from *Micrococcus lysodeikticus. FEBS Lett. 127:* 67-70.
58. ITOH, T.; SUGIYAMA, M. and HIGO, K.I. (1982) The primary structure of an acidic ribosomal protein from *Streptomyces griseus. Biochim. Biophys. Acta 701:* 164-172.
59. ITOH, T. and HIGO, K.I. (1983) Complete amino acid sequence of an L7/L12-type ribosomal protein from *Rhodopseudomonas spheroides. Biochim. Biophys. Acta 744:* 105-109.
60. BARTSCH, M.; KIMURA, M. and SUBRAMANIAN, A-R. (1982) Purification, primary structure, and homology relationships of a chloroplast ribosomal protein. *Proc. Natl. Acad. Sci. USA 79:* 6871-6875.
61. ITOH, T. and OTAKA, E. (1984) Complete amino acid sequence of an L7/L12-type ribosomal protein from *Desulfovibrio vulgaris,* Miyazaki. *Biochim. Biophys. Acta, 789:* 229-233.
62. MONTANDON, P.E. and STUTZ, E. (1983) Nucleotide sequence of *Euglena gracilis* chloroplast genome region coding for the elongation factor Tu: Evidence for a spliced mRNA. *Nucleic Acids Res. 11:* 5877-5892.
63. NAGATA, S.; TSUNETSUGU-YOKOTA, Y.; NAITO, A. and KAZIRO, Y. (1983) Molecular cloning and sequence determination of the nuclear gene coding for mitochondrial elongation factor Tu of *Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA 80:* 6192-6196.
64. LECHNER, K. and BOCK, A. (1987) Cloning and nucleotide sequence of the gene for an archaebacterial protein synthesis elongation factor Tu. *Mol. Gen. Genet. 208:* 523-528.
65. NAGATA, S.; NAGASHIMA, K.; TSUNETSUGU-YOKOTA, Y.; FUJIMURA, K.; MIYAZAKI, M. and KAZIRO, Y. (1984) Polypeptide chain elongation factor 1 (EF1-α) from yeast: nucleotide sequence of one of the two genes for EF1-α from *Saccharomyces cerevisiae. EMBO J. 3:* 1825-1830.
66. LENSTRA, J.A.; VLIET, A.V.; ARNBERG, A.C.; VAN HEMERT, F.J. and MÖLLER, W. (1986) Genes coding for the elongation factor EF 1-α in *Artemia. Eur. J. Biochem. 155:* 475-483.
67. BLACK, M.T.; MEYER, D.; WIDGER, W.R. and CRAMER, W.A. (1987) Light-regulated methylation of chloroplast protein. *J. Biol. Chem. 262:* 9803-9807.
68. FONZI, W.A.; KATAYAMA, C.; LEATHERS, T. and SYPHERD, P.S. (1985) Regulation of protein synthesis factor EF1-α in *Mucor racemosus. Mol. Cel. Biol. 5:* 1100-1103.
69. MARSHAK, D.R.; CLARKE, M.; ROBERTS, D.M. and WATTERSON, D.M. (1984) Structural and functional properties of calmodulin from the eukaryotic microorganism *Dictyostelium discoideum. Biochem. 23:* 2891-2899.
70. PONCZ, L. and DEARBORN, D.G. (1983) The resistance to tryptic hydrolysis of peptide bonds adjacent to N^{ϵ}, N-dimethyllysil residues. *J. Biol. Chem. 258:* 1844-1850.
71. COLSON, C. and SMITH, H.O. (1977) Genetics of ribosomal protein methylation in *Escherichia coli.* I. A mutant deficient in methylation of protein L11. *Mol. Gen. Genet. 154:* 167-173.
72. ROHL, R. and NIERHAUS, K.H. (1979) Methylgroups of ribosomal protein L11 are not related to the synthesis of ppGpp. *Mol. Gen. Genet. 170:* 187-189.

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The production of foreign proteins in *Saccharomyces cerevisiae*

La producción de proteínas extrañas en *Saccharomyces cerevisiae*

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Recombinant DNA technology can advantageously be used to produce medically and commercially important proteins. The ability to express eukaryotic genes in microorganisms allows the large-scale production of proteins which cannot be produced in significant amounts from natural sources.

This article describes part of the work that my colleagues and myself have carried out using the yeast system in the production of enzymes, growth factors and antigens for therapeutic, vaccine or diagnostic uses.

There are several reasons to select yeast as an important system in the biotechnology industry. Yeast cells can be genetically engineered toward the synthesizing of new and valuable proteins and to improve old strains or to develop new strains for industrial fermentation perhaps through sophisticated changes in metabolic pathways. Being a eukaryote, yeast can carry out a series of post-translational modifications which cannot be performed by bacteria. In addition, yeast is not a pathogen and it is widely used in the large scale production of food-related products. This feature should simplify its usé in the manufacturing scale and greatly assist the production of pharmaceutical products free from pyrogenic and toxic contaminants.

Yeast expression vectors

Genetic programming of yeast cells for the production of foreign proteins involve several steps. The first one is the construction of specialized plasmid expression vectors which contain the region coding for the protein of interest assembled together with the proper DNA regions necessary for expression such as promoters, enhancers, terminators and, if required, upstream regulatory sequences. The ideal plasmid vector for the expression of foreign proteins in yeast is one that can be used to transform cells at high frequency, can be maintained inside the cell at high copy number and is properly segregated during cell division. The type of vector that we have used employs a defective yeast LEU-2 gene as selectable marker for leu2 minus cells and contains most of the genetic elements of the yeast plasmid 2-micron (*Figure 1*). Among eukaryotes, the yeast *Saccharomyces cerevisiae* has unique advantages because it is the only organism that is known to carry a high copy number, autonomously replicating, extrachromosomal DNA element named the 2-micron DNA (1). The entire 2-micron DNA sequence has been determined (2). It is a 6218 bp double-stranded circular DNA molecular present at approximately 50 copies per haploid genome in most *Saccharomyces* strains. A unique structural feature is the presence is 2-micron circle of two regions of approximately 600 bp each which are precisely inverted repeats of each other. The 2-micron circle has a single efficient origin of replication which has been localized to a 350 bp region. Replication of the 2-micron circle is normally under cell-cycle control.

Although autonomous replication is conferred by the 2-micron circle origin, propagation at high copy number requires, in addition, other gene products. Two proteins REP1 and REP2 are required for the stable maintenance of the plasmid at high copy number (3). The genes encoding

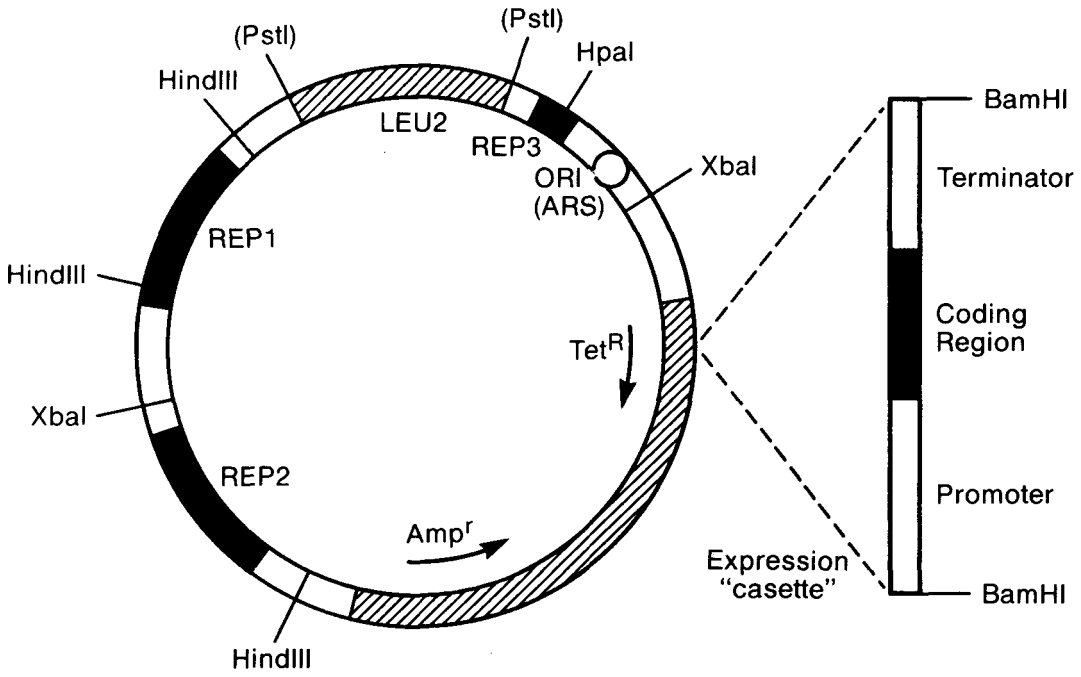

Fig. 1: A plasmid vector for the efficient expression of foreign proteins in yeast.

these functions have been identified by mutational analysis. The REP proteins of 2-micron circle apparently maintain high copy number by promoting amplification when the copy number is low. The mechanism by which the plasmid senses its own copy number and by which REP proteins induce multiple rounds of replication have not yet been identified. In addition to the REP1 and REP2 genes which act in trans, the amplification system requires a site designated REP3 which is located several hundred base pairs aways from the origin of replication, and which acts in cis.

For expression, a so-called "cassette" containing the gene to be expressed as well as appropriate yeast promoter and termination regions is inserted in the vector. Good expression requires the presence of an homologous yeast promoter. Several different yeast promoters have been used by workers in the field. The tendency is to employ the promoter region of a yeast glycolytic enzyme such as glyceraldehyde 3-P-dehydrogenase, P-glycerate kinase, or enolase which are expressed at high levels in yeast growing in glucose.

Regulation of expression

An important subject in the expression of foreign proteins in yeast is the area of regulation. In several instance the high-level expression of heterologous proteins may be deleterious to cell-growth either after genetic transformation or during fermentation. Several systems have been proposed for the regulation of expression in yeast. These involve changes in the concentration of glucose to regulate the PGK promoter in the synthesis of human inteferon α; changes in the concentration of Cu^{++} ions, galactose, and glucose, etc. in the synthesis of HBsAg, or EGF from the yeast copper-chelating, GAL-10 or ADR-2 promoters, respectively.

A particularly amenable system for large scale fermentation is that provided by yeast ADR-2 system. As demonstrated by Young and coworkers (5), the promoter of yeast alcohol dehydrogenase II is inactive at high concentrations of glucose and very active at low concentrations of the sugar. This is an optimal system for expressing the foreign proteins after the growth cycle has

been completed and the glucose concentration is low. J. Shuster and collaborators have demonstrated the utility of the ADR-2 system in the regulation of the synthesis and secretion of human epidermal growth factor, wich is high at low glucose concentrations and completely abolished at high glucose concentrations (6).

Other systems amenable for high scale production is that provided by changes in temperature. For example, A. Brake and collaborators have regulated the expression of hEGF from the α-factor promoter by using temperature sensitive sir3 mutants host which at the non-permissive temperature resemble diploid cells and do not express α or *a* specific gene products (7).

Another possibility is the use of Cu^{++} ions. Years ago J. Welch, S. Fogel and M. Karin isolated the yeast CUP1 gene which confers copper resistance to yeast (8). We later sequenced this gene and found out that it codes for a copper chelating protein of 60 amino acids which is transcriptionally induced by Cu^{++} (9). We have fused the 5' flanking region of this gene to foreign genes such as the HBsAg gene and shown that the synthesis of HBsAg is now dependent on the concentration of copper (10).

Integration and industrial strains

During our analysis of the expression of heterologous genes in yeast it has become evident that the levels of expression are not only dependent on the vector but also on the yeast strain. Another important areas of work is therefore the development of methods for transformation and efficient expression of foreign proteins in a broad range of yeast strains. Particularly important are the industrial strains which in contrast to laboratory strains grow several times more vigorously, to higher cell densities but which are genetically undefined.

Using the yeast CUP1 gene and the yeast tunicamycin resistance (TUN) gene, R. Bishop at Chiron has developed vectors for the transformation and stable high level of expression of heterologous proteins such as human superoxide dismutase in industrial yeast strains. These vectors contain both th TUN gene and the CUP1 gene in the same plasmid so both selective markers are used simultaneously. Expression is driven by the yeast glyceraldehyde 3-P-dehydrogenase promoter. Expression of human SOD by the Fleischman industrial strain is stable and of the order of at least 10% of the total cell protein (R. Bishop, unpublished results).

In addition to the development of plasmid episomal vectors, in attempts to improve yeast strains for industrial use, it will be crucial to be able to genetically modify the strains at the level of the chromosomes. R. Bishop and collaborators have developed methods to transform cells with integrating plasmids after which integration can be amplified and direct the synthesis of increased levels of foreign proteins such as human SOD. These integrating expression vectors contain first, a gene homologous to one present in the yeast chromosome (this sequence allows for integration and subsequent amplification by unequal crossing-over during mitosis); second, a gene providing resistance to drugs or heavy metals (CUP1, TUN, etc.) which allows for selective pressure; and third, an expression cassette for any foreign protein which will be coamplified with the above sequence. The integration and amplification level has been analyzed by Southern hydridization using DNA digested with restriction endonucleases which cut the vector but not within the sequences homologous to the integration site. As expected in the expression of the model protein, human SOD is largely increased after amplification of the integrated sequences.

Therapeutic enzymes

One particulary successful example of the use of the yeast system to produce foreign proteins of therapeutic potencial is the production of human superoxide dismutase (SOD). SOD is a cytoplasmic enzyme produced by the body to break down and scavenge free oxygen radicals (11, 12). Oxygen is obviously vital to the proper functioning of living tissue, but excessive oxygen supplied over a compressed period of time can be toxic. This often occurs after vital organs have been deprived of,

and then reperfused with oxygen. Examples might be heart muscle or brain tissue that has survived an infarct due to clots in arteries carrying oxygen-rich blood; successful organ transplants; patients coming off a heart/lung machine after bypass surgery; cancer patients undergoing experimental therapies aimed at depriving tumors of oxygen support; and premature infants or other ventilator-dependent patients receiving their primary source of oxygen by machine. SOD also may be indicated for treating rheumatoid arthritis, where the inflammation and destruction of joints may be mediated by release of free oxygen radicals in response to a cascade of events caused by the autoimmune process. R. Hallewell and collaborators (13) have produced this enzyme in amounts approaching 40% of the total yeast cell protein. These genetically engineered yeast cells make the authentic human protein. *S. cerevisiae* has a system that efficiently cleaves the N-terminal methionine and acetylates the next N-terminal residue, alanine. In contrast, *E. coli* is able to remove the methionine but does not acetylate the alanine residue. The human SOD made by yeast should be therefore a potentially better pharmaceutical product than the protein made by *E. coli.*

The ability to efficiently synthesize active foreign proteins and the easy of genetic manipulation of the yeast cells lend them as excellent system for protein engineering. S. Rosenberg, R. Hallewell and P. Barr and their collaborators (14) have engineered yeast cell for the production of human alpha$_1$-antitrypsin a protein of therapeutic potential in emphysema, through its inhibitory action on leukocyte elastase. However, oxidation of the methionine 358 located at the active site of alpha$_1$-antitrypsin results in a dramatic decrease of inhibitory activity. The availability of oxidation-resistant alpha$_1$-antitrypsin would make it possible to reduce the large doses required and provide a treatment for adult respiratory distress syndrome. S. Rosenberg and colleagues changed one base by in vitro mutagenesis and developed yeast cells making fully active, oxidation-resistant human alpha$_1$-antrypsin, having a valine instead of methionine at position 358 (14).

Secretion of foreign proteins

Yeast cells secrete very few proteins. In biotechnology it will be of obvious advantage to secrete the desired proteins into the medium. This can greatly facilitate purification and allow increased yields by using continuous fermentation techniques. Yeast secretes proteins by a general pathway similar to that of higher eukaryotes. The present knowledge of the events during the export of proteins in yeast is largely due to the work of R Schekman and collaborators who have developed an elegant genetic and biochemical approach to the study of this process (15). Secretory proteins enter the endoplasmic reticulum where the initial steps of glycosylation occur. Nine or more SEC gene products are required to transport the secretory proteins to the Golgi where further glycosylation occurs. Two or more gene products facilitate the packaging of nearly fully glycosylated proteins into secretory vesicles which are then transported into the bud where they fuse with the plasma membrane under the direction of at least 10 other gene products. Studies on the secretion of the vacuolar enzyme carboxypeptidase Y show that the vacuolar and secretory glycoproteins require the same cellular functions for transport at the level of the endoplasmic reticulum and the Golgi (15). The Golgi represents a branching point in the pathway. From this organelle vacuolar proenzymes are transported to the vacuole for proteolytic processing and secretory proteins are packaged into vesicles. The signals that make a secretory protein either to be inserted on the membrane or to establish an extracellular location at the cell wall or simply go freely into the medium are not yer understood. Based on the work of I. Herkowitz and J. Thorner and their colleagues (16, 17) Brake and coworkers have developed a method for the secretion of foreign proteins in yeast based on the α-factor pheromone (7). Mating in yeast is facilitated by oligopeptide hormones that cause arrest of the cells of the opposite mating type in

the G_1 phase of the cell division cycle. α-Cells produce and secrete the α-factor, a thirteen amino acid peptide which binds to specific receptors in the surface of *a*-cells to induce mating. Conversely, opposite mating type *a*-cells produce and secrete *a*-factor, an eleven amino acid peptide which binds α-cells to induce mating. The interest in these proteins stand from the efficiency by which they are secreted into the medium. The structure of the α-factor gene product deduced from the sequence of the gene isolated by J. Kurjan and I. Herkowitz (16) predicts that the α-factor protein is synthesized as a putative precursor of 165 amino containing a leader of approximately 85 amino acids (*Figure 2*). The leader has two domains. The N-terminal region, contains a canonical hydrophobic signal sequence of 20 or so residues. The remainder is hydrophilic and includes three potential glycosylation sites. The carboxyl terminal end of the precursor contains four exact occupies of the mature α-factor protein, which are linked by four short spacer peptides. The leader and spacer amino acids appear to contain the signals necessary for processing and secretion. Processing can occur by the combined action of specific proteolytic enzymes. A trypsin-like enzyme will act on the precursor and produce an intermediate with the spacer sequence $(Glu\text{-}Ala)_3$ still attached to the amino terminus of the α-factor and with Lys or Lys-Arg at its carboxyl terminus. This carboxyl terminus is probably trimmed by a carboxypeptidase B-like activity. The correct amino terminus of the α-factors protein could be generated by the action of a dipeptidyl aminopeptidase that specifically cleaves at the carboxyl side of the Glu-Ala sequences (18).

A. Brake, P. Barr and collaborators have engineered yeast to secrete several proteins using the α-factor system. Plasmids have been constructed in which the gene coding

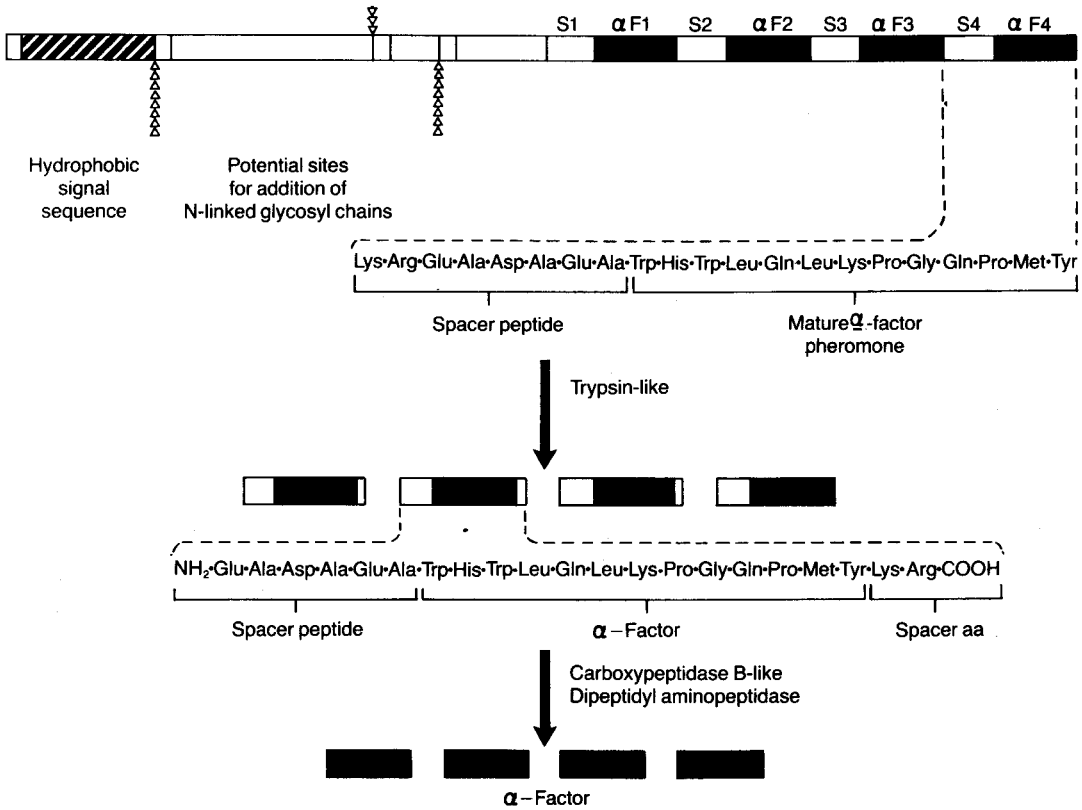

Fig. 2: Schematic representation of the α-factor secretion system in yeast.

for foreign proteins has been fused to the α-factor leader through a spacer region which contained slight sequence variations to allow the study of the proteolytic processing step. These studies indicate that the α-factor leader sequence is sufficient to drive the export of foreign proteins in yeast, that the processing enzyme dipeptidyl aminopeptidase which removes the Glu-Ala residues is limiting at high expression levels and third, that the system is general enough to allow the expression and secretion of several human proteins in yeast. These proteins include epidermal growth factor (7), growth hormone releasing factor (19), insulin-like growth factors (20), connective tissue activating peptide (21), interleukin-2 (22) and proinsulin (23).

Human epidermal growth factor

The protein that we have used as a model for the studies on secretion of heterogenous proteins in yeast is human epidermal growth factor (EGF) (24, 25). This hormone is a single chain polypeptide of 53 amino acids which stimulates the growth of skin and corneal epithelium in vivo and in organ cultures. In addition, EGF is a potent mitogen for certain human and mouse fibroblasts in culture and is a potent inhibitor of gastric acid secretion. The therapeutic potential of human EGF in the acceleration of repair of epithelial tissue traumatized by burns, accidents and surgery as well as in skin grafting and corneal surgery is significant. In ophthalmology hEGF may prove to be particularly versatile, accelerating healing not only in a few thousand serious burns and corneal transplants, but in hundreds of thousands of other procedures that involve manipulation of the cornea. These would range from conventional procedures like cataract surgery and the associated implantation of intra-ocular lenses to, perhaps most intriguing, the newly developing field to radial keratotomy, a minor surgical procedure for vision correction that unfortunately often requires an extended period of corneal repair.

The recombinant human EGF secreted by yeast cells has been purified to homogeneity and found to be identical in structure and biological activity to the hormone isolated from humans (7, 26). Recombinant human EGF has been tested with success in animal would repair models (27, 28) and it is presently being used in numerous burn centers around the world to accelerate the growth of skin cells in tissue culture prior to autologous skin grafting. Clinical studies in humans are presently underway for EGF as a wound healing agent in the donor site of autologous skin drafts, various skin ulcers and in corneal surgery.

Complex viral antigens

Finally, I would like to summarize our work on the use of yeast in the expression of complex viral antigens. As we have reported years ago, (29, 30) yeast cells transformed with specially designed plasmid expression vectors are able to synthesize and assemble a complex multimeric lipoprotein particle, the hepatitis B surface antigen (*Figure 3*). This particle is structurally very similar to the viral envelope and is highly antigenic upon injection into animals and man. Chimpanzees vaccinated with this material are totally protected against intravenous challenge with hepatitis B virus (31). After successful clinical studies in more than 2,000 individuals worldwide, this antigen has been approved by the FDA in the US last year as the first human vaccine produced by recombinant DNA methods.

Recently, the yeast system has been utilized for the synthesis of other surface antigens such as those from herpes virus (32), feline leukemia virus (33), AIDS virus (34), and malaria (35).

The most relevant property of the polymeric HBsAg particle is that basically it has solved the problem of antigen presentation which is one of the main problems of subunits vaccines made by recombinant DNA. It is a polymer of high MW, very stable and highly immunogenic. I have therefore argued that is should be possible to use the membrane insertion and in vivo assembly properties of the hepatitis B surface antigen protein in yeast for the in

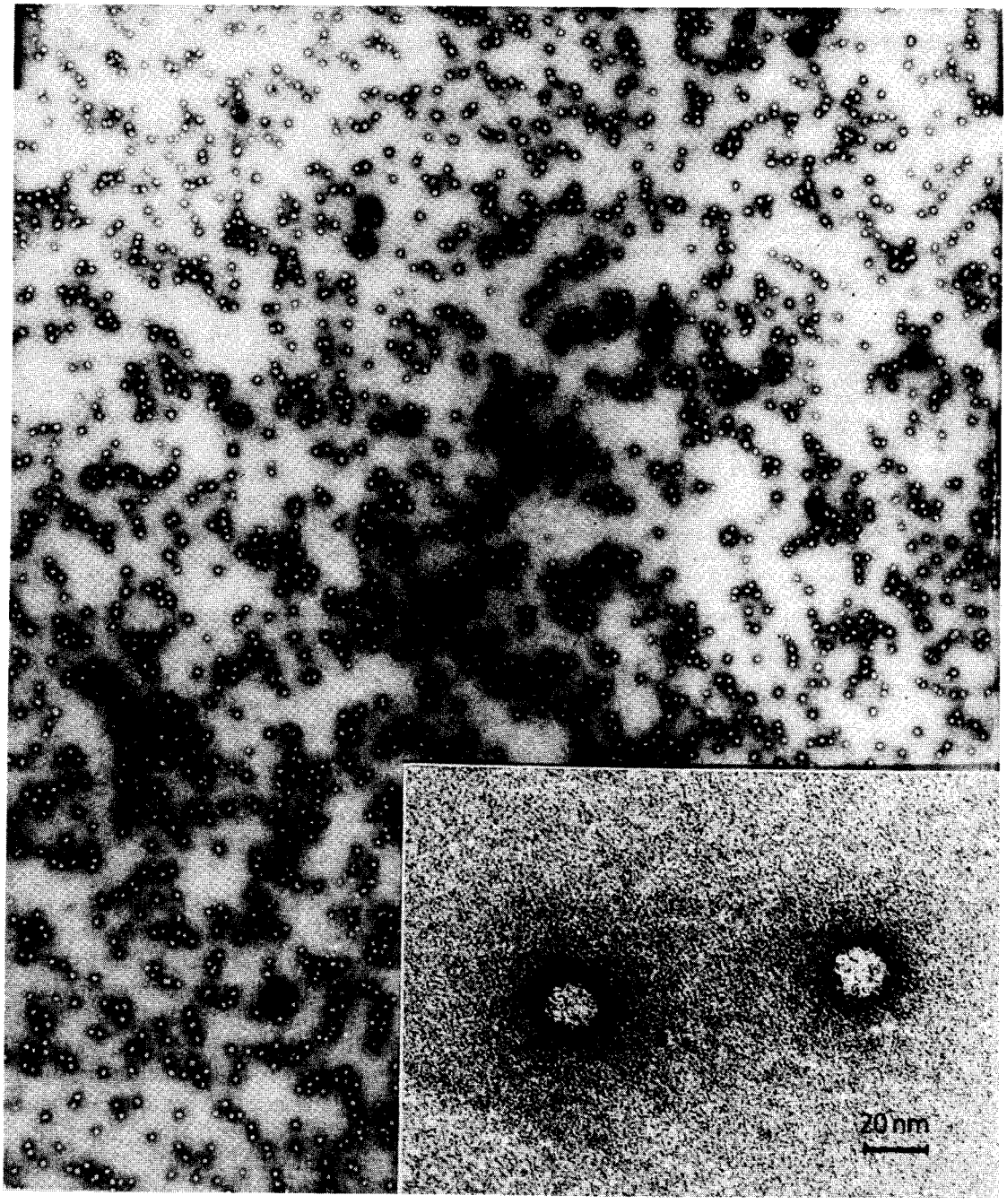

Fig. 3: Electron micrograph of the hepatitis B surface antigen particles made in yeast.

vivo assembly and presentation of other antigens (*Figure 4*). The main epitopes of the HBsAg including those determining the serotype specificities are located in the region betwen amino acids 110 and 156. There are other regions of importance such as the transmembrane protein region between amino acids 80 and 100. The first objective is therefore to identify those regions where new proteins can be inserted. These insertions should be such that 1) the assembly of the particle in yeast is not affected, 2) the HBsAg epitopes remain active (not essential but preferable) and 3) the new epitopes are exposed and accessible at the surface of the particle. The new epitopes can be introduced by genetically engineered hybrid genes.

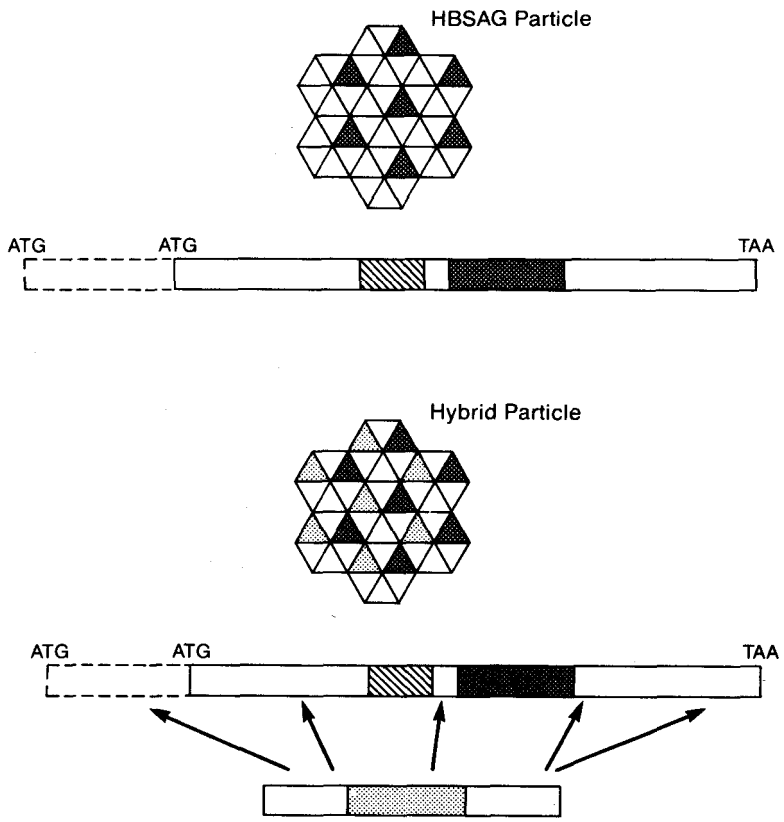

Fig. 4: Hybrid hepatitis B surface antigen particles containing "foreign" antigens. The segment coding for the 55 amino acids of the pre-S region is shown with an interrupted line. The region coding for the HBsAg transmembrane domain is shown filled with lines. The region coding for the main epitopes of HBsAg is shown in dark gray. The region coding for the new "foreign" epitopes is shown in light gray.

As a model system to test the concept and the properties of a hydrid particle, we have taken a segment of the gene coding for the herpes simplex-1 gD protein and inserted in phase into the region coding for the pre-S segment (36, 37). This fragment codes for 300 amino acids of the gDl protein, and does not contain the regions coding for the signal sequence at the N-terminus and the hydrophobic membrane-anchor region of the C-terminus. Both regions are obviously unnecessary for a construction of this type. This hybrid gene has been flanked by sequences required for expression such as the yeast glyceraldehyde 3-P-dehydrogenase promoter and terminator regions and introduced in a plasmid able to replicate and to be selected in yeast cells. This plasmid was introduced in yeast cells and extracts were analyzed for the presence of HBsAg epitopes and HSV-1 gD epitopes in the same molecule.

Results obtained in a sandwich assay using anti HSV-1 glycoprotein antibodies immobilized to polystyrene beads to capture the antigen and a second soluble anti-HBsAg antibody conjugated to HRP to develop color indicate that lysates from yeast cells transformed with the plasmid harboring the hydrid gene contain an antigen capable of reacting with both the immobilized HSV-1 gD and the soluble HBsAg antibodies (37). To further confirm this result, we also carried out the inverse experiment, a sandwich assay using anti-HBsAg immobilized to beads to capture the antigen and a second solute anti HSV-1 glycoprotein antibody to develop color. The results show again that yeast lysates contain an antigen able to react with both antibodies (37).

To further confirm that a fusion protein containing HSV-1 gD and HBsAg epitopes was being synthesized in yeast, we analyzed

the molecular weight of the monomer protein by gel electrophoresis and transfer to a nitrocellulose filter. A sample form lysates of a yeast strain synthesizing directly the 300 amino acid region of HSV-1 gD was also included in the experiment as control. The filter was treated with rabbit anti HSV-1 glycoproteins antibody and the color developed with a second antibody conjugated with HRP. A band of approximately 30 Kd as expected for the HSV-1 gD gene fragment used was obtained in the control lysates expressing this region (37). A much larger band, of approximately 65 Kd, very close to the expected size for the sum of HBsAg and HSV-1 gD region, was obtained in the lysates from cells expressing the hybrid gene. The assembly of the hybrid particle has also been demonstrated by electron microscopy (37).

These experiments demonstrate that the ability of yeast to synthesize and assemble the complex HBV antigens can be extended to the synthesis and presentation of other antigens through the assembly of a complex hybrid antigen particle in yeast. This will hopefully extend the use of yeast cells in the development of new vaccines, as well as polyvalent vaccines.

In conclusion: Some interesting and promising results have been already obtained but much work is ahead of us in order to extend the use of yeast in the industrial production of a wider range of proteins.

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REFERENCES

1. CLARK-WALKER, G.D. and MIKLOS, G.L.C. (1974) Localization and quantification of circular DNA in yeast. *Eur. J. Biochem. 41:* 359-365.
2. HARTLEY, J.L. and DONELSON, J.E. (1980) Nucleotide sequence of the yeast plasmid. *Nature 286:* 860-865.
3. JAYARAM, M.; LI, Y.Y. and BROACH, J.R. (1983) The yeast plasmid 2-micron circle encodes components required for high copy number propagation. *Cell 34:* 95-104.
4. BROACH, J.R. (1982) The yeast plasmid 2-micron circle. *Cell 28:* 203-204.
5. RUSSELL, D.W.; SITH, M.; WILLIAMSON, V.M. and YOUNG, E.T. (1983) Nucleotide sequence of the yeast alcohol dehydrogenase II gene. *J. Biol. Chem. 258:* 2674-2683.
6. SHUSTER, J.R. (1987) Regulated expression of heterologous gene products in the yeast *Saccharomyces cerevisiae. CRC Reviews* (in press).
7. BRAKE, A.J.; MERRYWEATHER, J.P.; COIT, D.; HEBERLEIN, U.; MASIARZ, F.R.; MULLENBACH, G.T.; URDEA, M.S.; VALENZUELA, P. and BARR, P.J. (1984) α-factor-directed synthesis and secretion of mature foreign proteins in *S. cerevisiae. Proc. Natl. Acad. Soc. USA 81:* 4642-4646.
8. FOGEL, S. and WELCH, J.W. (1982) *Proc. Natl. Acad. USA 79:* 5342-5346.
9. KARIN, M.; NAJARIAN, R.; HASLINGER, A.; VALENZUELA, P.; WELCH, J. and FOGEL, S. (1984) Primary structure and transcription of an amplified genetic locus: the CUP1 locus of yeast. *Proc. Natl. Acad. Sci. USA 81:* 337-342.
10. VALENZUELA, P.; MEDINA, M.A.; BISHOP, R.; NAJARIAN, R.C.; BARR, P.J. and KARIN, M. (1983) The structure of the yeast CUP1 locus and its use in the regulation of expression of foreign proteins in yeast. Cold Spring Harbor Symposium on Molecular Biology of Yeast, pp. 255.
11. McCORD, J.M.; KEELE, B.B. and FRIDOVICH (1971) *Proc. Natl. Acad. Sci. USA 68:* 1024-1028.
12. McCORD, J.M. (1985) Oxygen-derived free radicals in postischemic tissue injury. *N. Engl. J. Med. 312:* 159-165.
13. HALLEWELL, R.A.; MILLS, R.; TEKAMP-OLSON, P.; ROSENBERG, S.; BLACHER, R.; OETTING, F.; MASIARZ, F.; SCANDELLA, C. (1987) Amino terminal acetylation of authentic human Cu/Zn superoxide dismutase produced in yeast. *Bio/Technology. 5:* 363-366.
14. ROSENBERG, S.; BARR, P.J.; NAJARIAN, R.; HALLEWELL, R.A. (1984) Synthesis in yeast of a functional, oxidation resistant, active center mutant of human α_1-antitrypsin. *Nature 312:* 77-80.
15. SCHECKMAN, R. and NOVICK, P. (1982) The secretory process and yeast cell surface assembly. In *The Molecular Biology of the Yeast Saccharomyces,* Vol. 2 (J. Strathern, E.W. Jones and J.R. Broach, Eds.), pp. 361-398. Cold Spring Harbor Laboratories, New York.
16. KURJAN, J. and HERKOWITZ, I. (1982) Structure of a yeast pheromone gene: a putative α-factor precursor contains four tandem copies of mature α-factor. *Cell 30:* 933-943.
17. THORNER, J. (1982) Pheromonal regulation of the development of S. cerevisiae. In *Molecular Biology of the Yeast Saccharomyces,* Vol. 2 (J. Strahern, E.W. Jones and J.R. Broach, Eds.), pp. 143-180. Cold Spring Harbor Laboratories, New York.
18. JULIUS, D.; BRAKE, A.; BLAIR, L.; KUNISAW, R. and THORNER, J. (1984) Isolation of the putative structural gene for the lysine-arginine-cleaving endopeptidase required for processing of yeast prepro-α-factor. *Cell 37:* 1075-1084.
19. BARR, P.J.; MULLENBACH, G.T.; BRAKE, A.J. and VALENZUELA, P. (1983) The chemical synthesis and expression of genes for human growth hormone releasing factor (somatotropin). *Fed. Proc. Am. Sco. Exp. Biol. 42:* 1160.
20. MULLENBACH, G.T.; CHOO, Q.L.; URDEA, M.S.; BARR, P.J.; MERRYWEATHER, J.; BRAKE,

A.J. and VALENZUELA, P. (1983) The chemical synthesis, molecular cloning and expression in yeast of genes coding for human insulin-like growth factors I and II. *Fed. Proc. Fed. Am. Soc. Exp. Biol. 42:* 434.

21. MULLENBACH, G.T.; TABRIZI, A.; BLACHER, R. and STEIMER, K.S. (1985) Chemical synthesis and expression in yeast of a gene encoding connective tissue activating peptide-III, a major human platelet growth factor. *J. Biol. Chem. 261:* 719-722.
22. SHAW, A.R.E.; BLEACKLEY, R.C.; MERRYWEATHER, J.P.; BARR, P.J. (1985) Modulation of human natural killer cell activity by recombinant human interleukin-2. *Cell. Immunol. 4:* 117-128.
23. COUSENS, L.S.; URDEA, M.S.; GALLEGOS, C.; BRAKE, A.; SANCHEZ-PESCADOR, R.; STEMPIEN, M.; KU, L. and TEKAMP-OLSON, P. (1987) The alpha-factor secretion system for insulin expression in yeast. *Gene,* in press.
24. COHEN, S. (1962) Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. *J. Biol. Chem. 237:* 1555-1559.
25. GREGORY, H. (1975) Isolation and structure of urogastrone and its relation to epidermal growth factor. *Nature 257:* 325-327.
26. READ, L.C.; SUMMER, L.; GALE, S.M.; GEORGE-NASCIMENTO, C.; BALLARD, F.J. and WALLACE, J.C. (1986) Receptor-bindign and immunological properties of synthetic-gene recombinant human epidermal growth factor: Comparison with the natural growth factor from human urine and milk. *J. Endocrinology 109:* 245-250.
27. BRIGHTWELL, J.R.; RIDDLE, S.L.; EIFERMAN, R.A.; VALENZUELA, P.; BARR, P.J.; MERRYWEATHER, J.P. and SCHULTZ, G.S. (1985) Acceleration of primate corneal wound healing by human epidermal growth factor. *Invest. Ophthalmol. Vis. Sci. 26:* 105-110.
28. BROWN, G.L.; CURTSINGER, L. III; BRIGHTWELL, J.R.; ACKERMANN, D.M.; TOBIN, G.R.; POLK, H.C.; GEORGE-NASCIMENTO, C.; VALENZUELA, P.; SCHULTZ, G.S. (1986) Enhancement of epidermal regeneration by biosynthetic epidermal growth factor. *J. Exp. Med. 163:* 1319-1324.
29. VALENZUELA, P.; MEDINA, A.; RUTTER, W.J.; AMMERER, G. and HALL, B.D. (1982) Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature 298:* 347-350.
30. VALENZUELA, P.; TEKAMP-OLSON, P.; COIT, D.; HEBERLEIN, U.; KUO, G.; MASIARZ, F.R.; MEDINA, M.A.; ROSENBERG, S.; WHITNEY, J.; BURLINGAME, A. and RUTTER, W.J. (1984) Hepatitis B. Vaccine: Characterization of hepatitis B antigen particles produced in yeast. In *Modern Approaches to Vaccines. Molecular and Chemical Basis of Virus Virulence and Immunogenicity* (R.M. Chanok and R.A. Lerner, Eds.), pp. 209-213, Cold Spring Harbor Laboratories, New York).
31. McALEER, W.J.; BUYNAK, E.B.; MAIGETTER, R.Z.; MILLER, W.J. and HILLEMAN, M. (1984) Human hepatitis B vaccine from recombinant yeast. *Nature 307:* 178-180.
32. STANBERRY, L.R.; BERNSTEIN, D.I.; BURKE, R.L.; PACHL, C.A. and MYERS, M.G. (1987) Vaccination with recombinant herpes virus glycoproteins: protection against initial and recurrent genital herpes. *J. Infect. Dis. 255:* 914-919.
33. LUCIW, P.; PARKES, D.; VAN NEST, G.; DINO, D.; HENDRIX, K. and GARDNER, M.B. (1986) Recombinant DNA approaches to FELV immunization in Genetic Engineering of Animals: An Agricultural Perspective (Evans, J.W.; Hollander, A., eds.) Plenum Press, New York, pp. 207-215.
34. BARR, P.J.; GIBSON, H.L.; ENEA, V.; ARNOT, D.E.; HOLLINGALE, M.R. and NUSSENZWEIG, V. (1987) Expression in yeast of a Plasmodium vivax antigen of potential use in a human malaria vaccine. *J. Experimental Med., 165:* 1160-1171.
35. BARR, P.J.; STEIMER, K.S.; SAGIN, E.A.; PARKES, D.; GEORGE-NASCIMENTO, C.; STEPHANS, J.C.; POWERS, M.A.; GYENES, A.; VAN NEST, G.A.; MILLER, E.T.; HIGGINS, K.W. and LUCIW, P.A. (1987) Antigenicity and immunogenicity of domains of the human immunodeficiency virus (HIV) envelope polypeptide expressed in the yeast Saccharomyces cerevisiae. *Vaccine,* in press.
36. VALENZUELA, P.; COIT, D.; KUO, C.H. (1985) Synthesis and assembly in yeast of hepatitis B surface antigen particles containing the polyalbumin receptor. *Bio/Technology 3:* 317-320.
37. VALENZUELA, P.; COIT, D.; MEDINA-SELBY, M. A.; KYO, C.H.; VAN NEST, G.; BURKE, R.L.; URDEA, M.S. and GRAVES, P.V. (1985) Antigen engineering in yeast: synthesis and assembly of hybrid hepatitis B surface antigen-herpes simplex 1 gD particles. *Bio/Technology 3:* 323-326.

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The fate of sperm specific non-histone chromosomal proteins after fertilization in sea urchins

El destino de proteínas específicas cromosomales no histónicas de espermatozoide, después de la fertilización en el erizo de mar

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To determine the transitions of sea urchin sperm specific non-histone chromosomal proteins (Sp NHCP) following fertilization, their presence in zygotes during and after male pronuclear formation was investigated by comparing the electrophoretic patterns of NHCP obtained from gametes with those isolated from zygotes. Polyclonal antibodies directed against whole Sp NHCP were used to detect, by Western immunoblots, the Sp NHCP among the NHCP present in zygotes at different times post-insemination. The results obtained from immunological and electrophoretic studies indicate that shortly after fertilization the majority of Sp NHCP are lost. The electrophoretic patterns of NHCP obtained from unfertilized eggs were almost identical with those observed for NHCP isolated from zygotes. This similarity strongly suggests that the NHCP of unfertilized eggs are conserved after fertilization.

INTRODUCTION

After fertilization the sperm nucleus undergoes a series of morphological and structural changes leading to male pronuclear formation. The cone-shaped sperm nucleus is transformed into the spherical male pronucleus within the egg cytoplasm as the repressed sperm chromatin becomes disperse and undergoes derepression (1-3).

The morphological aspects of this process have been investigated by a great number of laboratories (1, 4, 5) and it has been suggested that the dispersion of the condensed sperm chromatin may be a structural manifestation of changes in the nucleoprotein content of the paternally derived chromatin. Biochemical investigations in sea urchin zygotes related to the changes of nucleoprotein composition following fertilization have demonstrated that the five sperm specific histones are lost shortly after fertilization (6, 7) and that their disappearance is preceded by their phosphorylation (8). The sperm specific histones are replaced by maternally stored and *de novo* synthesized cleavage stage histone variants (7, 9, 10). These biochemical changes are temporally correlated with the presence of nucleosomes exhibiting a shortest DNA repeat lenght than those derived from sperm nucleus digested with micrococcal nuclease (11, 12).

The fate of whole sperm chromosomal proteins after fertilization has been investigated previously by comparing their electrophoretic migration with that observed for unfertilized eggs or zygotes. The results of these studies indicate that after insemination paternally derived chromatin acquires proteins of molecular weight greater than 80,000 and a nuclear composition similar to that of the female pronucleus (13). Some of these proteins are already present in the unfertilized egg cytoplams and may be involved in male pronuclear formation (14, 15). But, due to the comigration of sperm specific non histone chromosomal proteins (Sp NHCP) with those present in unfertilized eggs, the Sp NHCP are electrophoretically indistinguishable from unfertilized egg non-histone chromosomal proteins (Ue NHCP) and newly synthesized zygote non-histone chromosomal proteins

This article is dedicated to the memory of Professor Osvaldo Cori.

(Z NHCP). Therefore the fate of Sp NHCP after fertilization remains unknown.

To obtain more direct information on the involvement of Sp NHCP in male pronucleus formation, we report here the immunological detection of Sp NHCP in zygotes using polyclonal antibodies directed against whole Sp NHCP.

METHODS

Gametes and embryos

Sea urchins, *Tetrapygus niger,* were collected from the bay of Concepción, Chile, and maintained at room temperature under constant aeration in an aquarium containing natural sea water.

Eggs were obtained by gently shaking the female gonads into sterile sea water, filtering through a 100 μm pore size plankton network, and washing several times with sterile sea water. Semen was shed directly into a Petri dish. For fertilization, 2 drops of undiluted semen were added to 20 ml of egg suspension. After 3 min, each culture was diluted to ten times its original volume. Zygotes were harvested at 3 min, 7 min, 20 min, and 30 min after insemination, transferred to sea water acidified with HCl to pH 4.0 to stop their metabolic activity, and centrifuged immediately at 750 g for 3 min.

Development took place at room temperature in sterile sea water with constant aeration. Only embryos with a synchronization over 95% were used and a part of each culture was allowed to develop up to pluteus stage as a control.

Preparation of non-histone chromosomal proteins

Chromatin was isolated according to the method described previously (6). Histones were extracted from the chromatin by stirring overnight in 0.4 N H_2SO_4. The acid insoluble material containing the non-histone chromosomal proteins and nucleic acids was dialyzed againt tridistilled water, lyophilized, and stored at −20°C.

SDS-polyacrylamide gel electrophoresis

The non-histone chromosomal proteins were analyzed by sodium-dodecyl-sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) according to the method described by Laemmli (16) and visualized after staining with Coomassie Brilliant Blue.

Preparation of antisera

Antisera against non-histone chromosomal proteins from sperm were prepared by immunizing rabbits with 6 mg of the antigen suspended in 1 ml complete Freund's adjuvant diluted 1:1 in 0.01 M sodium phosphate buffer pH 7.0, containing 0.1% SDS and 0.15 M NaCl. Inoculations were given at weekly intervals and the blood was collected from the ear veins 7 to 10 days after each inoculation. The antisera were examined with Ouchterlony plates.

Electrophoretic transfer, blotting, and immunodetection.

The procedure followed was basically that described by Towbin *et al.* (17) modified as follows: SDS/PAGE slab gels containing the NHCP isolated from sperm, unfertilized eggs, and zygotes were washed briefly with distilled water and then in 25 mM Tris, 0.2 M glycine pH 8.3 containing 0.01% SDS and 20% methanol. Electrotransfer to nitrocellulose sheets was performed for 2 hours at 200 mA in the same buffer with a Transblot Cell (Bio RAD, Richmond, California, USA). After transfer, the nitrocellulose sheet was saturated with Tris buffered saline (TBS; 0.5 M NaCl, 0.02 M Tris pH 7.5) containing 3% gelatin for 1 hour at 37°C. The sheet was then washed five times (5 min each) with TBS containing 0.05% Tween-20 and incubated for 1 hour at room temperature in TBS containing 1% gelatin supplemented with rabbit anti Sp NHCP serum (diluted 1:50). In the next step the sheet was washed five times (5 min each) with TBS-0.05% Tween 20 and incubated for 1 hour at room temperature in TBS containing 1% gelatin supplemented with anti-rabbit-biotinylated antiserum (diluted 1:250) (Amersham, Buckinghamshire England). Subsequently the sheet was washed five times (5 min each) with TBS-0.05% Tween-20 and incubated for 1 hour at room temperature with TBS containing 1% gelatin supplemented with streptavidin-biotinylated-peroxidase preformed complex (diluted 1:400) (Amersham, Buckinghamshire, England). Finally the sheet was washed three times for 5 min each with TBS-0.05% Tween 20 and immunodetection was achieved by incubating at room temperature for 5 to 15 min with HRP color, a chromogenic substrate of peroxidase (BIO RAD, Richmond, California USA). The peroxidase reaction was stopped by washing the sheet in distilled water.

RESULTS

Electrophoretic analysis of NHCP from gametes and zygotes

The electrophoretic patterns of Sp NHCP, Ue NHCP, and Z NHCP are compared in Fig. 1. The Z NHCP were isolated from zygotes harvested at varying times between insemination and the beginning of the first S phase (3 min, 7 min, 20 min and 30 min after insemination), estimated as the time

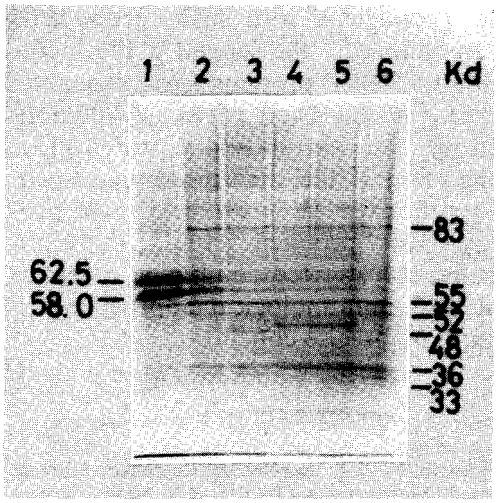

Fig. 1: SDS-PAGE profiles of total NHCP isolated from gametes and zygotes. *Lane 1,* Sp NHCP; *lane 2-5* NHCP from zygotes obtained 3 min, 7 min, 20 min and 30 min postinsemination; *lane 6,* Ue NHCP.

of male pronuclear formation according to data previously published and reviewed by Poccia (18).

As shown in Fig. 1 the electrophoretic patterns of the NHCP isolated from unfertilized eggs and zygotes are practically identical except for a band migrating at 48 Kd observed only in zygotes collected at 20 and 30 min post insemination and the two main Sp NHCP (62.5 Kd and 58 Kd) appearing as prominent bands shortly after fertilization (3 min and 7 min post insemination), and disappearing afterwards.

The major bands observed for both the Ue NHCP and the Z NHCP are located at 83 Kd, 55 Kd, 52 Kd, 36 Kd and 33 Kd in SDS/PAGE. These bands are not observed as major components among the Sp NHCP. The comparative analysis of minor bands observed for Sp NHCP is very difficult due to their comigration with Ue NHCP.

Immunobiochemical analysis of Sp NHCP in zygotes

To obtain more detailed information about the fate of Sp NHCP after fetilization, the Sp NHCP were followed in zygotes with polyclonal antibodies against Sp NHCP. As shown in Fig. 2 the anti Sp NHCP antiserum did not cross-react with Ue NHCP when assayed by Ouchterlony plates.

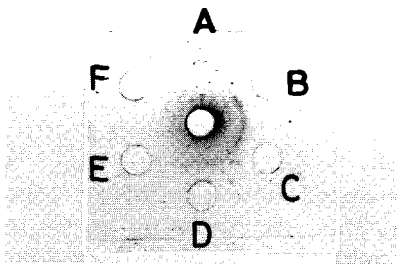

Fig. 2: Ouchterlony plates of NHCP stained with Amidoblack. The center well contained serum against whole Sp NHCP. *Well A,* contained buffer 0.01 M sodium phosphate pH 7.0, 0.1% SDS, 0.15 M NaCl; *Wells B-D,* contained 60 μg; 30 μg and 15μg of total Sp NHCP, respectively; *Wells E and F,* contained 100 μg and 50 μg of Ue NHCP, respectively.

To obtain more specific information, the NHCP obtained from both gametes and all zygotes were subjected to Western immunoblot analysis. As shown in Fig. 3, the sera obtained after the third inoculations of Sp NHCP showed positive reaction with the majority of the minor Sp NHCP and no apparent cross reaction with Ue NHCP except for two bands migrating at 58 Kd and 62 Kd. This cross reactivity might indicate either that these two proteins are present in both gametes and all zygotes, or that the antibodies are also detecting a post-translational modification. In effect, the antigenic capacity of ADP-ribose polymers or glycosylated proteins have been previously documented (19, 20). As shown in Fig. 3 the majority of Sp NHCP were only slightly detectable in zygotes harvested 3 min after insemination and disappeared afterwards.

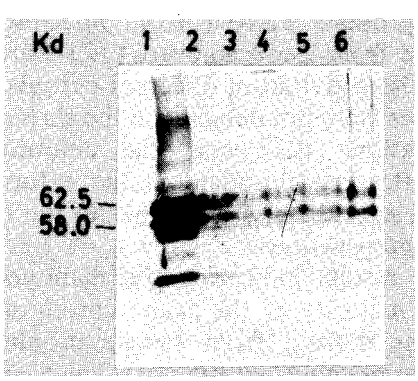

Fig. 3: Western immunoblot analysis of total NHCP from gametes and zygotes exposed to antiserum against Sp NHCP. *Lane 1,* Sp NHCP; *lanes 2-5,* Z NHCP from zygotes harvested 3 min, 7 min, 20 min and 30 min p.i., *lane 6,* Ue NHCP.

DISCUSSION

The results of the present study indicate that in sea urchins the majority of the sperm specific NHCP are lost shortly after fertilization. This finding is based on the immunological detection of Sp NHCP in zygotes only shortly after insemination. Additionally, the electrophoretic similarity of NHCP derived from unfertilized eggs with those obtained from zygotes harvested at varying times after insemination, strongly suggests that these proteins are maintained after fertilization.

One of the limitations of this study could present is the different antigenic capacity of the various Sp NHCP, however the majority of Sp NHCP comigrating with Ue NHCP in SDS/PAGE proved to be acceptable antigens. The second aspect that should be born in mind, is that in unfertilized eggs and zygotes it is technically very difficult to accurately determine the eventual contaminations of NCHP with cytoplasmic proteins, because of the very high cytoplasmic/nuclear ratio. Although, we cannot state conclusively that there is no cytoplasmic contamination, several lines of evidence indicate that the chromatin isolation method used in this study is reliable. Cleavage stage histone variants have been obtained and characterized from these chromatin preparations (10). Additionally, the electrophoretic patterns of these proteins are consistent with those obtained by other laboratories (7, 12) as well as the electrophoretic profiles observed for the basic proteins derived from nucleosomes isolated from 2-cell embryos (12). Furthermore the activities of different cytoplasmic enzymes were not detected in the chromatin preparations (21) and the amount of pigment, very abundant in the cytoplasm of this species, was negligible in the chromatin preparations.

Our results indicating the maintenance of the majority of the acidic chromosomal proteins derived from female pronucleus confirm previous reports (13). The eventual participation of Ue NHCP in the remodeling of the male pronucleus is still uncertain, since three possibilities may be considered. The first is a resettlement of NHCP from female pronucleus into male pronucleus after amphimixys, the second is the incorporation of NHCP proteins from oogenetic reservoirs, as has been previously demonstrated for basic chromosomal proteins (7); and the third is the incorporation of proteins synthesized *de novo* into male pronucleus. This problem should therefore be further investigated combining morphological and immunological approaches to reach a more precise conclusion.

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REFERENCES

1. LONGO, F.; ANDERSON, F. (1968) The fine structure of pronuclear development and fusion in sea urchin *Arbacia punctulata. J. Cell. Biol. 39:* 335-368.
2. MAXSON, R.; EGRIE, J.C. (1980) Expression of maternal and paternal histone genes during early cleavage stages of the echinoderm hybrid *Strongylocentrotus purpuratus* x *Lytechinus pictus. Dev. Biol. 74:* 335-342.
3. POCCIA, D.; WOLFF, R.; KRAGH, S.; WILLIAMSON, P. (1985) RNA synthesis in male pronuclei of the sea urchin. *Biochim Biophys Acta. 824:* 349-356.
4. ECKLUND, P.S., LEVINE, L. (1975) Mouse sperm basic nuclear protein. Electrophoretic characterization and fate after fertilization. *J. Cell. Biol. 66:* 251-262.
5. KOPECNY, V.; PAVLOK, A. (1975) Incorporation of arginine-^{3}H into chromatin of mouse eggs shortly after sperm penetration. *Histochemistry 45:* 341-345.
6. IMSCHENETZKY, M.; PUCHI, M.; MASSONE, R. (1980) Histone analysis during the first cell cycle of development of the sea urchin *Tetrapygus niger. Differentiation 17:* 111-115.
7. POCCIA, D.; SALIC, J.; KRYSTAL, G. (1981) Transitions in histone variants of the male pronucleus following fertilization and evidence for a maternal store of cleavage-stage histones in the sea urchin eggs. *Dev. Biol. 82:* 287-296.
8. GREEN, G.R.; POCCIA, D. (1985) Phosphorylation of sea urchin sperm H_1 and H_2B histones precedes chromatin decondensation and H_1 exchange during male pronuclear formation. *Dev. Biol. 108:* 235-245.
9. ARCECI, R.; GROSS, P. (1980) Histone variants and chromatin structure during sea urchin development. *Dev. Biol. 80:* 186-209.
10. IMSCHENETZKY, M.; PUCHI, M.; MASSONE, R. (1986) Characterization of cleavage stage chromosomal proteins from zygotes of the sea urchin *Tetrapygus niger. Comp Biochem and Physiol 84 B:* 23-27.

11. SAVIC, A.; RICHMAN, P.; POCCIA, D. (1981) Alterations in chromatin structure during early sea urchin embryogenesis. *Proc. Natl. Acad. Sci. USA 78:* 3706-3710.
12. SHAW, B.; COGNETTI, G.; SHOLES, W.M.; RICHARDS, R. (1981) Shift in nucleosome populations during embryogenesis: Microheterogeneity in nucleosomes during development of the sea urchin embryo. *Biochemistry 20:* 4971-4978.
13. KUNKLE, M.; LONGO, F.; MAGUN, B. (1978) Nuclear protein changes in the maternally and paternally derived chromatin at fertilization. *J. Exp. Zool 203:* 371-380.
14. KUNKLE, M.; MAGUN, B.; LONGO, F. (1978) Analysis of isolated sea urchin nuclei incubated in egg cytosol. *J. Exp. Zool 203:* 381-389.
15. LOHKA, M.; MASUI, Y. (1983) The germinal vesicle material required for sperm pronuclear formation is located in the soluble fraction of egg cytoplasm. *Exp. Cell. Res. 148:* 481-491.
16. LAEMMLI, V.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature 227:* 680-685.
17. TOWBIN, H.; STAEHELIN, T.; GORDON, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA 76:* 4350-4354.
18. POCCIA, D. (1986) Remodeling of nucleoproteins during gametogenesis, fertilization, and early development. In: Bourne C, Jeon, K.; Friedlander, M. (eds) International review of citology, Vol. 105. Academic Press, New York. p. 1-65.
19. KANAI, Y.; NIWA, M.; MATSUSHIMA, T.; SUGIMURA, T. (1974) Studies on antipoly (Adenosinediphosphate ribose) antibody. *Biochem. Biophys. Res. Commun. 59:* 300-306.
20. GOLDBERG, A.; JEOMAN, L.; BUSCH, H. (1978) Chromatin-associated glycoproteins of normal rat liver and Novikoff Hepatoma Ascites Cells. *Cancer Research 38:* 1052-1056.
21. IMSCHENETZKY, M.; ROJO, I.; PUCHI, M.; MASSONE, R. (1979) Relationship between basic proteins associated to DNA and replication during early development of Echinoderms. *Arch. Biol. Med. Exp. 12:* 433-438.

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Biochemical and genetic studies of bacteria metabolizing lignin-related compounds

Estudio bioquímico y genético de bacterias que metabolizan compuestos relacionados con la lignina

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The ability of bacterial strains to metabolize lignin model compounds was studied. Strains examined were non-filamentous bacterial isolates obtained from decaying wood and the actinomycete *Streptomyces viridosporus* T7A. Model compounds included dimers containing either the β-1 (1,2-diarylethane) or the β-O-4 (arylglycerol-β-aryl ether) type of linkage. *Pseudomonas fluorescens* biovar I A1 proliferated on anisoin (4,4'-dimethoxybenzoin) accumulating anisic acid temporarily. Cleavage at the β-1 bond was also observed with crude extracts prepared from the same strain. In turn, cleavage of the β-O-4 linkage of veratrylglycerol-β-guaiacyl ether was detected in cultures of *Pseudomonas acidovorans* D3. In this case, main degradation intermediates were β-hydroxypropioveratrone, acetoveratrone and guaiacol. *S. viridosporus* T7A reduced the carbonyl group of some β-1 dimers and did not modify the β-O-4 model compounds tested.

Attempts to ascribe a catabolic character to large molecular weight extrachromosomal DNA present in some strains were unsuccessful. Gene banks of *P. fluorescens* biovar I A1 and *P. acidovorans* D3 were prepared utilizing the broad host range cosmid pLAFR1 as vector.

INTRODUCTION

Lignin is distributed throughout the plant kingdom, representing about one quarter of the biomass on the earth. Having a complex irregular structure, lignin is formed by phenylpropanoic residues connected by various types of C-C and C-O-C covalent linkages (Figure 1).

Filamentous fungi, mainly those belonging to the group of white rot fungi, play a major role in the mineralization of lignin in natural environments. The isolation of ligninase from the basidiomycete *Phanerochaete chrysosporium* in 1983 marked a milestone in our understanding of this important catabolic process (1, 2). The extent to which bacteria can degrade lignin is still unknown. Filamentous bacteria (*Actinomycetes*) are clearly able to degrade

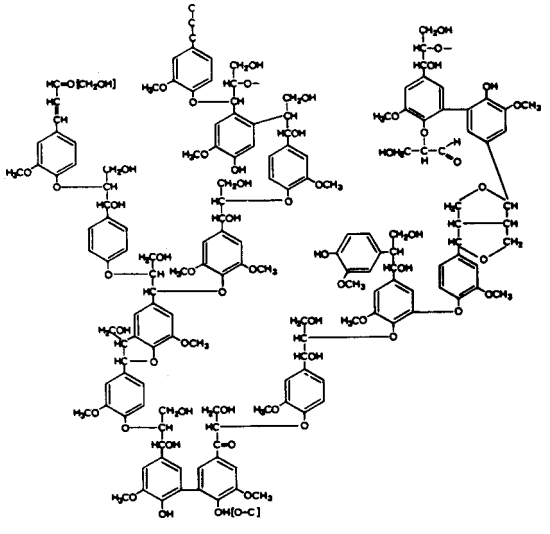

Fig. 1: **Proposed structure for lignin.** (Reproduced from Adler, E. *Wood Sci. Technol. 11:* 169-218, 1977.)

We dedicate this work to the memory of professor Osvaldo Cori, whose accomplishments honour the chilean scientific community. A sucessful researcher, he received worldwide recognition. For several generations he was the master of young biochemists, influencing them with his knowledge, style and strong personality. For his intellectual stature and all his efforts devoted in benefit of scientific development in our country, we feel for him great appreciation, admiration and gratitude.

lignocellulose, which decomposes to CO_2 and soluble lignin fragments (3). Depolymerization of the macromolecule by non-filamentous bacteria has rarely been reported (4, 5). Apparently, they contribute to the process of lignin degradation modifying byproducts of fungal metabolism. The overall picture emerging suggests that bacteria are able to metabolize lignin-derived compounds found in soil, degrade lignin oligomers released by filamentous fungi and in some cases mineralize lignin to a small extent.

Possibly, lignin resistance to attack by these microorganisms could be due to the absence of ligninolytic enzymes. An alternative explanation would be that lignin degrading enzymes do indeed exist in bacteria, but they are not secreted from the cell. It has been shown that some strains belonging to the groups of *Pseudomonas* (6, 7), *Nocardia* (8) and others are able to metabolize dimeric model compounds containing interunit bonds characteristic of lignin. In most cases, reaction intermediates have been identified, although to date the isolation of the enzymes involved has not been reported.

Genetic studies on this field are also very scarce. Some bacteria harbour plasmids coding for the enzymes of the degradation pathways of aromatic substances such as toluene, naphtalene and salicylate (9, 10). There are preliminary reports on the involvement of catabolic plasmids in the degradation of lignin model compounds (11, 12), while there are no studies on chromosomal genes related to lignin biodegradation.

MATERIALS AND METHODS

Chemicals

Vanillic, cinnamic, p-coumaric, syringic and protocatechuic acids were from Sigma Chemical Co., USA. Compounds derived from 1,2-diarylethane (propane) were obtained as reported previously (13). Guaiacylglycerol-β-guaiacyl ether (GGGE) and veratrylglycerol-β-guaiacyl ether (VGGE) were synthesized as described by Landucci *et al.* (14).

Microorganisms and culture conditions

Strains of non-filamentous bacteria were grown and selected in minimal medium (12) adjusted to pH 6.7, supplemented with a mixture of trace minerals (15) and with the lignin-related compound as the sole carbon source. Cultures were incubated at 30°C with agitation.

Streptomyces viridosporus T7A (Figure 2) was a generous gift from Dr. D.L. Crawford (Moscow, Ida.). Stock slants were kept at 4°C on Luria broth. Spores were suspended in sterile water, added to minimal medium (16) supplemented with 0.3% yeast extract and the aromatic substrate. Incubation was at 37°C with vigorous agitation. It must be stressed that, as opposed to cultures of non-filamentous bacteria, those of *S. viridosporus* T7A always included a small amount of yeast extract. Therefore, aromatic substrates tested in these experiments were not used as the sole carbon source. The same applies for β-O-4 substrates (see below).

The aromatic compounds were present in the cultures at a final concentration of 5 mM (monomers) or 1 mg/ml (dimers) in all experiments.

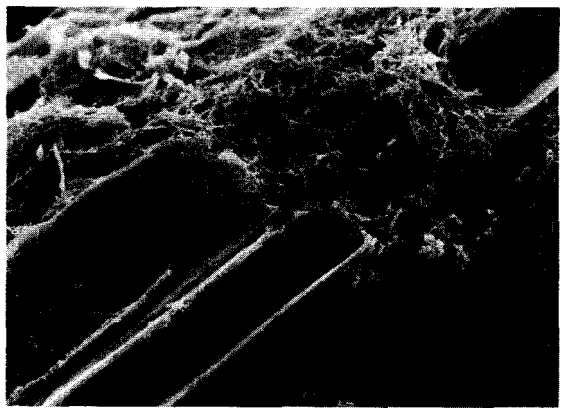

Fig. 2: ***Streptomyces viridosporus*** **T7A growing on wheat lignocellulose.** The sample was fixed in formaldehyde: ethanol 75%: acetic acid (1:18:1). Scanning electron microscope, magnification 470x.)

Analytical methods

Small samples (one ml) were withdrawn from cultures and centrifuged. Supernatants were acidified to pH 2.0 with 6 N HCl and extracted twice with diethyl ether. These ether extracts were evaporated to dryness for further analysis.

For spectrophotometric tracings, ether extracts were resuspended in water and absorption spectra were recorded between 200 and 400 nm in a Shimadzu UV-visible spectrophotometer.

Samples for thin layer chromatography (TLC) were prepared resuspending the dry extracts in ether and spotting small aliquots on silicagel 60 F_{254} plates. Developing solvent was chloroform: isopropanol: ammonia (12:1:1) for the

β-O-4 substrates and chloroform: acetone (9:1) for all other compounds.

For high performance liquid chromatography (HPLC) analysis, dried samples were resuspended in distilled water, injected in a Gilson System 41 Gradient Analytical HPLC apparatus and resolved with a methanol-water gradient (13).

Protocols used for gas chromatography/mass spectrometry are described elsewhere (17).

RESULTS AND DISCUSSION

1. *Choice of substrates*

Model compounds have been of great importance to clarify the mechanism by which filamentous fungi degrade lignin. Both the complex structure of the polymer and its insolubility in water-based solvents have driven investigators to use model substrates for biodegradation studies. Therefore, in order to assess the role of bacteria in this process, we have followed the same experimental approach.

In our work, both monomeric and dimeric compounds were used for the isolation of strains, as described below. The former included derivatives of benzoic and cinnamic acids, while two kinds of dimeric substrates were employed: i) those containing the β-1 bond, like anisoin and hydroxymethyl desoxyanisoin (HMDA), and ii) model compounds containing the β-O-4 linkage, such as the phenolic guaiacylglycerol-β-guaiacylether (GGGE) and the non-phenolic veratrylglycerol-β-guaiacylether (VGGE) dimers (Figure 3).

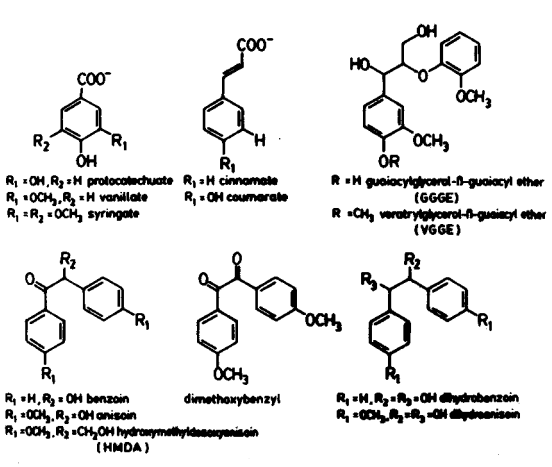

Fig. 3: **Molecular formulae of some model compounds used in this study.**

2. *Isolation and characterization of strains*

Samples from rotting wood, pulp mill effluents and forest litter were collected from different locations of central and southern Chile. Enrichment cultures containing a defined minimal medium and one lignin-related compound as the sole carbon source were inoculated with each of these samples.

Cultures were incubated at 30°C in a rotary shaker and after several transfers were plated on agar containing Luria medium. Isolated colonies obtained were grown again in minimal medium and screened for their ability to metabolize each of the different lignin-related substrates. Growth in each compound was recorded as optical density at 600 nm reached at stationary phase. Table I shows the ability of some bacterial isolates to proliferate on various carbon sources.

Starting from many original samples and using the substrates indicated above, about 100 different strains were isolated using a mixture of vanillate and coumarate. In turn, enrichment cultures in anisoin led to the isolation of only two bacterial strains, A1 and A2. Four isolates (D1 to D4) were found upon incubation of samples in GGGE (18). Strains A1 and D3 were subsequently identified as *P. fluorescens* biovar I A1 (19) and *P. acidovorans* D3 (17), respectively.

Some monomeric compounds are metabolized by most strains, such as vanillate and protocatechuate. Other substrates, like cinnamate and syringate, are decomposed by only a few strains (18). In general, the metabolic versatility of the strain is related to the structure or complexity of the carbon source used in its isolation (18). For example, the few strains isolated on synthetic dimers were able to grow on most monomers and in some dimeric model compounds, whereas none of the various isolates selected on vanillate plus coumarate grew on a dimeric model compound. To date we have not been able to isolate a strain in HMDA, which contains a hydroxymethyl group instead of the hydroxyl group in anisoin. On the other hand, strain D3 isolated on GGGE is the

TABLE I

Metabolic versatility of strains isolated on diverse lignin-related compounds

Strain	Initial selection	Va	Co	Pr	Ci	Sy	GGGE	VGGE	An	HMDA
A_{10}	Va/Co	+	+	+	–	–	–	–	–	–
S_{10}	Va/Co	+	+	+	–	–	–	–	–	–
C_2	Va/Co	+	+	+	+	–	–	–	–	–
M_5	Va/Co	+	+	+	–	+	–	–	–	–
A1	An	+	+	+	+	+	+	–	+	–
D3	GGGE	+	+	+	–	+	+	+	–	–

Growth was measured as optical density at 600 nm after 3 days of incubation at 30°C under continous agitation. Abbreviations are: Va = vanillate, Co = coumarate, Pr = protocatechuate, Ci = cinnamate, Sy = syringate, GGGE = guaiacylglycerol-β-guaiacylether, VGGE = veratrylglycerol-β-guaiacylether, An = anisoin, HMDA = hydroxymethyl-desoxyanisoin.

only strain able to grow on VGGE and does not consume β-1 dimers.

3. *Metabolism of β-1 model compounds*

Shimada and Gold (20) first described that anisoin behaves like a β-1 dimer in ligninolytic cultures of *Phanerochaete chrysosporium*. Since the bacterial strain *P. fluorescens* biovar I A1 proliferates on anisoin as the sole carbon and energy source, experiments to identify catabolic intermediates were designed. Aliquots of growth media were withdrawn at different times and the supernatants analyzed by spectrophotometric tracings, thin layer chromatography (TLC) on silica plates and high performance liquid chromatography (HPLC, see Methods). During early log phase of growth (5-15 hours), the transient accumulation of anisic acid was detected (19). This observation, which has been reproduced with partially purified fractions (B. González, unpublished results), indicates that the C-C intermonomeric bond of anisoin was cleaved. *In vitro*, the reaction proceeds in the absence of exogenous cofactors, yielding anisaldehyde as the only product. However, when NAD^+ is added, anisaldehyde is quantitatively oxidized to anisic acid.

Analogous *in vivo* experiments performed with benzoin instead of anisoin allowed the detection of benzoic acid, indicating again that cleavage of the dimer had occurred (19).

P. fluorescens biovar I A1 could not proliferate on HMDA nor on 2,3-diphenylpropenoic acid. To date, isolation of bacterial strains on the former has not been reported. With respect to the latter, resistance of the double bond to bacterial attack seems to be a general phenomenon, as suggested by results obtained by other workers using a consortium of bacteria (21). They observed that 2,3-diphenylpropanoic acid was readily degraded yielding single ring aromatic intermediates, while 2,3-diphenylpropenoic acid was scarcely metabolized.

When *S. viridosporus* T7A was incubated in the presence of anisoin, a reduction of the carbonyl group was detected. The reduced compound, dihydroanisoin, was identified by a characteristic UV spectrum and TLC analysis (13). Other substrates structurally related to anisoin, i.e. benzoin (Figure 4) and dimethoxybenzyl, were also reduced to the corresponding alcohols. In contrast, 1,2-diarylpropane model compounds such as HMDA were not modified. It is interesting to point out that a reducing activity of anisoin has also been found and partially purified in *P. fluorescens* biovar I A1 (19). Cleavage of bonds by *S. viridosporus* T7A was not detected with any of the substrates tested (13).

4. *Metabolism of β-O-4 model compounds*

The same experimental approach described above for β-1 compounds was used to analyze the metabolism of β-O-4 substrates by *P. acidovorans* D3 and *S. viridosporus* T7A. Both dimeric substrates, GGGE

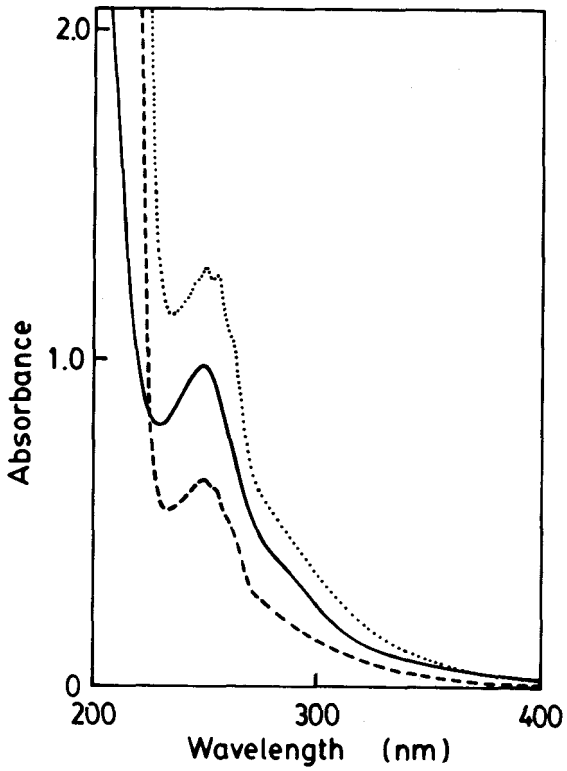

Fig. 4: **Reduction of benzoin to dihydrobenzoin by *Streptomyces viridosporus* T7A.** Spectrophotometric tracings of aliquots taken after 0 (——), 96 (– – –) and 220 (·····) hours of incubation.

and VGGE, are degraded quantitatively by the non-filamentous strain. Catabolic intermediates were detected using gas chromatography/mass spectrometry (GC/MS). Dissimilation of the two compounds followed parallel steps, as shown by early detection of guaiacol in both cultures. Additional intermediates identified in incubations using GGGE were β-hydroxypropiovanillone and acetovanillone. Analogously, β-hydroxypropioveratrone and acetoveratrone were identified when VGGE was added to cultures of *P. acidovorans* D3. Catabolism of GGGE and VGGE by this species has been studied in more detail and reported elsewhere (17). Major steps of the corresponding pathways are depicted in Figure 5.

Metabolism of β-O-4 model compounds by *S. viridosporus* T7A was investigated by different analytical techniques (UV spectrometry, TLC and HPLC) and no cleavage or modification of them could be detected (13). Experiments were also performed in the presence of purified wheat lignocellulose in order to activate the enzyme(s) that catalize lignocellulose breakdown (22), but no change was observed. Our results differ from those reported by Crawford *et al.* using the same model compound and the same culture conditions (23). The reasons for this discrepancy are not known to us.

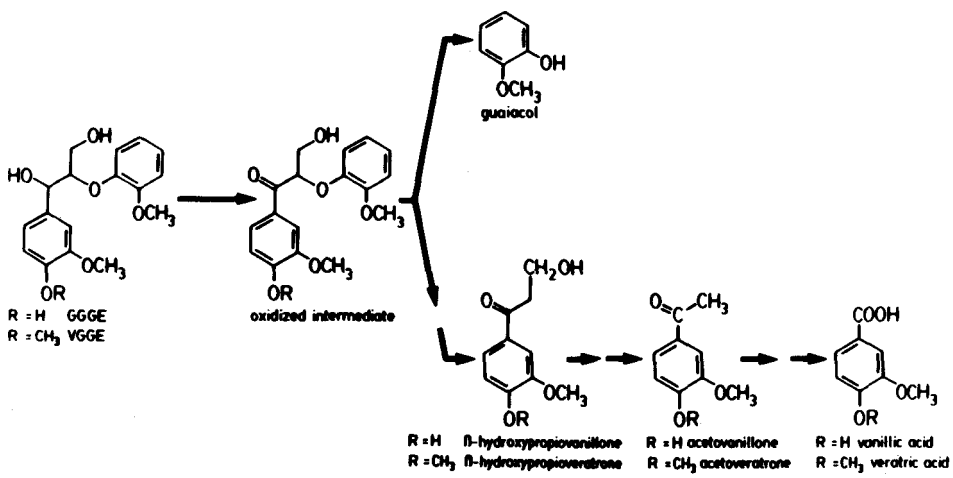

Fig. 5: **Major steps in the catabolism of GGGE and VGGE by *P. acidovorans* D3.**

5. *Genetic studies*

A. Search of plasmids involved in the degradation of lignin-related compounds

A large variety of aromatic substances, both xenobiotic and naturally occuring, are degraded by microorganisms belonging frequently to the Pseudomonaceae family. Genetic analysis of the pathways has shown that in several instances these are coded in extrachromosomal DNA. Well known examples are plasmids TOL (24), NAH (25) and SAL (10), which code for the degradation of toluene/xylene, naphthalene and salicylate, respectively. Based on this evidence, we proposed that the enzymes responsible for the degradation of monomeric lignin model compounds could also be plasmid encoded.

The strains isolated as described above were screened for the presence of plasmids according to the method of Kado and Liu (26). Approximately 20% of the strains contained one and in some cases two plasmids. Figure 6 shows plasmids from three different bacterial isolates (A_{10}, C_2 and S_{10}) after electrophoresis on a 0.8% agarose gel. Approaches designed to prove the above hypothesis included mating and curing experiments.

These and other strains were treated with curing agents that included mitomycin C, ethidium bromide, novobiocin, dipyridylamine and high temperature. Cultures subjected to these treatments were plated on rich medium and replica plated on selective medium containing one lignin-related compound as the sole carbon source. About 1.0% of the colonies grew well in the former but failed to proliferate in the latter. Analysis of extrachromosomal DNA in over 200 of these colonies did not yield a single cured culture, indicating that plasmids are stable. These results do not prove nor disprove the existence of plasmids involved in the degradation of small-MW lignin derived substrates. A summary of these assays is shown on Table II.

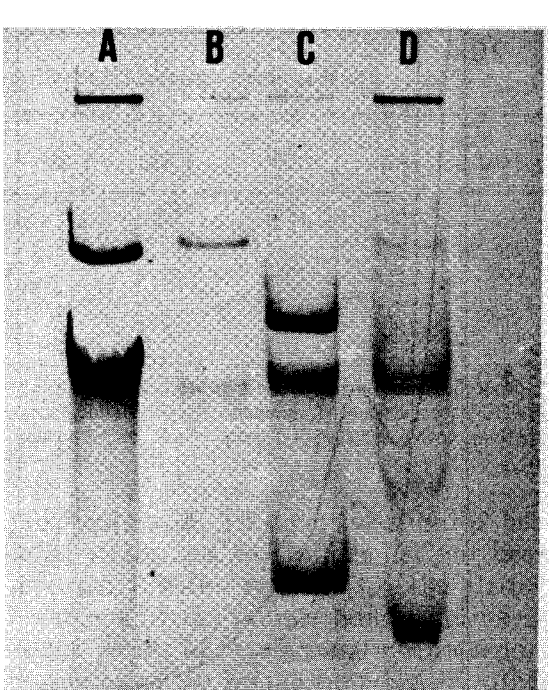

Fig. 6: **Agarose gel electrophoresis (0.8%) of plasmids from various strains.** Lane 1: standard pR_2 from *Salmonella typhi* (MW 62 Md); lane 2: strain C_2; lane 3: strain A_{10} and lane 4: strain S_{10}.

For conjugation experiments, strains were selected and mated with other *Pseudomonas* strains such as *P. aeruginosa* 2A (a generous gift from Dr. Oscar Grau, La Plata, Argentina) and some of our own isolates. Although different conditions and strains were used, our efforts were not successful. Mating assays using standard host strains such as *P. putida* 2440 (27) and *P. aeruginosa* PAO 1161 (28) could not be carried out because they grow on most of the monomeric aromatic compounds.

Plasmid involvement in the catabolism of lignin-related compounds has been proposed by Salkinoja-Salonen *et al.* (11, 12) based on observations of metabolic instability. Unfortunately, no direct evidence for plasmid presence was provided in this work. Only recently, the involvement of extrachromosomal elements in the dissimilation of cinnamate has been shown in one *Pseudomonas* strain (29).

TABLE II

Overview of curing experiments of strains A_{10}, C_2 and S_{10}

Strain	Curing agent	Conc.	Selective media	N° colonies
A_{10}	Mitomycin C	5-15 ug/ml	Co, Va, Fe	2,000
	T° (44°C)		Co, Va	200
	Dipyridyl	500 ug/ml	Co, Va	300
	Eth. bromide	450 ug/ml	Co	200
C_2	Mitomycin C	10-50 ug/ml	Co, Va, Fe, Ci	1,650
	Novobiocin	1-300 ug/ml	Ci	50
	T° (44°C)		Ci, Va	200
	Dipyridyl	50 ug/ml	Co, Ci	200
	Eth. bromide	350 ug/ml	Co	200
S_{10}	Mitomycin C	30-80 ug/ml	Co, Va, Fe	2,600
	Novobiocin	1.5-3 ug/ml	Co	500
	T° (44°C)		Co, Va	200
	Dipyridyl	250 ug/ml	Co, Va	200

Curing agents, lignin-related substrates used in the selective media and approximate number of colonies screened are indicated. Abbreviations are: Conc. = concentration, Dipyridyl = 2,2'dipyridylamine, Eth. bromide = ethidium bromide, Fe = ferulate. Other abbreviations are as in Table I.

B. Construction of gene banks

Having looked thoroughly for plasmid coded functions in numerous bacteria, we then decided to concentrate our efforts on chromosomal genes.

We have constructed gene banks of strains *P. fluorescens* biovar I A1 and *P. acidovorans* D3 in *E. coli* HB101 using the RK2 derived cosmid pLAFR1 (21.6 kb) as vector, following the procedure described by Friedman *et al.* (30). A random sample of clones from both banks was subjected to screening to verify the presence of recombinant cosmids. As expected, digestions with EcoR I confirmed the presence of the cosmid vector and inserts of different sizes (Figure 7).

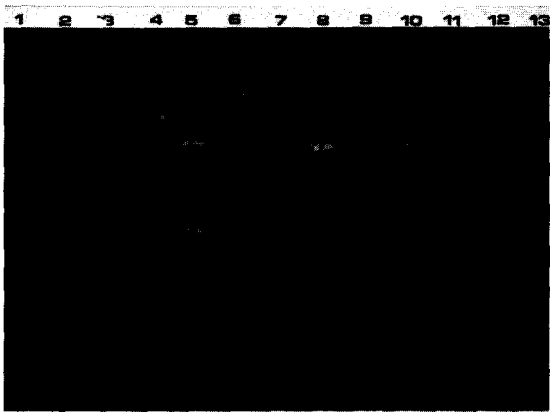

Fig. 7: **EcoRI-digested plasmid DNAs from *P. fluorescens* biovar I A1 gene bank.** Lanes 1-5 and 10-13: plasmid DNA from nine randomly chosen *E. coli* transformants; lane 6: intact chromosomal DNA; lane 7: DNA from phage lambda digested with Hind III; lane 8: pLAFR1 digested with EcoRI; lane 9: intact pLAFR1.

Since *E. coli* does not express efficiently genes of unrelated Gram negative bacteria (27), the expression analysis of the gene bank has to be done in a homologous species, e.g. *Pseudomonas.* Therefore, the genomic libraries were transfered to *P. putida* 2440 by triparental conjugation (31) using the helper plasmid pRK2013 (32). This element consists of the colE I replicon plus the transfer functions of RK2 and for this reason it may be used to mobilize recombinant cosmids from *E. coli* HB101 to the *Pseudomonas* recipient strain. We are presently attempting to isolate relevant clones by growing the conjugant bacteria in a culture medium having as sole carbon source a lignin-model compound of interest which is known to be degraded by the original donor and not by the recipient. This strategy seems appropiate, since it allows the possibility of isolating genes responsible for certain functions even before the corresponding enzymes have been characterized.

CONCLUDING REMARKS

Lignin biodegradation is one of the key steps in the recycling of carbon of the

biosphere. The role of bacteria in the complete breakdown of this complex macromolecule has to be defined in order to achieve a clear understanding of this vital process. Results obtained in this study, as well as others, show that catabolic reactions performed by non-filamentous eubacteria are quite diverse, but the versatily of each particular strain is relatively limited. However, cleavage of two important lignin linkages has been demonstrated. In this respect, bacteria appear to display a different strategy than fungi. Basidiomycetes secrete non-specific extracellular ligninases which, after catalizing one initial reaction involving aryl cation radical formation, produce a chain of random transformations of lignin products (33). Quite probably, the various genera of bacteria found in soil, sediments and aquatic environments have evolved strategies towards metabolization of aromatic compounds in general, including the products of lignin depolymerization by filamentous fungi.

Metabolic potential of the selected strains seems to be strongly dependent on the substrate employed initially for their isolation. This observation could be significant depending on the goals or future projections of the research undertaken. For example, natural isolates of non-filamentous eubacteria selected according to the above criteria might be of great advantage for specific bioconversion processes with industrial applications.

At this point it is clear that *S. viridosporus* T7A attacks lignocellulose efficiently, but there is no direct proof that it possesses a true ligninolytic enzymatic system. Our results indicate that it is not capable of cleaving β-O-4 linkage (by far the most frequent inter-monomeric bond found in lignin), nor β-1 or biphenyl bonds (13). The use of synthetic lignins (34) could help to reveal the mechanism by which actinomycetes attack the polymer.

The relatively low complexity of the bacterial genome and the techniques presently available for genetic manipulation make these microorganisms interesting subjects for both basic and applied research. To our knowledge, the construction of bacterial gene banks aimed to the isolation of genes relevant to lignin breakdown has not been reported in the literature. We hope to be able to find such a genes and transfer them among various strains in order to increase their metabolic efficiency.

ACKNOWLEDGMENTS

This investigation was supported by grants from Celulosa Arauco y Constitución, DIUC and Fondo Nacional de Ciencias. Studies on the degradation of β-O-4 dimers by *P. acidovorans* D3 were done in collaboration with Dr. Kent Kirk (Forest Products Laboratory, Madison), through a program financed by the National Science Foundation (grant 144X753). One of us (RV) was a John Simon Guggenheim fellow during 1986.

REFERENCES

1. TIEN, M. & KIRK, T.K. (1983) *Science 221:* 661-663.
2. GLENN, J.K.; MORGAN, M.A.; MAYFIELD, M.B.; KUWAHARA, M. & GOLD, M.H. (1983) *Biochem. Biophys. Res. Comm. 114:* 1077-1083.
3. McCARTHY, A.J.; PATERSON, A. & BRODA, P. (1986) *Appl. Microbiol. Technol. 24:* 347-352.
4. KERN, H.W. (1984) *Arch. Microbiol. 138:* 18-25.
5. KERR, T.J.; KERR, R.D. & BENNER, R. (1983) *Appl. Environ. Microbiol. 46:* 1201-1206.
6. CRAWFORD, R.L.; KIRK, T.K. & McCOY, E. (1975) *Can. J. Microbiol. 21:* 577-579.
7. KAWAKAMI, H. (1980) In *Lignin Biodegradation: Microbiology, Chemistry and Potential Applications* (T.K. Kirk, T.; Higuchi, H-m Chang, eds.). CRC Press Inc., Florida, pp. 103-125.
8. CRAWFORD, R.L. (1975) *Can. J. Microbiol. 21:* 1654-1657.
9. HUGHES, E.J.L.; BAYLY, R.C. & SKURRAY, R.A. (1984) *J. Bact. 158:* 79-83.
10. CHAKRABARTY, A.M. (1972) *J. Bact. 112:* 815-823.
11. SALKINOJA-SALONEN, M.S.; VAISANEN, E. & PATERSON, A. (1979) In *Plasmids of Medical, Environmental and Commertial Importance* (K.N. Timmis & A. Pühler, eds.), Elsevier, North-Holland Biomedical Press, pp. 301-314.
12. SALKINOJA-SALONEN, M.S. & SUNDMAN, V. (1980) In *Lignin Biodegradation: Microbiology, Chemistry and Potential Applications* (T.K. Kirk, T. Higuchi, H-m Chang, eds.). CRC Press Inc., Florida, pp. 179-198.
13. RUTTIMANN, C.; SEELENFREUND, D. & VICUÑA, R. (1987) *Enz. Microb. Technol., 9:* 526-530.
14. LANDUCCI, L.L.; GEDDES, S. & KIRK, T.K. (1981) *Holzforschung 35:* 67-70.
15. MATELES, R.I.; BARNAH, J.N. & TANNENBAUM, S.R. (1967) *Science 157:* 1322-1323.
16. CRAWFORD, D.L. (1978) *Appl. Environ. Microbiol. 35:* 1041-1045.
17. VICUÑA, R.; GONZALEZ, B.; MOZUCH, M. & KIRK, T.K. (1987) *Appl. Environ. Microbiol., 53:* 2605-2609.
18. GONZALEZ, B.; MERINO, A.; ALMEYDA, M. & VICUÑA, R. (1986) *Appl. Environ. Microbiol. 52:* 1428-1432.

19. VICUÑA, R.; GONZALEZ, B.; OLAVE, I.; RUTTIMANN, C.; SAPAG, A. & SEELENFREUND, D. (1987) In *Les Colloques de l'INRA,* París, *40:* 231-236.
20. SHIMADA, M. & GOLD, M.H. (1983) *Arch. Microbiol. 134:* 299-302.
21. KATAYAMA, Y. & FUKUZUMI, T. (1979) *Mokuzai Gakkaishi 25:* 67-76.
22. CRAWFORD, D.L.; POMETTO, A.L. & CRAWFORD, R.L. (1983) *Appl. Environ. Microbiol. 45:* 898-904.
23. CRAWFORD, D.L.; POMETTO, A.L. & CRAWFORD, R.L. (1983) In *Recent Advances in Lignin Biodegradation Research* (T. Higuchi, H-m Chang, T.K. Kirk, eds.), Unpublishers, Ltd., Tokio, pp. 78-95.
24. WILLIAMS, P.A. & MURRAY, K. (1974) *J. Bact. 120:* 416-423.
25. DUNN, N.W. & GUNSALUS, I.C. (1973) *J. Bact. 114:* 974-979.
26. KADO, C.I. & LIU, S.T. (1981) *J. Bact. 145:* 1365-1373.
27. FRANKLIN, F.C.H.; BAGDASARIAN, M.; BAGDASARIAN, M.M. & TIMMIS, K.N. (1981) *Proc. Nat. Acad. Sci. USA 78:* 7458-7462.
28. HOSOYA, H. & TOMIZUKA, N. (1984) *Report of the Ferm. Res. Inst. Japan 61:* 53-55.
29. ANDREONI, V. & BESTETTI, G. (1986) *Appl. Environ. Microbiol. 52:* 930-934.
30. FRIEDMAN, A.M.; LONG, S.R.; BROWN, S.E.; BUIKEMA, W.J. & AUSUBEL, F.M. (1982) *Gene 18:* 289-296.
31. RUVKUN, G.B. & AUSUBEL, F.M. (1981) *Nature 289:* 85-88.
32. FIGURSKI, D.H. & HELINSKI, D. (1979) *Proc. Nat. Acad. Sci. USA 76:* 1648-1652.
33. HAMMEL, K.E.; TIEN, M.; KALYANARAMAN, B. & KIRK, T.K. (1985) *J. Biol. Chem. 260:* 8348-8353.
34. KIRK, T.K.; CONNORS, W.J.; BLEAM, R.D.; HACKETT, W.F. & ZEIKUS, J.G. (1975) *Proc. Nat. Acad. Sci. USA 72:* 2515-2519.

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Síntesis y secreción del antígeno de superficie del virus de Hepatitis B en cultivo de células animales*

Synthesis and secretion of the surface antigen from Hepatitis B virus in animal cell cultures

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Mediante técnicas de ingeniería genética se ha logrado obtener líneas estables de células animales que sintetizan y secretan eficientemente partículas de antígeno de superficie del virus de la hepatitis B. Estas partículas presentan al microscopio electrónico un tamaño de 22 nm y son estructuralmente e inmunogénicamente similares a las obtenidas de plasma de enfermos crónicos, por lo que constituyen una excelente fuente de antígeno para la elaboración de una vacuna contra el virus.

Stable mammalian cell lines synthesizing and secreting Hepatitis B surface particules have been obtained through genetic engineering techniques. These particules show by electron microscopy a size of 22 nm, they are structurally and immunochemically similar to the particules present in the plasma from chronic hepatitis B patients. Therefore these particules are an excelent source for the preparation of a vaccine against the virus.

INTRODUCCION

El virus de la hepatitis B es el agente causal principal de las enfermedades al hígado. Luego de la fase aguda, la infección con este virus puede dar origen a hepatitis crónica, cirrosis y carcinoma hepático. La hepatitis crónica ocurre en un 5-15% de los adultos y en más de un 90% de los niños menores de 5 años, luego de la infección aguda (1). En forma conservadora, se estima que un 5% de la población mundial (200 millones de personas) son portadores crónicos de este virus (2). El carcinoma hepático es uno de los cánceres más corrientes en el mundo, con más de 250 mil casos nuevos cada año. Al menos un 80% de ellos es secuela directa de la hepatitis B (3). En Chile un 0,4% de la población, es decir aproximadamente 50.000 personas, son portadores crónicos de hepatitis (4). El grupo familiar inmediato de estos portadores, que constituyen el de más alto riesgo, es de aproximadamente 200.000 personas. La hepatitis B es, por lo tanto, un grave problema de salud pública mundial. Al no haber un método efectivo para su tratamiento, su prevención mediante una vacuna eficaz y libre de riesgo es esencial.

El antígeno de superficie de la hepatitis B, que ha sido aislado de la sangre de portadores crónicos, es una partícula no infecciosa, de 22 nm de diámetro compuesta de glicoproteínas insertadas en una bicapa lipídica (5). El clonamiento molecular y la secuencia de nucleótidos del genoma viral (6, 7) y el análisis de sus proteínas por electroforesis (8, 9) han revelado la estructura de las proteínas que forman la partícula de 22 nm. La proteína principal tiene 226 aminoácidos y está codificada por el gen S. Esta proteína tiene segmentos hidrofílicos expuestos al exterior de la partícula, que contiene los principales determinantes antigénicos del virus, y segmentos hidrofóbicos que probablemente atraviesan la bicapa lipídica. Existe en forma glicosilada (GP-27) y no-glicosilada (GP-24). El otro componente proteico importante en la partícula viral es una molécula de 281 aminoácidos, codificada por los genes S + pre-S2. La región pre-S2 codifica una zona de 55

* Dedicado con respeto y admiración al profesor Osvaldo Cori M., formador de numerosas generaciones de científicos.

** A quien dirigir la correspondencia.

aminoácidos de naturaleza hidrofílica que posee un epítope inmunodominante y una zona con capacidad de unirse a polialbúmina humana (10) y se localiza en la superficie de la cubierta viral. Esta proteína existe también en forma glicosilada (GP-36) y no glicosilada (GP-33) (9).

Mediante ingeniería genética se ha logrado la síntesis del antígeno de superficie de hepatitis B en levaduras (11, 12) y en varios tipos de células animales (13, 15). El antígeno producido está ensamblado en partículas de 22 nm de diámetro similares a las obtenidas de plasma de pacientes crónicos (11, 12). Las partículas obtenidas en levaduras han resultado ser altamente inmunogénicas en animales y humanos, y constituyen el componente principal para la elaboración de una vacuna recientemente aprobada en 1986 en Alemania y los Estados Unidos (16, 17).

En este trabajo nosotros comunicamos la síntesis, secreción y ensamble de partículas de antígeno de superficie de hepatitis B producida por células animales modificadas mediante ingeniería genética. Células de ovario de hámster (CHO) deficientes en dihidrofolato reductasa (DHFR–) (18) han sido transformadas con el gen que codifica el antígeno de superficie (S + pre-S2) y luego amplificadas usando metotrexato (19). Las células así obtenidas pueden mantenerse en cultivo indefinidamente y secretan, en forma eficiente, partículas de antígeno de superficie de estructura e inmunogenicidad semejantes a las de partículas obtenidas de plasma. Estas partículas constituyen un excelente material de partida para la elaboración de una vacuna efectiva contra la hepatitis B.

MATERIALES Y METODOS

Los reactivos utilizados en este estudio son de grado analítico y fueron obtenidos de Sigma Chem. Co., St. Louis Missouri, Merck Darmstadt, Bio-Rad Labs., Richmond, Ca. y BRL, Maryland. Las enzimas de restricción se obtuvieron de BRL, Maryland. El plásmido pSKV-10 fue obtenido de Pharmacia Inc., Piscataway. Las células CHO DHFR– fueron proporcionadas por la Dra. Leslie Rall. Los medios de cultivo fueron obtenidos de Difco Labs.

Las células CHO deficientes en dihidrofolato reductasa (DHFR–) se cultivaron de acuerdo al protocolo de Urlaub y Chasin (18). El crecimiento en medio no selectivo se suplementó con suero fetal de bovino al 5%. La transfección de células CHO se realizó con DNA purificado en gradientes de CsCl, usando el método de Graham y Van der Erb (20). Las células CHO DHFR+ fueron seleccionadas en medio mínimo Eagle modificado por Dulbecco, suplementado con suero fetal de bovino al 10% y prolina 35 ug/ml.

El contenido de HBsAg, en el medio de las células CHO, luego de centrifugación en gradiente de CsCl, fue medido usando el ensayo AUZYME de Abbott.

La inmunopotencia del antígeno se determinó de acuerdo al procedimiento recomendado por la OMS (21). Con este propósito se inyectaron intraperitonealmente 10 ratones BALB/c, con 1 ml de diluciones seriadas, ya sea con el antígeno obtenido del cultivo de células CHO o de suero humano (Hepatovax, Merck) en alumbre 0,1%. El nivel de anticuerpos alcanzado por los ratones luego de cuatro semanas fue determinado usando el ensayo AUSAB de Abbott.

RESULTADOS

1. *Construcción de plásmidos para la tranfección de células de ovario de hámster y expresión del antígeno de superficie de hepatitis B (HBsAg).*

El plásmido pBIOS-10, que contiene el gen del antígeno de superficie de hepatitis B (HBsAg) bajo el control de secuencias regulatorias apropiadas para su expresión en células animales (Figura 1A), se construyó mediante la inserción de un fragmento EcoRI-HhaI de 1235 pares de bases en el sitio BglII del plásmido pKSV-10 (22). Este fragmento contiene la región que codifica los 55 aminoácidos de la región pre-S2 y los 226 aminoácidos de la proteína S (7). Para lograr la inserción se usaron dos oligonucleótidos adaptadores con las secuencias terminales BglII-EcoRI y BglII-HhaI (Figura 1A). El plásmido pKSV-10 contiene el origen de replicación y el gen de betalactamasa de pBR-322 y la unidad activadora de la transcripción del virus SV-40. La región 5' de esta unidad contiene el origen de replicación y el promotor temprano de SV-40. La región 3' de esta unidad contiene el intrón del antígeno T mayor de SV-40 y el sitio de poliadenilación de la región temprana. El plásmido resultante de la fusión entre pKSV-10 y el gen preS2+S del virus de la hepatitis B se ha denominado pBIOS-10.

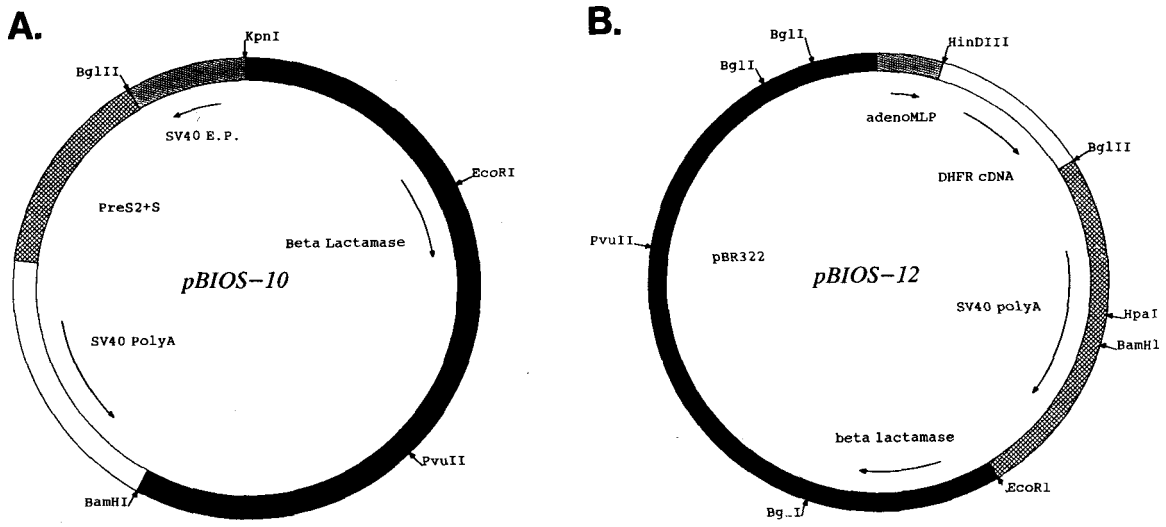

Fig. 1: Estructura de los plásmidos pBIOS-10 y pBIOS-12 usados en la transfección de células CHO DHFR– para obtener las células HEP-30.

El plásmido pBIOS-12 (Figura 1B) fue usado en cotransformación de las células CHO con el objetivo de seleccionar células capaces de crecer en medio selectivo. Este plásmido fue obtenido mediante la inserción de un fragmento HindIII-EcoRI (que contiene el cDNA que codifica la dihidrofolato reductasa (DHFR) de ratón y la señal de poliadenilación de la región temprana de SV-40) en un plásmido que contiene el promotor de la región tardía mayor de adenovirus 2 en pBR322 (pAML).

2. *Transfección de células de ovario de hámster (CHO) DHFR- con pBIOS-10 y pBIOS-12 y selección de líneas celulares productoras de HBsAg.*

Se transfectaron células CHO DHFR– con DNA de los plásmidos pBIOS-10 y pBIOS-12 en razón molar 2:1 y fueron seleccionadas en base al fenotipo DHFR+.

El HBsAg producido fue ensayado en el medio de cultivo de clones individuales. Alrededor de 30% de los clones DHFR+ analizados produjeron HBsAg en cantidades de 5 a 120 ng por 10^6 células por día. Cinco clones que producían más de 100 ng por 10^6 células por día fueron seleccionados y establecidos como líneas celulares paternas.

Con el fin de aumentar el nivel de producción de HBsAg, las líneas celulares paternas fueron crecidas en medio de cultivo con concentraciones crecientes de metotrexato (MTX) para aislar clones resistentes que hubieran coamplificado las secuencias codificadoras del HBsAg junto con las secuencias codificadoras de la DHFR. Los cinco clones paternos fueron tratados con MTX 0,5, 50 y 100 uM. Uno de estos clones, denominado HEP-30, tratado con MTX 0,5 uM, aumentó la producción de HBsAg a 2 ug por 10^6 células por día y en una segunda secuencia de amplificación, con MTX 50 uM, a 8 ug de HBsAg por 10^6 células por día. El clon HEP-30 fue aislado y cultivado en botellas rodantes con el objeto de estudiar la producción de HBsAg. Esta línea celular es estable en presencia de MTX 0,5 uM y produce HBsAg en forma constante por períodos de 4-6 semanas.

3. *Caracterización de HBsAg producido por línea celular HEP-30*

El HBsAg producido por las células HEP-30 puede ser purificado hasta homogeneidad por un procedimiento basado en distribución por partición en polietilén glicol, cromatografía de inmunoafinidad y centrifugación en gradiente de CsCl (A. De Ioannes, resultados no publicados).

El análisis de los componentes polipeptídicos de las partículas purificadas, usando electroforesis en geles con SDS, revela la presencia de dos proteínas de tamaños de

33.000 y 36.000 (Figura 2) que corresponden a los péptidos GP-34 y GP-36, respectivamente, codificados por los genes S y pre-S2. Ambas proteínas son inmunoprecipitadas por suero anti-HBsAg (resultados no publicados).

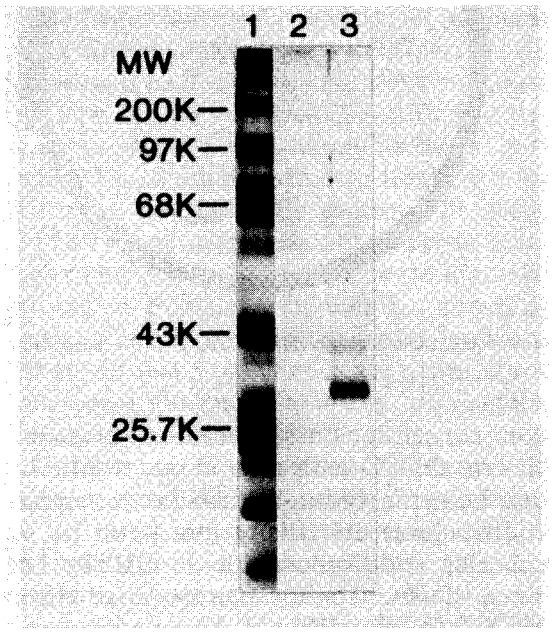

Fig. 2: Análisis electroforético de los polipéptidos componentes de las partículas de HBsAg.
Los polipéptidos fueron analizados en un gel de poliacrilamida al 10% con SDS 0,1%. Línea 1: estándar de pesos moleculares; línea 2: sobrenadante de medio de cultivo de células CHO transfectadas con pBIOS-12; línea 3: partículas purificadas de células CHO transfectadas con pBIOS-10. El gel se tiñó con nitrato de plata.

Las partículas del medio de cultivo de las células HEP-30 sedimentan en una gradiente de CsCl a una densidad de 1,2 g/cm^3 (Figura 3) que es idéntica a la densidad de las partículas de HBsAg aisladas de plasma (5) o producidas por levaduras (11).

El antígeno de superficie secretado por las células HEP-30 fue también analizado por microscopia electrónica (Figura 4). Los resultados muestran que el antígeno aparece formando partículas relativamente homogéneas, con un diámetro de 22 nm.

La inmunogenicidad de las partículas de HBsAg secretadas por las células HEP-30 fue estudiada en ratones y comparada con partículas de HBsAg purificadas de plasma humano. Como se muestra en la Tabla I, la inmunopotencia de ambas preparaciones es la misma. La cantidad de antígeno necesaria para producir una seroconversión en el 50% de los animales (ED50) es de 40 ng de HBsAg derivado de células HEP-30 o 35 ng de HBsAg derivado de plasma de pacientes crónicos.

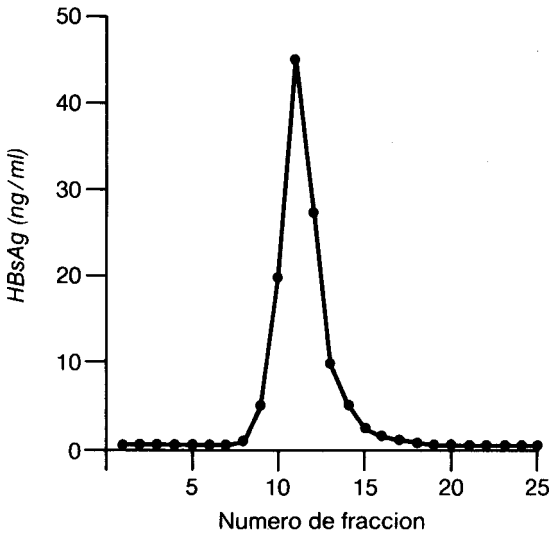

Fig. 3: Análisis del HBsAg producido por células CHO mediante centrifugación en gradiente de CsCl. Alícuotas de HBsAg purificadas a partir de medio de cultivo de células HEP-30 fueron depositadas sobre 5,3 ml de una solución de CsCl de 1,1 g/cc, la que a su vez fue depositada sobre 5,3 ml de una solución CsCl de 1,4 g/cc. Los tubos fueron centrifugados en un rotor SW-41 a 41.000 rpm por 38 horas. Las fracciones fueron colectadas y ensayadas para detectar HBsAg. El pico de HBsAg se ubica en una concentración de CsCl de 1,22 g/cc.

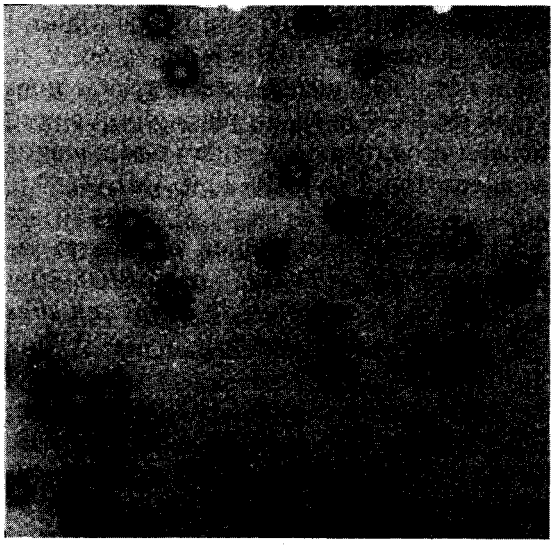

Fig. 4: Fotografía al microscopio electrónico de partículas de HBsAg producidas por células HEP-30. Las partículas purificadas fueron adsorbidas en grillas cubiertas con colodión y teñidas negativamente con acetato de uranilo al 2%.

TABLA I

Respuesta inmune frente a HBsAg producida por células HEP-30 en comparación con HBsAg aislado de plasma humano (Hepatovax)

Dosis por inyección (ug)	HBsAg-HEP-30 Ratones seroconvertidos (positivos/total)	HBsAg-Plasma Ratones seroconvertidos (positivos/total)
0,640	10/10	10/10
0,160	8/10	9/10
0,040	5/10	6/10
0,010	1/10	2/10
	ED50 40 ng	ED50 35 ng

DISCUSION

Este trabajo describe la manipulación genética de células animales y la obtención de una línea celular que sintetiza y secreta en forma estable altos niveles de antígeno de superficie de hepatitis B (HBsAg). Los plásmidos pBIOS-10 y pBIOS-12 fueron construidos y usados con el objeto de integrar los genes de HBsAg y de la DHFR respectivamente, en células de hámster deficientes en DHFR. Los clones iniciales fueron tratados posteriormente con metotrexato con el objeto de aislar líneas celulares resistentes a esta droga mediante la amplificación paralela de los genes de HBsAg y DHFR. Este procedimiento ha sido usado por varios autores anteriormente. McCormick y colaboradores lo han usado para obtener líneas celulares que expresan interferón beta (23) y Haynes y Weisman han reportado el uso de este procedimiento en la obtención de células CHO que sintetizan altos niveles de interferón alfa y gamma (24).

Las partículas de HBsAg producidas por las células HEP-30 son relativamente homogéneas y tienen el mismo diámetro y densidad que las partículas obtenidas de plasma humano. Ellas contienen dos polipéptidos de pesos moleculares aparentes de 33.000 y 36.000, que corresponden a las dos formas (no-glicosilada y glicosilada) de la proteína codificada por la región preS2+S.

Las partículas sintetizadas por las células HEP-30 contienen los 55 aminoácidos de la región pre-S2. Se ha descrito que esta región contiene un epítope dominante del virus de hepatitis B (25) y el receptor de polialbúmina humana (10). Se podría postular que este receptor podría jugar un papel importante en la especificidad y unión del virus a la célula hepática. Anticuerpos contra este receptor de polialbúmina podrían interferir directamente con este proceso y prevenir las etapas iniciales de la infección viral.

La inmunogenicidad de las partículas de HBsAg producidas por las células HEP-30 se demostró en ratones. La dosis necesaria para seroconvertir un 50% de los animales a anti-HBsAg es similar a la dosis de partículas de origen humano requerida para producir el mismo efecto. Esto sugiere que el antígeno producido por las células HEP-30 debe producir una respuesta inmune semejante en humanos.

Los resultados presentados aquí, demuestran el gran potencial de la modificación genética de células animales para lograr la obtención de altos niveles de expresión y secreción de proteínas foráneas. En especial, las partículas de antígeno de superficie descritas aquí pueden ser extremadamente valiosas en la preparación de una vacuna eficaz contra la infección causada por el virus de la hepatitis B.

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REFERENCIAS BIBLIOGRAFICAS

1. GUST, I.D. (1982) Comparison of the epidemiology of hepatitis A and B (Szmuness, W.; Alter, H.J.; Maynard, J.E. Eds.): Viral Hepatitis, Philadelphia, Franklin Institute Press, pp. 129-143.
2. FRANCIS, D.P. (1980) Selective primary health care: Strategies for control of disease in the developing World. III Hepatitis B virus and its related diseases. *Infect. Dis. Rev. 5,* 322-329.
3. BEASLEY, R.P. y HWANG, L.Y. (1984) (G.N. Vyas Ed.): Viral Hepatitis and Liver Disease, Grune and Stratton Press, New York, pp. 209-224.
4. VELASCO, M. y KATZ, R. (1970) Antígeno Australiano en muestras de población chilena y en diversos cuadros patológicos. *Rev. Med. Chile 98,* 1-5.
5. TIOLLAIS, P.; PURCEL Y.C. y DEJEAN, A. (1985) The hepatitis B virus. *Nature 317,* 489-495.
6. VALENZUELA, P.; GRAY, P.; QUIROGA, M.; ZALDIVAR, J.; GOODMAN, H.M. y RUTTER, W.J. (1979) Nucleotide sequence of the gene coding for

the major protein of hepatitis B virus surface antigen. *Nature 280,* 815-819.

7. VALENZUELA, P.; QUIROGA, M.; ZALDIVAR, J.; GRAY, P. y RUTTER, W.J. (1980) The nucleotide sequence of the hepatitis B viral genome and the identification of the major viral genes. (Fields B.N.; Jaenish, R.; Fox, C.J. Eds.) Animal Virus Genetics, New York, Academic Press, pp. 55-57.
8. STIBBE, W. y GERLICH, W.H. (1983) Structural relationships between minor and major proteins of hepatitis B surface antigen. *J. of Virol. 46,* 626-628.
9. HEERMANN, K.H.; GOLMAN, U.; SCHWARTZ, W.; SEYFFART, T.; BAUMGARTE, H. y GERLICH, W.H. (1984) Large surface proteins of hepatitis B virus containing the pre-S sequence. *J. of Virol. 52,* 396-402.
10. MACHIDA, A.; KISHIMOTO, S.; OHNUMA, H.; BABA, K.; ITO, Y.; MIYAMOTO, H.; FUNATSA, G., ODA, K.; USUDA, S.; TOGAMI, S.; NAKAMURA, T.; MIYAKAWA, Y. y MAYUMI, M. (1984) A polypeptide containing 55 amino acid residues coded by the pre-S region of hepatitis B virus DNA bears the receptor for polymerized human as well as chimpanzee albumins. *Gastroenterology 86,* 910-918.
11. VALENZUELA, P.; MEDINA, A.; RUTTER, W.J.; AMMERER, G. y HALL, B.D. (1982) Synthesis and assembly of hepatitis B Virus Surface antigen particles in yeast. *Nature 298,* 347-350.
12. VALENZUELA, P.; COIT, D. y KUO, C.H. (1985) Synthesis and assembly in yeast of hepatitis B surface antigen particles containing the polyalbumin receptor. *Bio/Technology 3,* 317-320.
13. CROWLEY, C.W.; LIU, C.C. y LEVINSON, A.D. (1983) Plasmid-directed synthesis of hepatitis B surface antigen in monkey cells. *Mol. Cell. Biol. 3,* 44-45.
14. LAUB, O.; RALL, L.B.; TRUETT, M.; SHAUL, Y.; STANDRING, D.N.; VALENZUELA, P. y RUTTER, W.J. (1983) Synthesis of hepatitis B surface antigen in mammalian cells: Expression of the entire gene and the coding region. *J. of Virol. 48,* 271-280.
15. DUBOIS, M.F.; POURCEL, C.; ROUSSET, S.; CHANY, C. y TIOLLAIS, P. (1980) Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA. *Proc. Natl. Acad. Sci. USA 77,* 4549-4553.
16. SCOLNICK, E.M.; McLEAN, A.A.; WEST, D.J.; McALEER, W.J.; MILLER, W.J. y BUYNAK, E.B. (1984) Clinical evaluation in healthy adults of a hepatitis B vaccine made by recombinant DNA. *JAMA 251,* 2812-2815.
17. STEVENS, C.E.; TAYLOR, P.E.; TONG, M.J.; TOY, P.T.; VYAS, G.M.; NAIR, P.V.; WEISSMAN, J.Y. y KRUGMAN, S. (1987) Yeast-recombinant hepatitis B vaccine. Efficacy with hepatitis B immune globulin in prevention of perinatal hepatitis B virus transmission. *JAMA 257,* 2612-2616.
18. URLAUB, G. y CHASIN, L.A. (1980) Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc. Natl. Acad. Sci. USA 77,* 4216-4220.
19. SCHIMKE, R.T. (1980) Gene amplification in cultured animal cells. *Cell 37,* 705-713.
20. GRAHAM, F. y VAN DER ERB. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology 52,* 456-467.
21. World Health Organization Technical Report Series, Nº 658, (1981).
22. Catalogo Pharmacia. Cloning Vectors, pp. 62. (1986).
23. McCORMICK, F.; TRAHEY, M.; INNIS, M.; DICKMANN, B. y RINGOLD, G. (1984) Inducible expression of amplified human beta interferon genes in CHO cells. *Mol. Cell. Biol. 4,* 166-172.
24. HAYNES, J. y WEISSMANN, C. (1983) Constitutive, long-term production of human interferons by hamster cells containig multiples copies of a cloned interferon gene. *Nucl. Acids Res. 11,* 687-705.
25. NEURATH, A.R.; KENT, S.B.H. y STRICK, N. (1984) Location and chemical synthesis of a pre-S gene-coded immunodominant epitope of hepatitis B virus. *Science 244,* 392-395.

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