TYROSINE ACTIVATION AND TRANSFER TO SOLUBLE RIBONUCLEIC ACID

BY

JAIME EYZAGUIRRE PHILIPPI
B.S., Universidad Catolica de Chile, 1958

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Chemistry
in the Graduate College of the
University of Illinois, 1963

Urbana, Illinois

UNIVERSITY OF ILLINOIS

THE GRADUATE COLLEGE

OCTOBER, 1962

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY JAIME EYZAGUIRRE PHILIPPI

ENTITLED TYROSINE ACTIVATION AND TRANSFER TO SOLUBLE RIBONUCLEIC ACID

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSPHY IN CHEMISTRY

John M Clark Jr.

In Charge of Thesis

Head of Department

Recommendation concurred in†

Carl S. Vestling

B. D. Hall

Harry Fenn

Richard F. Dickerson

Committee on Final Examination†

† Required for doctor's degree but not for master's.

D517

ACKNOWLEDGMENT

I wish to express my appreciation to Dr. John M. Clark, Jr. for his help as a teacher and friend during the course of this work.

I would like to thank Miss Carol Sherman and Mr. David Lloyd for their skillful technical assistance, and all professors and graduate students in the Biochemistry Division for their helpful suggestions or direct contributions to this thesis.

I also wish to acknowledge the financial support granted during my stay in the United States by the International Educational Exchange Service, the National Institutes of Health and the University of Illinois, and a travel grant from the Fulbright Commission.

TABLE OF CONTENTS

Page

Page

CHAPTER I

INTRODUCTION

The mechanism by which proteins are biosynthesized represents one of the most interesting and challenging problems in biology today. In recent years, great advances have been made in our understanding of protein biosynthesis, due to the availability of radioisotopes and modern research techniques. Two major concepts concerning protein biosynthesis have evolved with this work. First, protein biosynthesis from constituent amino-acids is an endergonic or energy-requiring process. Thus, cells must overcome this thermodynamic barrier by use of energy-coupled reactions. Second, there is a well defined genetic control of the eventual sequence of amino acids in biosynthesized protein. Cells, therefore, must possess amino acid organizing mechanisms or "templates" that translate genetic information into defined amino acid sequences in proteins.

The evidence for these two aspects of protein biosynthesis and their relationship to the experimental work presented in this thesis are discussed in the following introductory sections.

A. The Activation of Amino Acids and Transfer to SRNA

In 1955, Hoagland (55) showed that the 100,000 x G supernatant of a rat liver homogenate catalyzes an L-amino acid dependent exchange of PP^{32} into ATP in the presence of Mg^{++}. When hydroxylamine (which reacts with esters and anhydrides) is added, amino acid hydroxamates are formed. On the other hand, AMP failes to exchange with ATP or even to inhibit the reaction in the same system. These findings, similar to those of Maas & Novelli (84) for the activation of pantoate in the synthesis of pantothenate and Berg (6) for the activation of acetate (where acetyl-AMP is formed), suggested the formation of an anhydride or acyl adenylate bond. Hoagland (55,56) formulated the following mechanism with his data:

$$AA + ATP + \text{Enzyme} \rightleftharpoons \text{Enzyme} < \begin{matrix} AMP - AA + PP \\ \text{(Reaction I)} \end{matrix}$$

In 1956 Hoagland et al. (56) showed that this enzymatic activity (Reaction I) could be precipitated from the 100,000 x G supernatant fraction of rat liver by lowering the pH to 5.1. The resultant "pH 5 enzyme" obtained by resuspension of the precipitate, was shown to contain separate amino acid specific activating enzymes.

These findings were extended by De Moss et al. (34,35,36) and Nismann et al. (96) to bacteria, by Berg (8) to yeast, and by Clark (24), Marcus (85) and Davis & Novelli (33) to

plant tissue. The evidence obtained by these workers agrees with the mechanism postulated by Hoagland in reaction I.

First attempts to demonstrate the existence of free AMP - AA failed (35,56). De Moss et al. (35) emphasized the difficulty of such work by showing that the amino acyl adenylates are very unstable at physiological pH. Insight into the mechanism was provided by these workers who observed that there is no net breakdown of ATP during the enzymatic reaction of ATP and leucine, even in the presence of pyrophosphatase (35). This information supported the hypothesis that the AMP - AA is strongly bound to the enzyme surface (35,56). Direct evidence in this respect was furnished by Krishnaswamy & Meister (76) and Kingdon et al. (73), who were able to isolate enzymatically synthesized AMP-tryptophan using substrate levels of enzyme. Also, the addition of amino acyl adenylates to the enzyme in the presence of PP produces a net synthesis of ATP (9,35,76).

Further evidence for the existence of AMP - AA during amino acid activation comes from O^{18} studies (14,57), which showed that O^{18} from the carboxyl of tryptophan or alanine is transferred to the phosphate of AMP by the respective amino acid activating enzyme.

Although much of the above described work has been done with crude extracts or "pH 5 enzymes," many laboratories have

engaged in the study of amino acid specific activating enzymes. Enzymes following Hoagland's mechanism (Reaction I), and specific for the following amino acids, have been isolated: tryptophan (25,32,73), methionine (8,9,13), leucine (13,35,99), alanine (65,127), phenylalanine (26), serine (129), threonine (1,68,82), tyrosine (68,106,120), glycine (44), valine (13) and isoleucine (13,99). Of all these preparations, only the alanine (127) and tryptophan (32) enzymes have been obtained with a high degree of purity, as expressed by a high specific activity. These observations indicate that there are specific enzymes for every amino acid. Study of such enzymes reveals that they have narrow substrate specificity, for only certain amino acid analogs which can be incorporated into proteins and act as competitive growth inhibitors are activated. For example, the growth inhibitors 7-azatryptophan and tryptazan, are activated by the purified tryptophan activating enzyme. These compounds are also incorporated into proteins (109). 5-Methyl tryptophan on the other hand, also a growth inhibitor, is not incorporated into proteins, but acts as an inhibitor of the tryptophan activating enzyme.

The only exception observed so far to Hoagland's mechanism is a glycine activating enzyme found in Photobacterium fischeri, which forms glycine hydroxamate when incubated with

ATP, glycine and hydroxylamine, but which is observed to exchange iP rather that PP into ATP (27,28).

The fate of the activated amino acid was investigated as a natural follow-up to the activation studies. Holley (63), using the analogy of acetate activation (7), proposed the following mechanism:

$$AA + ATP + Enzyme \rightleftharpoons Enzyme < AMP - AA + PP \quad \text{(Reaction I)}.$$

$$Enzyme < AMP - AA + X \rightleftharpoons Enzyme + AA - X + AMP \quad \text{(Reaction II)}$$

in which X would be an undefined amino acid acceptor. He was able to demonstrate the existence of such a scheme by showing an alanine dependent, ribonuclease inhibited conversion of AMP to ATP, in the "pH 5 enzyme" from rat liver (63). This work, later verified with a partially purified alanine activating enzyme (65), implicated an RNA as the amino acid acceptor.

Shortly thereafter, Hoagland et al. (58) and Ogata & Nohara (98) used direct means to show that an amino acid acceptor RNA was present in the "pH 5 enzyme." Further studies revealed that the incorporation of amino acids into this "soluble RNA" (SRNA) is reversible; that PP accelerates the loss of C^{14} amino acid from pre-labeled SRNA, and that C^{12} amino acids, in the presence of ATP, dilute the radioactivity in the SRNA (59). Berg & Ofengand (10) showed that the activating enzymes are involved in the transfer of the activated amino acid to the acceptor SRNA (transfer reaction), and that the overall reaction requires the presence of ATP and Mg^{++}.

Studies with partially purified activating enzyme, showed that they possess a high substrate specificity, transferring only the amino acid that they specifically activate (10).

The nature of the linkage between the amino acid and the SRNA was determined from several lines of evidence. This linkage was found to be acid stable and alkali labile (59). The amino acid was shown to be linked through its carboxyl groups, for NH_2OH treated SRNA - AA resulted in the formation of amino acid hydroxamate (58). Zachau et al. (135) used the observations that amino acids can be released from SRNA - AA by the action of RNAase (10) and that SRNA - AA is not attackable by periodate (135) to show that the amino acids of SRNA - AA were bound as esters to either the 2' or 3' position of the ribose in the terminal 5' adenylic acid of the SRNA chain. Such an ester linkage was shown to be a "high energy" bond, for amino acid charged SRNA will synthesize ATP in the presence of PP and AMP (45).

Further support of this hypothesis was obtained from the observations in several species that the removal of the 3' terminal nucleotides in the SRNA chain causes a complete loss in the ability to incorporate amino acids (51,53,102). The addition of ATP, CTP and a specific enzyme system restores this incorporation ability. These nucleotide restoring enzymes, which lack amino acid activating activity (20,102) restore the ability of the SRNA to incorporate amino acids by adding on

a pCpCpA sequence on the end of the chain. Such evidence serves as proof that the amino acid-binding end of SRNA terminates in this sequence (52,102).

The equilibrium constant of the overall amino acid incorporation reaction into SRNA was found to give values ranging from 0.3 (79,101) to 0.7 (82), thus substantiating that the ester bond between SRNA and amino acid is a high energy bond with an energy similar to the anhydride linkage in ATP.

Hoagland et al. (59) found that the SRNA isolated from the "pH 5 enzyme" in considerably smaller than that of other kinds of SRNA. They obtained a sedimentation coefficient ($S_{20,w}$) of 1.85 S. The molecular weight of SRNA from various preparations has been determined in different laboratories and the values obtained range from 15,500 (17) to as high as 35,000 (139). A sedimentation coefficient of 4 S and a molecular weight of 25-30,000 (chain length of approximately 80 nucleotides) are most generally accepted today (19).

The base composition of SRNA has been found to be remarkably different from that of the RNA obtained from ribosomes. The ratio of purines to pyrimidines is close to 1 as in the case of DNA (91,139,140), and a comparatively high level of unusual nuclectides, especially methyl purines and pseudouridine, has been found (41,91).

Studies of the specific rotation and optical density of SRNA as a function of temperature, suggest that SRNA has regions

of a secondary structure, possibly in a helix (18,29,97). This secondary structure may be that of an intramolecular double helix as indicated by X-ray patterns (18) but not necessarily with regular base pairings, for sharp melting curves are not obtained (140). The fact that heating and rapid cooling under certain conditions produces an increase in the molecular weight supports the intramolecular double helix structure in that one can envision intermolecular hydrogen bond formation as the cause of the molecular weight increase (18). This secondary structure of SRNA may not be essential for its activity to incorporate amino-acids, since this activity is almost unaffected by heating and rapid cooling (18), although, since SRNA is a comparatively small polynucleotide, the possibility of a re-formation of enough secondary structure under these conditions cannot be discarded.

As seen earlier, the terminal sequence of the amino acid acceptor end in all nucleotide chains in SRNA is pCpCpA (20, 52). The nucleotide in the opposite end is a 5' guanylic acid (111,139). The chain lengths of the SRNA molecules are similar, as shown by Klee & Cantoni (74). However, it was observed early that when several amino acids are incorporated into SRNA, the extent of incorporation is additive, and no competition is observed between the different amino acids (107). This suggested the possibility that SRNA corresponds to an heterogeneous population of polynucleotides, differing in specificity for every amino acid. Some preliminary studies using $(NH_4)_2SO_4$

fractionation and ion exchange columns (113) showed enrichment of the ability of SRNA to incorporate certain amino acids. Definite proof of the hypothesis has come with the development of several procedures for the purification of amino acid specific SRNAs . Holley's group pioneered the use of countercurrent distribution (40,64,66,67). With this method they have been able to obtain histidine, tyrosine, valine and alanine acceptor SRNAs of a purity ranging from 23 to 66% (4). Zamecnik's group developed an SRNA fractionation method involving periodate treatment of SRNA labeled with one amino acid followed by coupling of a dye to the IO_4^- generated aldehyde groups. Solubility properties then allowed the separation of the SRNA - AA from the dye SRNA complex (137,138). Further fractionation with a DEAE Sephadex column has allowed the recovery of valine specific SRNA to a purity of 80% (115). Zubay (140), with a similar procedure has obtained 70% pure leucine specific SRNA. Brown et al. (17) reported fractionation of tyrosine and histidine specific SRNA's by selective binding of the respective RNA - AA's to polydiazostyrene. Recently, Sueoka & Yamane (116) have developed a methylated albumin column that has proved to be effective in the separation of certain amino acid specific SRNAs.

The difference between the amino acid specific SRNAs must be due either to differences in the nucleotide composition and/or sequence of nucleotides in the chain. If there are

differences in sequence, they must be located in the interior of the polynucleotide chain. It has been suggested (11) that since the methylated purines found in SRNA are present at levels of less than one per chain, they may participate in determining the specificity of certain chains. Holley et al. (69) have studied the nucleotide composition of some countercurrent distribution purified SRNAs. Alanine specific SRNA contains a higher level of Gp and a lower level of Ap when compared to valine and tyrosine specific SRNAs. Yet, although these latter two SRNAs show similar nucleotide composition, there are great differences in their partition coefficients as seen by their properties upon countercurrent distribution (4). This suggests major differences in the nucleotide sequences of these two SRNAs.

Attempts to determine the nucleotide sequence of SRNA chains beyond the three common terminal nucleotides indicate differences among the SRNAs . Lagerkvist et al. (77) established that the fourth nucleotide of the chain varies, with 68, 24, 8 and <1% of the chains containing adenylate, guanylate, uridylate and cytidylate respectively. The fifth nucleotide is in most cases a pyrimidine.

Recently, McCully & Cantoni (90) have studied the composition of oligonucleotides obtained by hydrolysis of SRNA with takadiastase T_1 ribonuclease, that specifically cleaves the polynucleotide chain between 3' guanylic acid and the adjacent nucleotide. These investigators have found that certain

sequences exceed the theoretical value obtained if a random sequence is expected.

It has also been found that there are chain differences even between SRNA molecules specific for one amino acid. This has been observed for leucine by the use of countercurrent distribution (40,130). The implications of this finding will be discussed later.

An SRNA chain must be able to recognize its specific amino acid and activating enzyme. Since both the structure of the enzyme and RNA are under genetic control, they are subject to genetic modifications (mutations), that could lead to the appearance of species specificities. Thus, the findings by Berg, Benzer and collaborators (5,12) that E. coli and yeast SRNA show species specificities dependent upon enzyme source and amino acid tested, is not surprising. These properties of SRNA are probably due to differences in nucleotide composition and/or sequence in the incompatible species. Preliminary experiments with partially purified tyrosine specific SRNA from yeast and E. coli indicate that there are differences in their nucleotide composition (19).

B. The Assembly of a Protein on the Template

It has been known for several years that C^{14} amino acids can be incorporated into proteins and subcellular particles using various cell free extracts and an ATP generating system (136). Simplification of the system through the years has

lead into the following general conclusions: the activated amino acids, attached to the SRNA are transferred to microsomal particles in the presence of a complex GTP requiring preparation called "transfer enzyme" (46,59,72,133):

$$\text{SRNA - AA} + \text{ribosomes} \xrightarrow[\text{transfer enzyme}]{\text{GTP}} \text{ribosomes - SRNA - AA} \qquad \text{(Reaction III)}$$

This transfer enzyme has been partially purified (42); it is activated by sulfhydryl compounds (15); it is common for all amino acids (93) and has no amino acid activating activity (93,122). The role of GTP is at present unknown, but Allende et al. (3) have recently isolated two fractions from the transfer enzyme that are complementary in the incorporation reaction, one of which was found to present GTPase activity. Puromycin and chloramphenicol have been found to inhibit Reaction III (93), but their mechanism of action is not clear.

While these incorporation studies were in progress, an entirely different line of work was being developed. This work investigated the mechanism by which genetic information expresses itself in the protein synthesizing machinery. The basic theoretical backgroupd of this approach was developed by Crick in his "adaptor hypothesis" (30,61). According to this hypothesis, genetic information contained in the base sequence of a polynucleotide chain is passed unidirectionally from DNA to RNA to protein. The sequence of amino acids in a protein is thus determined by an RNA template. Amino acids are brought to this template by "adaptors" that can translate

the genetic information contained in it. Specific adaptors, that link to the template by hydrogen bonding would be required for every amino acid, and these adaptors could subsequently be released to accept another identical amino acid.

The evidence available today is in very good agreement with the adaptor hypothesis. The experiments discussed above suggest that SRNA plays the role of the adaptor. The most widely accepted ideas on a genetic code propose that a sequence of three nucleotide bases (triplets) is sufficient to code one amino acid (30). Since SRNAs have common terminal nucleotide sequences, it is logical to assume that there must be an internal triplet with the code letters for one particular amino acid. Perhaps the pCpCpA common end of all chains is only involved in bringing the amino acids together to form a polypeptide chain (61,126). The cyclic or catalytic role of SRNA has been confirmed by the observations of Hoagland & Comly (62) and Nathans & Lipmann (93). They showed that SRNA becomes firmly but transitorily attached to the ribosomes during amino acid transfer (62), and can be subsequently isolated intact without loss of its activity to incorporate amino acids (93).

The template was originally believed to be ribosomal RNA because of its predominance in cells, but many inconsistencies to this idea were apparent. Ribosomal RNA has a markedly uniform size, a very constant base composition, not reflecting that of the respective DNA, and it is remarkably stable (16). This does not agree with what would be expected of a template

under the conditions where protein synthesis is known to take place. These discrepancies made Jacob & Monod (71) suggest, and other biochemists look for, a special type of RNA that would have a DNA like composition, be very heterogeneous in size, be associable with ribosomes and have a high rate of turnover. An RNA with these characteristics has been found in several laboratories (50,121,134), and has been called "messenger RNA."

This prompted Nirenberg & Matthaei (95) to try the effects of synthetic polynucleotides as "messengers." This work has lead to the discovery of many messenger RNA triplets, each specific for a particular amino acid (80,89).

The study of the manner by which messenger RNA carries the information of DNA is one of the most active fields of biochemistry today. Exact sequence of the triplet codes are not known, but some parts of the mechanism have become clear. First, it is apparent that the SRNA attaches to a specific point in the messenger, and "translates" the code spelled by the messenger. This is shown by the work of Chapeville et al. (21,22) who altered amino acids bound to SRNA (oxidation of cysteine to cysteic acid; conversion of cysteine to alanine). Subsequent messenger controlled transfer of the modified amino acid from the SRNA to protein or amino acid polymer showed that the modified amino acid was incorporated as if it were the original amino acid, amino acid. Since the SRNA was specific for the original amino acid, this gave further support to the adaptor hypothesis.

Second, there are indications that the genetic code is degenerate, and that several triplets may correspond to one amino acid. As it was discussed above, there are two types of leucine specific SRNA in E. coli. Degeneracy is indicated by the observation that a different synthetic polynucleotide is required to enhance the incorporation of leucine from these SRNAs to amino acid polymer (130).

Least is known about the last stages of protein synthesis, although some significant breakthroughs in this area have been made with a reticulocyte system (94,108). This system has provided the first direct evidence that amino acids from SRNA - AA are incorporated into soluble protein. Further, C^{14} containing peptides of a particular protein (hemoglobin) can be identified by "fingerprinting," from this system (108,123). Such properties have allowed the discovery that hemoglobin is not assembled randomly, but rather is assembled by a steady sequential addition of amino acids from the free amino end (37,108). Finally, studies of hemoglobin synthesis in intact cells indicate a "turnover" on the ribosomes, or that messenger RNA containing ribosomes can synthesize many hemoglobin chains in succession (108). Similar studies in cell-free extracts reveal a loss in ability to synthesize hemoglobin chains continuously, perhaps because the extract is unable to build more messenger RNA to direct the synthesis.

Preliminary evidence points toward an ATP requiring enzymatic release of the ribosome-bound polypeptide chains (70,92,110,128). A non enzymatic release has been induced in the presence of puromycin (70,92).

Little is known about the mechanism by which newly synthesized proteins attain their final secondary and tertiary structure.

C. Scope of this Thesis

This thesis presents further insight into the details of the amino-acid activating reaction, (Reaction I) and subsequent transfer of this amino acid to SRNA (Reaction II). For this purpose a highly purified tyrosine activating enzyme has been obtained from pig pancreas. Special emphasis has been put in the kinetics and properties of Reaction II, and some preliminary information has been obtained with respect to the structural requirements of the SRNA molecule in the incorporation reaction.

CHAPTER II

METHODS

A. Abbreviations

The abbreviations commonly used and their meanings are listed below:

Tris-Cl:	Tris-(hydroxymethyl)-aminomethane titrated with HCl to desired pH
ATP:	Adenosine triphosphate
K-ATP:	ATP titrated with KOH to desired pH
ADP:	Adenosine diphosphate
AMP:	Adenosine monophosphate
GTP:	Guanosine triphosphate
CTP	Cytosine triphosphate
RNA:	Ribonucleic acid
SRNA:	Soluble RNA
AA:	Amino acid
SRNA - AA:	Amino acid attached to SRNA
AMP - AA:	Amino acyl adenylate
DNA:	Deoxyribonucleic acid
Pi or iP:	Orthophosphate
PP:	Pyrophosphate
Enz:	Enzyme
RNAase:	Ribonuclease
DEAE-cellulose:	Diethyl-amino ethyl cellulose
TCA:	Trichloroacetic acid
EDTA:	Ethylene diaminetetraacetate
BSA:	Bovine serum albumin
SPDE:	Spleen phosphodiesterase
TAE:	Tyrosine activating enzyme
M.W.:	Molecular weight
O.D.:	Optical density
c.p.m.:	counts per minute
R.P.M.:	revolutions per minute
G:	gravity

The other abbreviations used are the internationally used chemical symbols.

B. Preparative Methods

1. Purification of the pig pancreas tyrosine activating enzyme

All operations performed at 0-2°C unless stated otherwise.

a. Preparation of the acetone powder

Fresh pig pancreas, obtained from W. M. Davis and Co., Danville, Illinois, was quickly cooled to 0°C in ice just after slaughter. All further operations were performed as soon as possible.

300 g of defatted tissue were ground with 2,200 ml cold (-15°C) reagent grade acetone in a large Waring blendor for 2 minutes and then vacuum filtered through Whatman No. 1 paper in a large stainless steel Buchner funnel. During the final stages of filtration, the cake was washed with 400-500 ml of cold acetone to enhance water removal. The resultant cake was reground for an additional 2 minutes in 1,100 ml of cold acetone, filtered as before, and subsequently ground for 30 seconds with one pound of cold (-15°C) reagent grade ether followed by filtration and final washing of the dry cake with 100 ml of ether. The final cake was air dried at room temperature while being worked with the hands. The light tan powder obtained was allowed to remain at room temperature for an additional hour to remove all traces of ether and then stored at -15°C.

The powder was kept for periods of over 6 months without appreciable loss of activity. The yields obtained were in

the order of 10-20% of the original weight of the pancreas. The purification was started with 150 g batches of acetone powder.

b. Adsorption with calcium phosphate gel

One batch of acetone powder was slowly added to 1,500 ml of 0.02 M tris-Cl buffer, pH 7.5, and stirred until a thick suspension was obtained. No special effort was made to disrupt big lumps. The mixture was allowed to sit for 15 minutes and then centrifuged for 1 hour at 15,000 x G (maximum speed) in the GSA head of the Servall RC-2 refrigerated centrifuge.

Aged $Ca_3(PO_4)_2$ gel prepared according to the method of Singer & Kearney (112) or obtained from Sigma Chemical Co., St. Louis, Mo., was added to the supernatant (Fraction I, about 1,200 ml). 6 mg (dry weight) of gel were used per ml of supernatant. Prior to addition, the gel was centrifuged for 5 minutes at 3,000 x G to eliminate most of the water.

Lumps within the gel slurry were disrupted by homogenization. The slurry was adjusted to pH 7.5 with 1 N KOH (approximately 12 ml), and after gentle stirring for 30 minutes, the gel was removed by centrifugation for 10 minutes at 5,000 x G in the Servall RC-2. The supernatant was discarded. The precipitated gel was washed by resuspension in 750 ml of 0.2 M potassium phosphate buffer, pH 6.0, using a teflon tipped Potter-Elvehjem homogenizer, followed by 30 minutes of gentle

stirring and final resedimentation of the gel by centrifugation at 10,000 x G for 10 minutes.

The supernatant was discarded, and the gel precipitate was resuspended by homogenization in 750 ml of 2 M potassium phosphate buffer, pH 8.1. This gel slurry was stirred for 30 minutes and then centrifuged (30 minutes, 15,000 x G). The gel precipitate was discarded. The supernatant was suction-filtered through Whatman No. 50 paper to eliminate any residual gel particles. The resultant filtrate was dialyzed against at least 10 liters of 0.02 M tris-Cl, pH 7.5, for 12 hours to yield 1200 ml (Fraction II). No changes of the buffer were made during the dialysis.

c. Ammonium sulfate fractionation

120 g of "enzyme grade" ammonium sulfate (Mann Research Laboratories, Inc., New York, N. Y.) was slowly added to each 200 ml of Fraction IV. During the salt addition, the pH of the solution was kept close to pH 8 (pH paper) by the dropwise addition of 1 N KOH (about 60 ml for the whole batch). The final solution was adjusted to pH 8.0 (pH meter, 1:5 dilution of the solution, measured at room temperature). The mixture was allowed to stand for 3 hours followed by centrifugation at 15,000 x G for 15 minutes to sediment the insoluble proteins. The supernate was carefully decanted and discarded. The precipitate was dissolved in 50 ml of 0.1 M tris-Cl, pH 7.5, centrifuged (10 minutes, 5,000 x G) to eliminate

undissolved materials, and then dialyzed for 12 hours against 4 liters of 0.02 M tris-Cl pH 7.5, to yield about 55 ml of Fraction III.

d. Acid precipitation

While stirring constantly, approximately 0.4 ml of ice cold 1 N acetic acid was slowly added to the approximately 55 ml of Fraction III, until the pH dropped to 5.4-5.6 (pH meter in the cold room, temperature setting 5°C). The precipitate was centrifuged down (5 minutes, 5,000 x G) and discarded. The supernatant solution was titrated back to pH 7.5 with approximately 0.2 ml of 1N KOH, to yield about 53 ml of fraction IV.

e. DEAE-cellulose column

DEAE-cellulose (Selectacel, purchased from Brown & Co., Berlin, N. H.) was treated as follows before use. DEAE powder, screened through a 50 mesh screen, was suspended in 10-20 volumes of water, suction filtered and then washed twice (suspension and vacuum filtration) with 10-20 volumes of 95% ethanol. The filter cake was subsequently washed twice with 10-20 volumes of 1N NaOH and then repeatedly washed with water until the pH of the supernatant dropped below 10. The DEAE was then washed once with 10-20 volumes of 1M tris-Cl, pH 7.5, and twice with a similar volume of 0.02 M tris-Cl, pH 7.5, to bring the resin to the desired pH and ionic strength.

The prepared resin was resuspended in 0.02 M tris-Cl pH 7.5 (about 40 mg dry weight of resin per ml) and stored at 4°C until used.

3 grams (dry weight) of DEAE were packed under pressure (50 inches of water) in 0.02 M tris-Cl, pH 7.5, in a 1.5 cm diameter column. One batch of fraction IV (about 53 ml) containing about 1 mg/ml of protein was added to the column. The ratio of protein to dry weight of resin was always kept under 1:30. A flow rate of about 12 drops per minute was kept and 10-15 ml fractions were collected. O.D. at 280 mμ was recorded during the run with a U. V. monitor (Gilson Medical Electronics, Middleton, Wisconsin). After the enzyme entered the resin, the column was washed with 0.02 M tris Cl pH 7.5 until the monitor showed that no more 280 mμ absorbing material was eluted. 10-15 ml fractions eluted by 500 ml of a 0.04 M tris Cl, pH 7.5, to 0.3 M tris-Cl, pH 7.5 linear gradient, were then collected (Figure 1). All fractions were analyzed for protein concentration and hydroxamate forming activity (see later in ASSAY METHODS). Fractions with a specific activity of 30 and over (hydroxamate assay) were combined (Fraction V) and saved for further purification. Sometimes the salt concentration was determined with a Radiometer type CDM2 conductivity meter, using solutions of tris Cl, pH 7.5, of known concentration as standards.

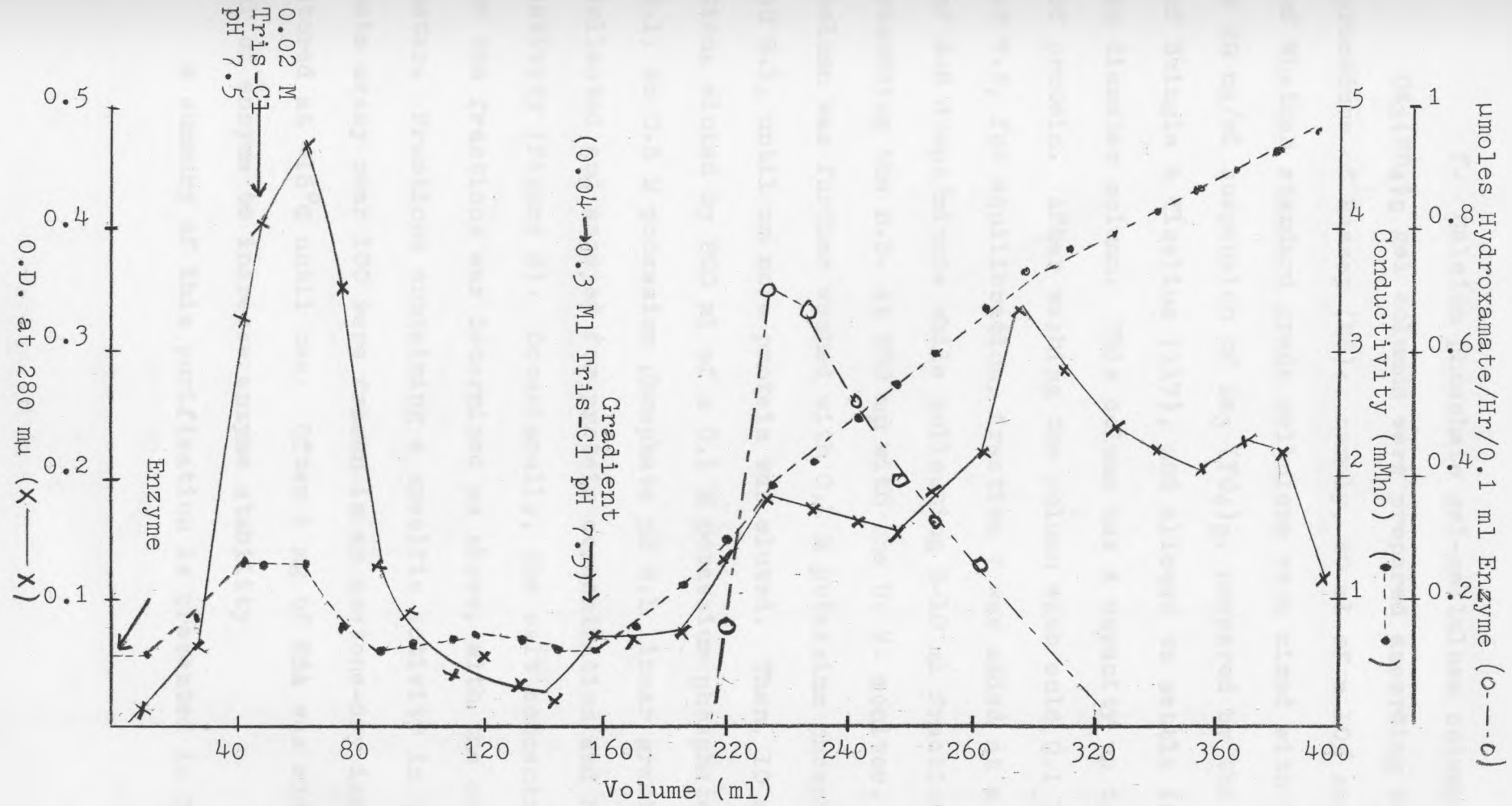


Figure 1. Elution Pattern of the Pig Pancreas Tyrosine Activating Enzyme from a DEAE-Cellulose Column.

Procedure as described in the text. O.D. at 280 mμ determined in each fraction with the Zeiss spectrophotometer. Conductivity: 0.2 M tris-Cl pH 7.5 correspond to 4.58 mMho.

f. Calcium phosphate gel-cellulose column

$Ca_3(PO_4)_2$ gel columns were prepared according to the procedure of Massey (88): namely, 60 ml of a 10% suspension of Whatman standard grade cellulose were mixed with 20 ml of a 28 mg/ml suspension of $Ca_3(PO_4)_2$, prepared by the method of Swingle & Tiselius (117), and allowed to settle in a 1.5 cm diameter column. This column has a capacity up to 100 mg of protein. After washing the column with cold 0.1 M tris Cl, pH 7.5, for equilibration, fraction V was added at a flow rate of 4-8 drops/minute while collecting 5-10 ml fractions, and recording the O.D. at 280 mμ with the U. V. monitor. The column was further washed with 0.1 M potassium phosphate buffer, pH 8.1, until no more protein was eluted. Then, 10 ml fractions eluted by 200 ml of a 0.1 M potassium phosphate, pH 8.1, to 0.5 M potassium phosphate pH 8.1 linear gradient were collected and assayed for protein concentration and hydroxamate activity (Figure 2). Occasionally, the salt concentration of the fractions was determined as above, with the conductivity meter. Fractions containing a specific activity in the hydroxamate assay over 100 were frozen in an acetone-dry ice bath and stored at -15°C until use. Often 1 mg of BSA was added per ml of enzyme to increase enzyme stability

A summary of this purification is presented in Table I.

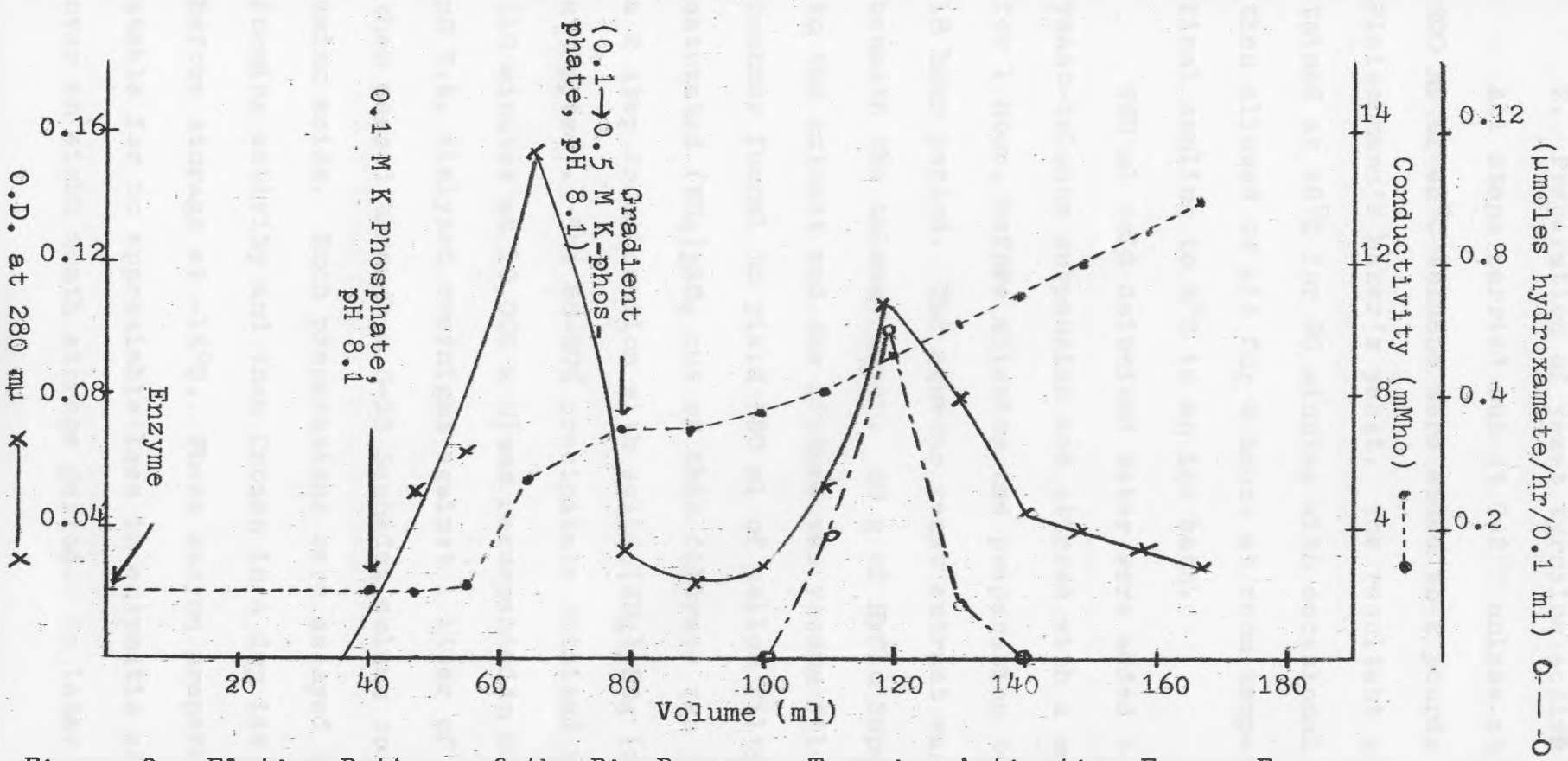


Figure 2. Elution Pattern of the Pig Pancreas Tyrosine Activating Enzyme From a $Ca_3(PO_4)_2$ Gel Cellulose Column.

Procedure as described in the text. O.D at 280 mμ determined with the Zeiss spectrophotometer. Conductivity: 0.1 M K-phosphate pH 8.1 corresponds to 7.1 mMho.

2. Preparation of yeast tyrosine activating enzyme

All steps carried out at 0-2°C unless stated otherwise. 800 ml of 45°C toluene were added to 2 pounds of minced Fleischmann's baker's yeast. The resultant slurry was maintained at 45°C for 90 minutes with occasional stirring, and then allowed to sit for 2 hours at room temperature before final cooling to 5°C in an ice bath.

960 ml cold deionized water were added to the autolyzed yeast-toluene suspension and stirred with a magnetic stirrer for 1 hour, before allowing the preparation to settle over an 18 hour period. The aqueous yeast extract was removed from beneath the toluene layer. 60 g of Hyflo Super Cel were added to the extract and the mixture was vacuum filtered through a Buchner funnel to yield 650 ml of yellow filtrate. A 50-60% saturated $(NH_4)_2SO_4$ cut of this filtrate was prepared using a 2 step fractionation with solid $(NH_4)_2SO_4$ (no pH alteration attempted). The 50-60% precipitate obtained by centrifugation (10 minutes at 10,000 x G) was resuspended in 50 ml 0.02 M tris Cl, pH 7.5, dialyzed overnight against 1 liter of the same buffer, and then passed through a G-25 Sephadex column to eliminate free amino acids. Such preparations were assayed for hydroxamate forming activity and then frozen in a dry ice acetone bath before storage at -15°C. These enzyme preparations are quite stable for no appreciable loss in enzymatic activity occurs over an eight month storage period. In later work the Sephadex

G-25 step was eliminated for very little free amino acid was found in the dialyzed fraction before Sephadex treatment.

Further purification attempts using DEAE columns, $Ca_3(PO_4)_2$ adsorption, heat or pH fractionations proved of little success, for this enzyme becomes very unstable and loses its activity. This enzyme has no noticeable hydroxamate activity towards valine or alanine.

3. Preparation of a yeast extract with a "broad spectrum" of amino acid activating activity

50 g of Fleischman's baker's yeast were mixed with 60 g of 150 μ glass beads and 50 ml of 0.05 M tris Cl pH 7.5, 0.001 M $MgCl_2$, 0.001 M mercaptoethanol and ground for 10 minutes in an ice jacketed cup of a colloid mill ("Mini-Mill," Gifford-Wood Co., Hudson, N. Y.). The resultant slurry was centrifuged for 10 minutes at 15,000 x G (Servall SS-1 centrifuge) in the cold and then the supernate obtained was recentrifuged for 1 hour at 105,000 x G in the Spinco model L preparative ultracentrifuge. The 105,000 x G supernatant obtained by decantation was dialyzed against 1 change of 50 volumes of 0.01 M tris-Cl, pH 7.5, 0.0002 M mercaptoethanol, 0.0001 M K-EDTA for 27 hours. The dialyzed enzyme was then centrifuged for 1 hour at 105,000 x G in the Spinco model L to clarify the enzyme, and aggregate lipids on the surface of the preparation. After careful removal of the lipid layer, the preparation was frozen in a dry ice-acetone bath and kept at -15°C. Such preparation retains sufficient activity for periods of 2 months

and longer and was used in experiments where activating activity towards several amino acids was needed.

4. Preparation of E. Coli tyrosine activating enzyme

All operations carried out at 0-4°C. 33 g net weight of frozen E. Coli B cells grown on Difco Nutrient Broth medium were thawed, suspended in 40 ml of 0.01 M tris-Cl pH 7.5, and sonically ruptured by treatment for 15 minutes in a 10 KC Raytheon sonicator Model DF 101 (Raytheon Manufacturing Co., Waltham, Mass.). After centrifugation of the ruptured cell suspension (15 minutes at 30,000 x G), the clear supernatant obtained was fractionated in a 2 step fractionation with solid $(NH_4)_2SO_4$ (no pH adjustment during the salt addition) to yield a 35-55% saturated $(NH_4)_2SO_4$ fraction. This precipitate was resuspended in 35 ml of 0.02 M tris-Cl, pH 7.5, and dialyzed overnight against 1 liter of 0.01 M tris-Cl, pH 7.5. Acid precipitation by adjustment to pH 5.0 with 1 N acetic acid gave an inactive precipitate (upon resuspension) while the supernatant, which was titrated back to pH 7.5 with 1 N KOH, retained activity. This fraction was frozen in small aliquots in a dry ice-acetone bath and stored at -15°C until needed. Just before use, this enzyme was passed through a G-25 Sephadex column to free it of endogenous amino acids.

Further attempts to purify this enzyme with DEAE columns, $Ca_3(PO_4)_2$ gel cellulose columns or heating at 55°C, all resulted in complete loss of activity.

5. Preparation of soluble RNA

All operations at 0-4°C unless stated otherwise. SRNA was prepared from pig liver, yeast, E. coli, broccoli and rat liver. In all cases the procedure used was a slight modification of that described by Holley et al. (67). The following procedure, as applied to 6 pounds of yeast, illustrates the method.

Six pounds of Fleischman's baker's yeast were suspended in 3.6 liters of water; 5.4 liters of 78:28 phenol-water mixture (prepared by addition of water to Mallinckrodt chromatography grade 88% liquid phenol) were added to the yeast slurry, followed by incubation for one hour at room temperature with occasional shaking. The mixture was then chilled in ice and centrifuged at 15,000 x G for 15 minutes in the RC-2 Servall. The upper or aqueous phase and the turbid phenol-water interface were carefully decanted and recentrifuged as above. The phenol layer from the first centrifugation and the precipitate from the second centrifugation were discarded. 0.1 volume of 20% potassium acetate, pH 5.0, and 2.5 volumes 95% ethanol were added to the clear, yellow supernate (4400 ml). The precipitate was allowed to settle overnight. Most of the clear supernate was then siphoned off, after which the precipitate was collected by centrifugation (10 minutes, 15,000 x G), washed once by resuspension and recentrifugation with 300 ml 95% ethanol and finally dried under vacuum.

Often at this stage the SRNA preparations were stored for several days at 2°C.

The dry crude SRNA obtained from 6 lb. of yeast was dissolved in 800 ml of 0.1 M tris-Cl, pH 7.5, and extracted one time with 250 ml prechilled ether. The solution was then mixed with chunks (approximately 48 g dry weight) of washed DEAE cellulose resin (see page 21) obtained by vacuum filtration of DEAE suspended in 0.1 M tris Cl pH 7.5. This allowed 75% of the O. D. at 260 mμ of the solution to be adsorbed by the resin as measured by O. D. determinations on supernatant aliquots of the resin-SRNA solution slurry. The RNA-containing DEAE was washed twice by suspension in 2 liters of 0.1 M tris-Cl pH 7.5 followed by vacuum filtration. The washed resin was then suspended in a minimum of 0.1 M tris Cl pH 7.5 and packed into a column with 50 inches of water pressure.

The SRNA was eluted from the DEAE column with 1 M KCl, 0.1 M tris Cl, pH 7.5. Those fractions containing the majority of the 260 mμ absorbing material were combined (approximately 2200 ml obtained); 2.5 volumes of 95% ethanol were added, and the precipitate was allowed to settle overnight. The precipitate, obtained by centrifugation, was washed once (resuspension followed by centrifugation) with 200 ml 80% ethanol and once with 200 ml 95% ethanol before being vacuum-dried. Final yield: 4 g. Some of the later experiments used commercial

yeast SRNA (General Biochemical Industries, Chagrin Falls, Ohio), prepared in a manner similar to the above procedure.

6. Purification of the SRNA by means of a G-75 Sephadex column

SRNA prepared by the above method contains a measurable quantity of nucleotides and oligonucleotides. Certain experiments, e.g. phosphatase and phosphodiesterase treatment of SRNA require the removal of these nucleotides. The following procedure using a G-75 Sephadex column accomplished this task.

Fine particles were removed from 15 g of G-75 Sephadex (Pharmacia Fine Chemicals, Inc., Rochester, Minn.) by repeated suspension of the Sephadex granules in water followed by gravity sedimentation and decantation of the turbid supernatant. The Sephadex was then washed several times with 0.01 M NH_4OOCH, residual air bubbles were removed with vacuum, and the remaining large gel particles were packed in a 3 cm diameter column by the gradual addition of small increments of gel to a flowing column filled with an agitated solution of 0.01 M NH_4OOCH. After packing, the column was placed in the cold room (0-4°C) where all further operations were performed, and washed with several volumes of cold 0.01 M NH_4OOCH, before use. The flow rate was about 15 drops per minute.

A typical run was as follows, 100 mg SRNA were dissolved in 6 ml 0.01 M NH_4OOCH and centrifuged to eliminate insoluble materials. The solution was placed on the column and allowed

to enter the gel. 5 ml 0.01 M NH_4OOCH were then passed into the column to assure complete entry of the SRNA. The SRNA was eluted with 0.01 M NH_4OOCH, 10-15 ml fractions were collected, and the O. D. at 260 mμ was determined with the Zeiss spectrophotometer. Fractions containing the first, or major peak of 260 mμ absorbing material (see Figure 3) were pooled, lyophilized to 20% of their original volume, and while ice cooled, mixed with 0.1 volumes of cold 20% potassium acetate, pH 5, and 2.5 volumes of cold 95% ethanol. The precipitate was allowed to settle overnight at 0-2°C before isolation by centrifugation and vacuum drying (yield about 70 mg).

In some experiments, potassium phosphate or C^{14} leucine were added to the SRNA before gel filtration in order to serve as small molecule markers. Their elution patterns (determined by phosphate assay (48) or radioactive counting) matched those of the small nucleotides, established by determination of O. D. at 260 mμ. Occasionally catalase (M.W. 248,000) was used as a large molecule marker. In these instances, catalase eluted slightly ahead of the SRNA (Fig. 3) (for catalase assay, see page 43). Thus, as expected, SRNA is not completely excluded from Sephadex G-75 particles, whose advertised exclusion limit is 40,000 M.W.

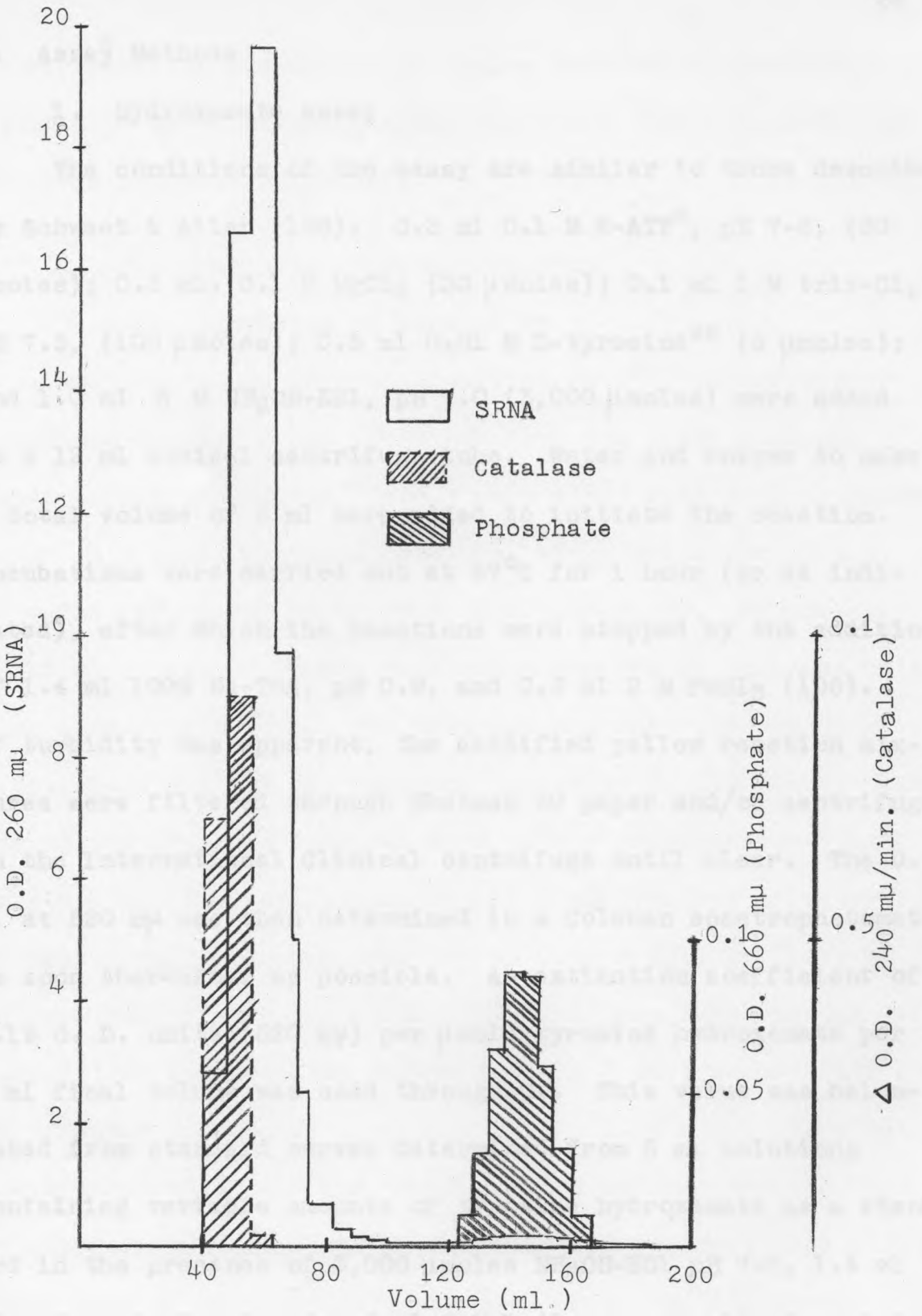


Figure 3. Profile of the Elution of SRNA From a 15 g. G-75 Sephadex Column.

Elution and Assays as described in the text. 1.5 ml 0.01 M NH_4OOCH containing 20 mg SRNA, 200 μg catalase and 28 μmoles phosphate added to the column.

C. Assay Methods

1. Hydroxamate assay

The conditions of the assay are similar to those described by Schweet & Allen (106). 0.3 ml 0.1 M K-ATP*, pH 7-8, (30 μmoles); 0.3 ml. 0.1 M $MgCl_2$ (30 μmoles); 0.1 ml 1 M tris-Cl, pH 7.5, (100 μmoles); 0.5 ml 0.01 M L-tyrosine** (5 μmoles); and 1.0 ml 3 M NH_2OH-KCl, pH 7.0 (3,000 μmoles) were added to a 12 ml conical centrifuge tube. Water and enzyme to make a total volume of 3 ml were added to initiate the reaction. Incubations were carried out at 37°C for 1 hour (or as indicated), after which the reactions were stopped by the addition of 1.4 ml 100% Na-TCA, pH 0.9, and 0.6 ml 2 M $FeCl_3$ (105). If turbidity was apparent, the acidified yellow reaction mixtures were filtered through Whatman 50 paper and/or centrifuged in the International Clinical centrifuge until clear. The O. D. at 520 mμ was then determined in a Coleman spectrophotometer as soon thereafter as possible. An extinction coefficient of 0.19 O. D. units (520 mμ) per μmole tyrosine hydroxamate per 5 ml final volume was used throughout. This value was calculated from standard curves determined from 5 ml solutions containing variable amounts of tyrosine hydroxamate as a standard in the presence of 3,000 μmoles NH_4OH-KCl pH 7.0, 1.4 ml 100% Na TCA pH 0.9 and 0.6 ml 2 M $FeCl_3$. One unit of activity

---

*Obtained from Sigma Chemical Co.

**Obtained from California Corp. for Biochemical Research.

is expressed as the amount of enzyme required to synthesize 1 μmole of tyrosine hydroxamate per hour. Specific activity is given in units/mg protein.

2. Pyrophosphate exchange reaction

a. Synthesis of labeled PP

Small aliquots (2-4 millicuries) of P^{32} labeled orthophosphate in dilute HCl (obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn.) were mixed with 2 ml deionized water in a clean platinum crucible, after which the pH was adjusted (pH paper) to about 9 with 0.25 N KOH. 45 μmoles (0.15 ml of 0.3 M) KH_2PO_4 were added and the mixture was dried down with a heat lamp. The crucible was then covered with a porcelain top and its contents pyrolyzed by a 5 minute heating of the crucible to a red heat with a Bunsen flame. Both crucible and top were washed with 0.5 ml aliquots of water several times, and once with 0.5 ml 0.01 M HCl, in order to extract most of the radioactivity. The resultant extract was poured into a Dowex 1-Cl column (0.75 x 7 cm); washed with 10 ml of water and eluted with 0.01 N HCl, 0.05 M KCl (flow rate 11-16 drops/minute). The radioactivity of the fractions was detected with a conventional radioisotope monitor. The first sharp peak of radioactivity composed of Pi was discarded, while fractions from the second or broad peak of radioactivity containing the pyrophosphate were pooled. The purity of the PP^{32} was then checked by ascending chromatography using known

$K_4P_2O_7$ and K_2HPO_4 with aliquots of the radioactive peaks in a 60:40 acetone: 35% monochloroacetic acid solvent system. Phosphate compounds were located on the paper by use of a $HClO_4$-phosphomolybdate spray and compared with the radioactivity detected with a conventional radioisotope monitor. The PP^{32} containing fraction was then brought to pH 7-8 with 1 N KOH (pH paper) and added to a 0.5 x 2 cm column of Dowex 1-Cl (at this pH there is quantitative absorption of the PP). The absorbed PP^{32} was then completely eluted with 3 ml of 0.1 M HCl, 0.1 M KCl; titrated to pH 7-8 with 1 M KOH and stored at -15°C until use.

b. Exchange reaction: according to Clark (24)

The PP^{32} prepared above was diluted with cold K-PP pH 7.5 to obtain about 500,000 c.p.m./ml at a concentration of 0.05 M. 0.1 ml of this 0.05 M K-PP^{32} (5 μmoles) and 0.5 ml of a mixture containing 10 μmoles KF; 50 μmoles tris Cl, pH 7.5; 10 μmoles K-ATP, pH 7.5; 5 μmoles $MgCl_2$ and 2 μmoles of L-amino acid (usually L-tyrosine) were added to a 12 ml conical centrifuge tube. Enzyme and water to make a final volume of 1 ml were added to initiate the reaction. Incubations were for 10 minutes unless otherwise indicated. The reactions were stopped by the addition of 1 ml 12% TCA to the tube placed in ice. 2 ml 6% TCA were subsequently added and if an appreciable precipitate was obtained, the tubes were centrifuged in a clinical centrifuge until clarified.

The supernatant was then decanted to 12 ml centrifuge tubes containing about 300 mg Norit A and stirred to allow adsorption of the ATP to the charcoal. The stirring rods were removed with the addition of a few drops of alcohol to insure wetting of the charcoal, after which the charcoal precipitate containing ATP was obtained by centrifugation in the clinical centrifuge and decantation of the supernatant. The charcoal was then washed 4 times (suspension and centrifugation) with 2 ml aliquots of water.

2 ml 1 N HCl were added to the charcoal (now freed of unbound PP^{32} as a result of the washing); the tubes were boiled for 10 minutes, and after cooling, 2 ml of water were added. The charcoal was sedimented by centrifugation and the supernatant containing the 2 terminal phosphates hydrolyzed from the bound ATP was decanted into 10 ml calibrated tubes. The charcoal precipitates were then each washed with a 4 ml aliquot of water; these aliquots were combined with the original extracts and the volumes of the combined extracts and washes were made up to 10 ml with water. After good mixing of each sample to assure even distribution of counts, 0.5 ml from each sample was plated on stainless steel planchets, dried and counted in a thin window gas flow counter (Model D 47, Nuclear Chicago Corp., Des Plaines, Ill.). The inorganic phosphate present in each 10 ml sample was determined by the method of Fiske and Subbarow (43), modified with the use of

Elon as described in (48). A unit of enzyme activity is 1 μmole of PP^{32} exchanged per hour. Specific activity is expressed in units per mg of protein.

c. Calculations

The counts obtained from the 0.5 ml aliquot (after subtraction of background) were multiplied by 20 to obtain the total counts per 10 ml. This value, the results of the inorganic phosphate determination, and a knowledge that 10 minutes boiling in 1 N acid releases only 2 moles of Pi per mole of ATP were then used to calculate the counts per minute per 10 μmoles of ATP. This allowed the calculation of the "fraction exchanged," given by the formula:

$$\text{fraction exchanged} = \frac{\text{c.p.m. per 10 } \mu\text{moles ATP}}{2/3 \times \text{c.p.m. in initial 5 } \mu\text{moles } PP^{32}}$$

By definition, there is 100% exchange when there is complete equilibration of inorganic PP with the terminal PP in the ATP. Such equilibration would place 2/3 of the counts originally in 5 μmoles of PP into the 10 μmoles of ATP in this assay. Hence the denominator in the above equation.

The "fraction exchanged" does not take into account the chances that some of the label from ATP may be re-exchanged back into PP. The following formula, or coincidence correction (23,124) was used to obtain values of the actual μmoles of PP exchanged:

$$\mu\text{moles exchanged (actual exchange)} = -7.67 \log (1 - \text{fraction exchanged})$$

3. Incorporation of radioactive amino acids to soluble RNA

The assay is based on the method of Holley et al. (67). Each reaction mixture contained 0.05 ml 0.05 M K-ATP pH 7.5, (2.5 μmoles); 0.05 ml 0.01 M K-EDTA, pH 7.0, (0.5 μmoles); 0.05 ml 0.1 M $MgCl_2$ (5 μmoles) in 0.5 M tris Cl, pH 7.5) (25 moles); 0.1 ml 0.0005 M C^{14} amino acid (usually L-tyrosine*) (0.05 μmoles, about 40,000 c.p.m. per assay tube), and sufficient enzyme and water to bring the volume to 1 ml. The reaction was started by the addition of enzyme to the incubation tube in the 37°C bath, and allowed to proceed for the lengths of time indicated in individual experiments.

The reaction was stopped by placing the tubes in an ice bucket and adding 0.3 ml of cold 2% casein, pH 7.0, and 3 ml of cold 0.6 M $HClO_4$. After stirring, followed by centrifugation in the clinical centrifuge (maximum speed, 3-5 minutes), the supernatant was decanted and discarded. The precipitate was washed (at room temperature, by resuspension and centrifugation), twice with cold 0.2 M $HClO_4$, once with 4 ml cold 95% ethanol, 0.2 M $HClO_4$ (5.1), once with cold 95% ethanol and once with 95% ethanol-ether (3:1). The washed precipitate was then (in the order indicated) air dried, dissolved in 1 ml of 0.1 M NH_4 acetate, NH_4OH pH 9.0, treated with 0.1 ml of 0.05 mg/ml pancreatic RNAase at toom temperature for 15 minutes, and reprecipitated with the addition of 0.6 ml of a

---

*Obtained from Nuclear Chicago Corp.

1:1 mixture of 95% alcohol--2.5 M acetic acid. This suspension was then centrifuged and the supernatant containing the amino acids and nucleoside amino acids released by the alkali and RNAase, was plated on planchets, dried and counted in the thin window gas flow counter (Nuclear Chicago Corp.). The results of equilibrium studies are expressed in mμmoles of amino acid incorporated per mg SRNA present. Rate values are expressed in mμmoles of amino acid incorporated per minute or mμmoles of amino acid incorporated per hour. Specific activity is expressed in mμmoles of amino acid incorporated per hour per mg of protein.

4. Assay for spleen and snake venom phosphodiesterase activity

The spleen phosphodiesterase preparation used (Worthington Biochemical Co., Freehold, N. J.) is that of Hilmoe (54). 3 ml of glass distilled water were added to a vial containing 15-20 enzyme units (54) of lyophilized enzyme. Such enzyme solutions retained their activity when stored at -15°C, therefore the same vial was often used in several experiments.

Various amounts of G-75 Sephadex treated yeast SRNA were dissolved in 0.1 M K succinate buffer pH 6.7, and different amounts of enzyme were added. The mixtures were incubated in a water bath at 37°C, with samples removed at different times for assay.

When the extent of hydrolysis was measured, samples (usually 0.5 ml) of the incubated mixture were added to 0.5 ml 0.6 M $HClO_4$ and the precipitates were removed by centrifugation (10 minutes, 10,000 x G) and decantation. The supernatants were then titrated to pH 6.7 (pH paper) with 1 N KOH and after removal of the precipitated $KClO_4$ by centrifugation and decantation, the volume and the O.D. at 260 mμ of each supernatant was determined.

The per cent hydrolysis of the SRNA by spleen phosphodiesterase was calculated in the following manner. A known amount of SRNA was completely hydrolyzed to nucleotides by incubation for 48 hours at 37°C in 1 N KOH. The mixture was then titrated with 0.6 M $HClO_4$ to pH 6-7, the $KClO_4$ eliminated by centrifugation and decantation, and the O.D. at 260 mμ of the supernatant determined. Assuming a molecular weight of 27,600 and a chain length of 80 nucleotides for the SRNA, an extinction coefficient of 9.88 O.D. units per μmole/ml of RNA nucleotide was calculated, and 2.9 μmoles of nucleotides per mg of SRNA should be obtained with 100% hydrolysis. Knowing the O.D. at 260 mμ, the volume and amount of SRNA present per tube, the number of μmoles of nucleotide released per mg of SRNA could be calculated in the samples from diesterase assay. The value obtained from a sample not treated with diesterase was substracted and the percent hydrolysis calculated.

The amount of amino acid incorporating ability in SRNA partially hydrolyzed by spleen phosphodiesterase was determined by first, stopping enzymatic hydrolysis of SRNA with 5 minutes treatment at 100°C. This caused a complete loss of the diesterase activity but only slightly affected the ability of the SRNA to incorporate amino acids. The incorporation ability (i.e., the extent of amino acid incorporation at equilibrium) was then measured directly on the boiled SRNA samples containing the denatured diesterase. The percent of incorporation ability recovered was determined by comparison with the incorporation ability of untreated but boiled (5 minutes) SRNA.

Snake venom phosphodiesterase treatments of SRNA were similar to those described above using spleen phosphodiesterase. 0.5 mg/ml or 1 mg/ml stock solutions of purified snake venom phosphodiesterase (75,132) (obtained from Worthington Biochemical Co.) were used in the assays in the presence of SRNA in 0.1 M tris Cl buffer pH 7.5.

5. Density gradient centrifugation

The sucrose density gradient centrifugation procedure used is basically that described by Martin and Ames (87).

The apparatus to prepare the gradient is based on that described by these authors (87) except that the outflow tube is a No. 20 needle. 4.6 ml of a gradient (5% sucrose to 20% sucrose, all in 0.05 M tris Cl pH 7.5) was added to No. 5050 Spinco lusteroid-cellulose tubes over a 5-6 minute period,

after which the tubes were allowed to sit at 0-4°C for 4 hours. 0.1 ml of the sample to be analyzed (lacking sucrose) was carefully layered on the top of the gradient; then the tubes were centrifuged at 3°C in the SW 39 head of the Spinco model L ultracentrifuge at approximately 38,000 R.P.M. The time of centrifugation was generally 12 hours.

A special device consisting of a greased metal cup with a needle center connected to a No. 20 needle plugged with a thin wire was used to puncture the bottoms of the assay tubes containing the samples. With subsequent removal of the wire and concommitant draining of the tube, 30 or 31 fractions of 12 drops each were collected. Under these conditions the distance traveled by the meniscus when 1 drop is removed (using a No. 20 needle) is $9.45 \cdot 10^{-3}$ cm (47).

This method was used to estimate the sedimentation coefficient and molecular weight of the pig pancreas tyrosine activating enzyme, and to estimate the purity of the yeast SRNA used in these studies. Beef liver catalase and rabbit muscle lactic dehydrogenase were used as standards.

Catalase activity was determined by measuring the decrease in O.D. at 240 mμ with the Zeiss spectrophotometer in a mixture containing 2 ml 0.05 M tris Cl buffer pH 7.5, 1 ml 0.018 M H_2O_2 and 0.05 ml of enzyme. Before addition of the enzyme, the O.D. scale of the spectrophotometer was arbitrarily set at 0.3 for the reaction mixture. O.D. readings were taken

every 15 seconds. The enzymatic activity was expressed as the decrease in O.D. at 240 mμ per minute.

Lactic dehydrogenase activity was determined by measuring the increase in O.D. at 340 mμ in a reaction mixture containing 1 ml 0.1 M sodium veronal buffer pH 8.6, 1 ml 6 x 10^{-4} M DPN, 1 ml 0.33 M sodium lactate and 0.05 ml of enzyme. O.D. readings were taken every 15 seconds and the enzymatic activity was expressed as the increase in O.D. at 340 mμ per minute.

Tyrosine activating enzyme activity was measured with the PP exchange reaction (see page 35). The SRNA concentration was determined by measuring the O.D. at 260 mμ of the unknown samples.

6. Protein determination

In all instances protein determinations were performed according to the spectrophotometric or 280/260 ratio method of Warburg & Christian (125) as described in (78). In some experiments, the Folin-Ciocalteu method (78) was used to confirm protein determinations obtained with the Warburg & Christian method, giving results 10-20% lower than those obtained with the spectrophotometric procedure. BSA was used as a standard in the Folin-Ciocalteu assays and may account for the slight discrepancies observed.

7. Electrophoresis with cellulose acetate strips

Purified tyrosine activating enzyme was concentrated up to 1 mg protein per ml by placing a dialysis bag containing the enzyme in a Biodryex* bed in the cold room for 2-5 hours. 20-30 µg of the concentrated enzyme were put on moist 18 x 5 cm cellulose acetate strips (Consolidated Laboratories Inc., Chicago Heights, Ill.). After the protein application, the samples were submitted to electrophoresis in 0.05 M potassium phosphate buffer at pH's 5.7, 8.0, or 9.0 for 5 hours with a current of 0.4 m A per cm width (approximately 150 Volts) at room temperature. The strips were then stained overnight in 0.01% nigrosin in 2% acetic acid, followed by removal of excess dye with repeated water washing.

8. Separation of the RNAase hydrolysis products of C^{14} tyrosine SRNA with high voltage electrophoresis

20 mg of SRNA were labelled with highly radioactive C^{14} L-tyrosine (400,000 cpm per 0.05 µmoles) as described on page 39, without adding casein when stopping the incorporation reaction, and drying the product with air after all the washings. This C^{14} tyrosine SRNA was resuspended in 0.5 ml of 0.001 M NH_4OOCH, pH 5.0, using 0.1 N NH_4OH to titrate the excess acid. 0.05 ml of a 2 mg/ml solution of RNAase was added; the mixture was incubated for 30 minutes at room temperature and then pipetted onto a 0.5 x 3 cm Dowex 1-X8

*Obtained from Svenska Cellulosa Aktiebolaget, Kemiska Produkter, Essvik, Sweden.

-OOCH (200-400 mesh) column. The column was washed with water and then eluted with 0.005 N HCOOH. This eluate was lyophilized to dryness; dissolved in a small volume of water (~0.05 ml) and run on high voltage paper electrophoresis (2 hours, 1,200 Volts) on 60 x 11 cm Whatman No. 1 filter paper strips in 0.005 M NH_4OOCH buffer pH 5.0 according to the method of Markham & Smith (86). C^{14} tyrosine, AMP and adenosine were used as standards. After electrophoretic separation, the paper was dried and the radioactivity (measured with a Vanguard model 800 strip scanner, Vanguard Instrument Co., La Grange, Ill.) and U.V. quenching of the test sample was compared with the standards on the same paper strip.

9. Hydroxylamine trapping of C^{14} tyrosine from C^{14} tyrosine SRNA

C^{14} tyrosine was incorporated into 4 mg SRNA by the procedure described on page 39, without addition of casein. The dried, labeled SRNA was resuspended in 0.1 ml of cold water, and 0.1 ml of 3.0 M salt free hydroxylamine pH 7.5 was added. The mixture was incubated 5 minutes at 30°C and then 0.2 ml of 1:1 95% ethanol 2.5 M acetic acid were added. After cooling down in ice, the precipitate was centrifuged down and the supernatant was removed and lyophilized to dryness. The dry material obtained was resuspended in 0.5 ml of water and lyophilized again. The sample was redissolved in 0.05 ml of water and spotted on Rohm & Haas SA -2 ion exchange paper.

Tyrosine C^{14} was used as a control. The paper was developed with 0.05 M K-phosphate buffer, pH 7.0, in ascending direction, dried and counted in a Vanguard strip scanner. This procedure is a modification of that described by Loftfield & Eigner (83).

CHAPTER III

EXPERIMENTAL RESULTS

A. Properties of the Purified Tyrosine Activating Enzyme from Hog Pancreas

1. Enzyme purification

Table I shows the results obtained in the purification of the enzyme. The specific activities of the acetone powder extract are, at best, approximations, for they are obtained from an impure preparation that contains considerable amounts of endogenous free amino acids.

The specific activity for Fraction VI of 132 in the hydroxamate assay represents a value obtained from a mixture of fractions whose specific activities range from 100-147. This represents a purification of almost 5 fold over that reported by Schweet and Allen (106) using the same assay. This also represents a major advance in the availability of highly purified activating enzymes, for only two others, the tryptophan activating enzyme of beef pancreas (32) and the alanine activating enzyme of hog liver (127) have been purified to this range of specific activity.

A comparison of the purity of these different enzymes is difficult because the data reported for the specific activities of the alamine and tryptophan enzymes are not complete.

Table I

Purification of tyrosine-activating enzyme

Fraction	Vol.	Protein Content	Specific Activity			Hydroxamate Total Activity	Yield
			Hydroxamate Assay	PP Exchange	Tyrosine Incorp.		
	ml.	mg/ml	units/mg	units/mg	units/mg	units	%
I. Acetone powder extract (unclarified)	1250	47.6	0.012	0.17	--	700	100
II. $Ca_3(PO_4)_2$ gel eluate	1160	0.21	2.15	40	--	512	73
III. $(NH_4)_2SO_4$	55	1.60	5.87	54	0.61	517	74
IV. pH 5.3 supernate	53	0.84	9.55	75	0.96	425	61
V. DEAE column eluate	56	0.12	56.0	485	5.06	376	54
VI. $Ca_3(PO_4)_2$ column eluate	23	0.10	132	886	9.00	293	42

The values given for Fraction I are those obtained after subtraction of values obtained without added tyrosine. Endogenous activity in all assays in Fractions II-VI is negligible.

The alanine enzyme has been obtained to a specific activity of 6840 μmoles PP exchanged per hour per mg protein (127), while a value of 86 μmoles of hydroxamate per hour per mg protein is reported for the tryptophan enzyme (32). Since there is no parallelism between pyrophosphate exchange and hydroxamate activity in different activating enzymes (56), this makes a comparison of their specific activities difficult. The tyrosine activating enzyme reported here represents the most thoroughly characterized purified activating enzyme available.

Table I also shows that the ratio of specific activities for hydroxamate formation, PP exchange and tyrosine incorporation remain nearly constant during the purification. This supports the idea that the same enzyme catalyzes both the activation of an amino acid and its subsequent transfer to SRNA.

2. Enzyme purity

In a successful purification starting with 150 g of acetone powder, 0.8 mg of purified enzyme with a hydroxamate specific activity over 140 can be obtained. Determinations of the purity of the enzyme by use of boundary electrophoresis requires at least 20 mg of protein per run and an ultracentrifugation run with schlieren optics a minimum of 5 mg. Due to the lack of sufficient protein for such studies, electrophoresis on cellulose-acetate was used (see METHODS).

Experiments performed at pH's 5.7, 8.0 and 9.0 reveal the presence of only one component (Fig. 4), with an isoelectric point of approximately pH 7.

Fractions of the tyrosine enzyme with a specific activity in the hydroxamate reaction of over 100 are free of nuclease activity, for prolonged preincubation of SRNA with such enzyme fractions do not interfere with the ability of the SRNA to incorporate C^{14} tyrosine (Table II). Such fractions are also free of adenylic kinase, for they fail to exchange C^{14} AMP into ATP (Table XIV).

3. Stability of the enzyme

The purified tyrosine enzyme is considerably more stable than both the alanine and tryptophan enzymes. For example, half of the activity can be recovered when the enzyme is kept at -15°C for two months. Freezing in liquid nitrogen does not further stabilize the activity nor does the presence of 10^{-3} M glutathione, substrates or cofactors (Table III). Bovine serum albumin protects the enzyme and is therefore often added to purified enzyme fractions that are subsequently used in activity assays. The presence of 10^{-3} M redistilled mercaptoethanol causes a considerable loss in activity (see Table III).

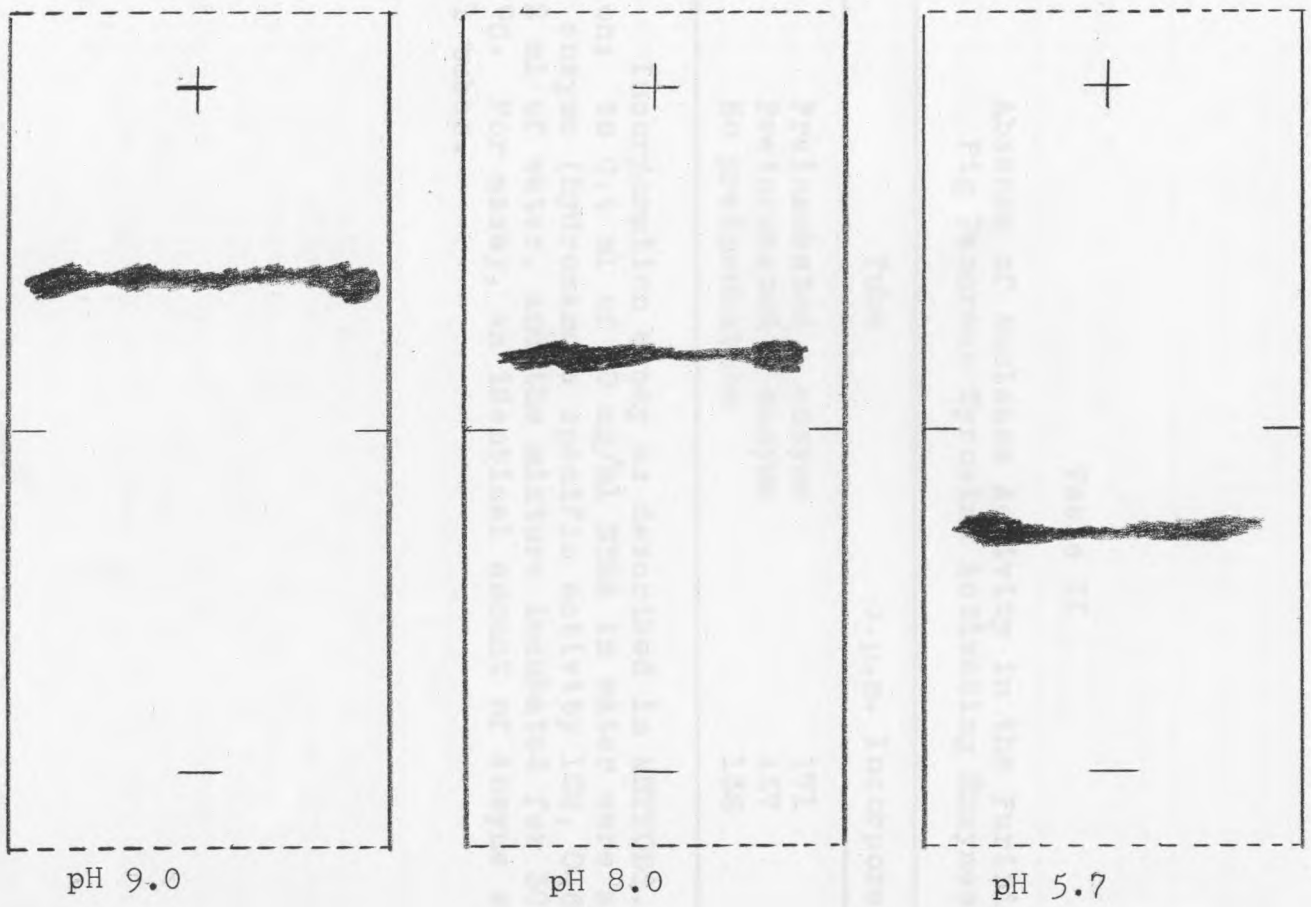


Figure 4. Tracing of Cellulose Acetate Electrophoresis Runs of the Purified Pig Pancreas Tyrosine Activating Enzyme.

Procedure as described in "Methods". 30 μg of enzyme of a specific activity of 143 (hydroxamate) used in each experiment.

Table II

Absence of Nuclease Activity in the Purified Pig Pancreas Tyrosine Activating Enzymes

Tube	c.p.m. Incorporated
Preincubated + enzyme	171
Preincubated - enzyme	157
No preincubation	138

Incorporation assay as described in METHODS. Preincubation: to 0.4 ml of 10 mg/ml SRNA in water were added 0.2 ml of enzyme (hydroxamate specific activity 102, 0.62 mg/ml) or 0.2 ml of water, and the mixture incubated for 30 minutes at 37°C. For assay, an identical amount of enzyme was added to all tubes.

Table III

Stability of the Purified Tyrosine Activating Enzyme in the Presence or Absence of Several Reagents

Enzyme	μmoles Hydroxamate/hr		
	Before Freezing	1 Day After Freezing	9 Days After Freezing
No Additions	0.90	0.87	0.47
Mercapto-ethanol	0.88	0.30	0.15
Glutathione	0.95	0.73	0.65
Tyrosine Mg^{++}, ATP Tris-Cl	0.89	0.85	0.62
BSA 0.5 mg/ml	1.12	1.18	0.83
BSA 5.8 mg/ml	1.21	1.34	0.81

Incorporation assay as described in METHODS. The reagents listed were added to samples of the enzyme preparation and the final concentrations were: mercaptoethanol, 10^{-3} M; glutathione, 10^{-3} M; ATP, 10^{-2} M; $MgCl_2$, 10^{-3} M; tyrosine, 5×10^{-4} M; Tris-Cl pH 7.5, 5×10^{-3} M. 0.2 ml of enzyme (hydroxamate specific activity 70, 0.09 mg/ml) were used in every assay. The enzyme samples were frozen in acetone-dry ice and kept at $-15^{\circ}C$.

4. Presence of nucleotide material

The purified tyrosine activating enzyme contains no detectable amount of nucleotide material, for the 280/260 ratio is 1.8. This is in contrast to both the highly purified tryptophan (32) and alanine (127) activating enzymes which contain approximately 4% nucleotide material even in their most purified state. The contaminating nucleotide material of the hog pancreas preparations is largely removed during the DEAE fractionation for material prior to this stage has a 280/260 ratio of 0.7-0.9 while aliquots obtained from the DEAE fractionation have a 280/260 ratio of over 1.5.

5. Estimation of the molecular weight and turnover number

Precise molecular weight determinations by sedimentation velocity-diffusion or sedimentation equilibrium require considerably larger amounts purified enzyme than were available in these studies. Therefore, the less precise sucrose density gradient centrifugation procedure (87) which offers the advantage of enzyme detection by means of enzyme activity, was used (see METHODS).

Beef liver catalase (M.W. 248,000) (39) and rabbit muscle lactic dehydrogenase (M.W. 130,000) (131) were used as standards (Figure 5). Table IV shows the values obtained for the molecular weight and sedimentation coefficient of the pig pancreas tyrosine activating enzyme.

Figure 5. Sucrose Density Gradient Centrifugation Pattern of the Pig Pancreas Tyrosine Activating Enzyme.

Procedure as described in "Methods". 0.1 ml 0.01 M tris-Cl pH 7.5, containing 35 µg tyrosine activating enzyme (hydroxamate specific activity 100, 1 mg/ml), 8 µg beef liver catalase and 100 µg rabbit muscle lactic dehydrogenase placed on top of the gradient before centrifugation. Catalase and lactic dehydrogenase were obtained from Sigma Chemical Co.

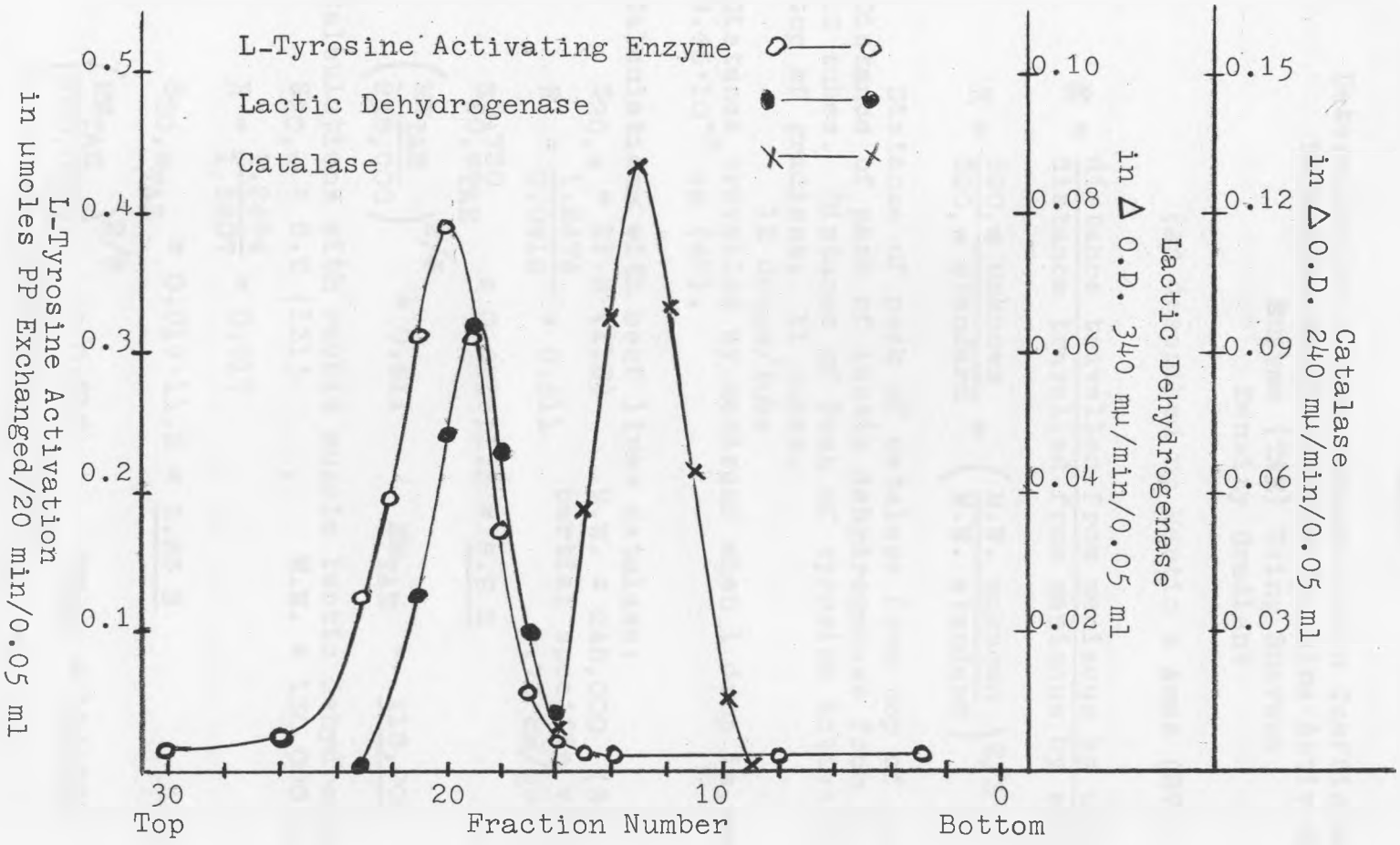

L-Tyrosine Activating Enzyme
Lactic Dehydrogenase
Catalase
L-Tyrosine Activation
in µmoles PP Exchanged/20 min/0.05 ml
Lactic Dehydrogenase
in Δ O.D. 340 mµ/min/0.05 ml
Catalase
in Δ O.D. 240 mµ/min/0.05 ml
0.5
0.4
0.3
0.2
0.1
0.10
0.08
0.06
0.04
0.02
0.15
0.12
0.09
0.06
0.03
30
20
10
0
Top
Fraction Number
Bottom

Table IV

Determination of the Sedimentation Coefficient and Molecular Weight of the Tyrosine Activating Enzyme (TAE) Using Sucrose Density Gradient

(As described by Martin & Ames (87))

$$K = \frac{\text{distance travelled from meniscus by unknown}}{\text{distance travelled from meniscus by standard}}$$

$$R = \frac{S20,w \text{ unknown}}{S20,w \text{ standard}} = \left(\frac{\text{M.W. unknown}}{\text{M.W. standard}}\right)^{2/3}$$

Distance of peak of catalase from top of gradient: 18 tubes
Distance of peak of lactic dehydrogenase from top of gradient: 12 tubes. Distance of Peak of tyrosine activating enzyme from top of gradient: 11 tubes.

12 drops/tube

Distance travelled by meniscus when 1 drop is removed: $9.45 \cdot 10^{-3}$ cm (47).

Calculations with beef liver catalase:

$S_{20,w} = 11.3$ (118) M.W. = 248,000 (39)

$R = \frac{1.2474}{2.0412} = 0.611$ partial specific volume: 0.73 cm/gr (118)

$S^{0.730}_{20,w_{TAE}} = 0.611 \cdot 11.3 = \underline{6.9\ S}$

$\left(\frac{MW_{TAE}}{248,000}\right)^{2/3} = 0.611 \qquad MW_{TAE} = \underline{118,000}$

Calculations with rabbit muscle lactic dehydrogenase:

$S_{20,w} = 6.9$ (131) , M.W. = 130,000 (31)

$R = \frac{1.2474}{1,3607} = 0.917$

$S_{20,w_{TAE}} = 0.917 \cdot 11.3 = \underline{6.33\ S}$

$\left(\frac{MW_{TAE}}{130,000}\right)^{2/3} = 0.917 \qquad MW_{TAE} = \underline{114,000}$

Several limitations have to be kept in mind when using this procedure. First, a true $S_{20,w}$ value (and hence, M.W.) can only be obtained if the partial specific volumes are known. The partial specific volume for catalase is 0.730 (118), but those of the other enzymes used here are unknown. For the calculations, the assumption is made that the partial specific volumes of standard and unknown are the same. This may account for part of the differences observed between the values obtained with the two standards. According to Martin & Ames (87), since most proteins have a partial specific volume in the range of 0.700-0.750, the errors will probably be small. Second, the validity of the formula

$$\frac{S_1}{S_2} = \left(\frac{MW_1}{MW_2}\right)^{2/3}$$

is dependent on the assumption that all the proteins in question are spherical in shape, an assumption not proven in this instance. Third, inaccuracies in the reported molecular weights and sedimentation coefficients of the standards, will influence the values obtained.

Using the value 118,000 as the molecular weight of the enzyme, the turnover numbersof the most purified fraction, Fraction VI are 1740 and 17.7 for the PP exchange and the L-tyrosine incorporation reactions respectively (see Table V). The values are small when compared to those of many other enzymes (38). The low turnover numbers calculated may reflect, in part, the presence of protein impurities in the

Table V

Calculation of the Turnover Number of the Tyrosine Activating Enzyme

1. M.W. of the enzyme: 118,000 (Table IV)

2. 1 mg Enzyme = $\frac{1{,}000{,}000}{118{,}000{,}000}$ = 0.0085 μmoles

A. Turnover number for the PP exchange reaction:
Specific Activity = 886 μmoles PP exchanged per hour per mg protein (Table I)

886 μmoles/hr = 14.8 μmoles/min

turnover number = $\frac{14.8}{0.0085}$ = 1740 μmoles exchanged per minute per μmole of enzyme

B. Turnover number for C^{14} tyrosine incorporation:
Specific Activity = 9.0 μmoles incorporated per hour per mg of protein (Table I)

9.0 μmoles/hr = 0.15 umoles/min

turnover number = $\frac{0.15}{0.0085}$ = 17.7 μmoles incorporated per minute per μmole of enzyme

enzyme preparation, for Fraction VI represents a combination of aliquots with varied specific activities. Nevertheless, the enzyme purity assays and the total degree of purification dictate that the turnover numbers for this enzyme are relatively low.

6. Substrate specificity

The purified activating enzyme shows a defined specificity for the activation of only certain L-amino acids. Thus, of the 18 naturally occurring amino acids tested, only tyrosine, and to a lesser extent phenylalanine, and possibly threonine support the PP exchange into ATP (Table VI).

No studies were made to determine the cause of the slight threonine-dependent exchange. The observed L-phenylalanine dependent PP exchange into ATP is not due to contamination of the L phenylalanine with traces of L-tyrosine, for the phenylalanine used in these experiments was shown to be tyrosine-free by paper chromatography in a solvent that effectively separates tyrosine from phenylalanine (N butanol saturated with 2 N NH_4OH), and by the use of the amino acid analyzer. Phenylalanine chromatographed in the above solvent showed identical activity than non-chromatographed material.

The structural similarities of phenylalanine and tyrosine suggest that the L-phenylalanine and L-tyrosine dependent exchanges are catalyzed by the same enzyme. A Lineweaver-Burk analysis (81) of these amino acid dependent exchanges (Figure 6)

Table VI

Substrate Specificity of the Purified Pig Pancreas Tyrosine Activating Enzyme, Measured with the PP Exchange Reaction

Amino Acid	μmoles PP Exchanged/hr	% of Tyrosine Activity
L-tyrosine	6.36	100%
L-glycine	--	
L-alanine	--	
L-leucine	--	
L-isoleucine	--	
L-valine	--	
L-serine	--	
L-threonine	0.161	2%
L-phenylalanine	0.943	15%
L-tryptophan	--	
L-glutamic acid	--	
L-aspartic acid	--	
L-proline	--	
L-lysine	--	
L-histidine	--	
L-arginine	--	
L-cysteine	0.003	
L-methionine	--	

0.5 ml 0.02 M amino acids (except tyrosine, 0.01 M) used in the assays, with 0.2 ml enzyme (hydroxamate specific activity 120; 0.08 mg/ml). All amino acids obtained from California Corporation for Biochemical Research, Los Angeles, California.

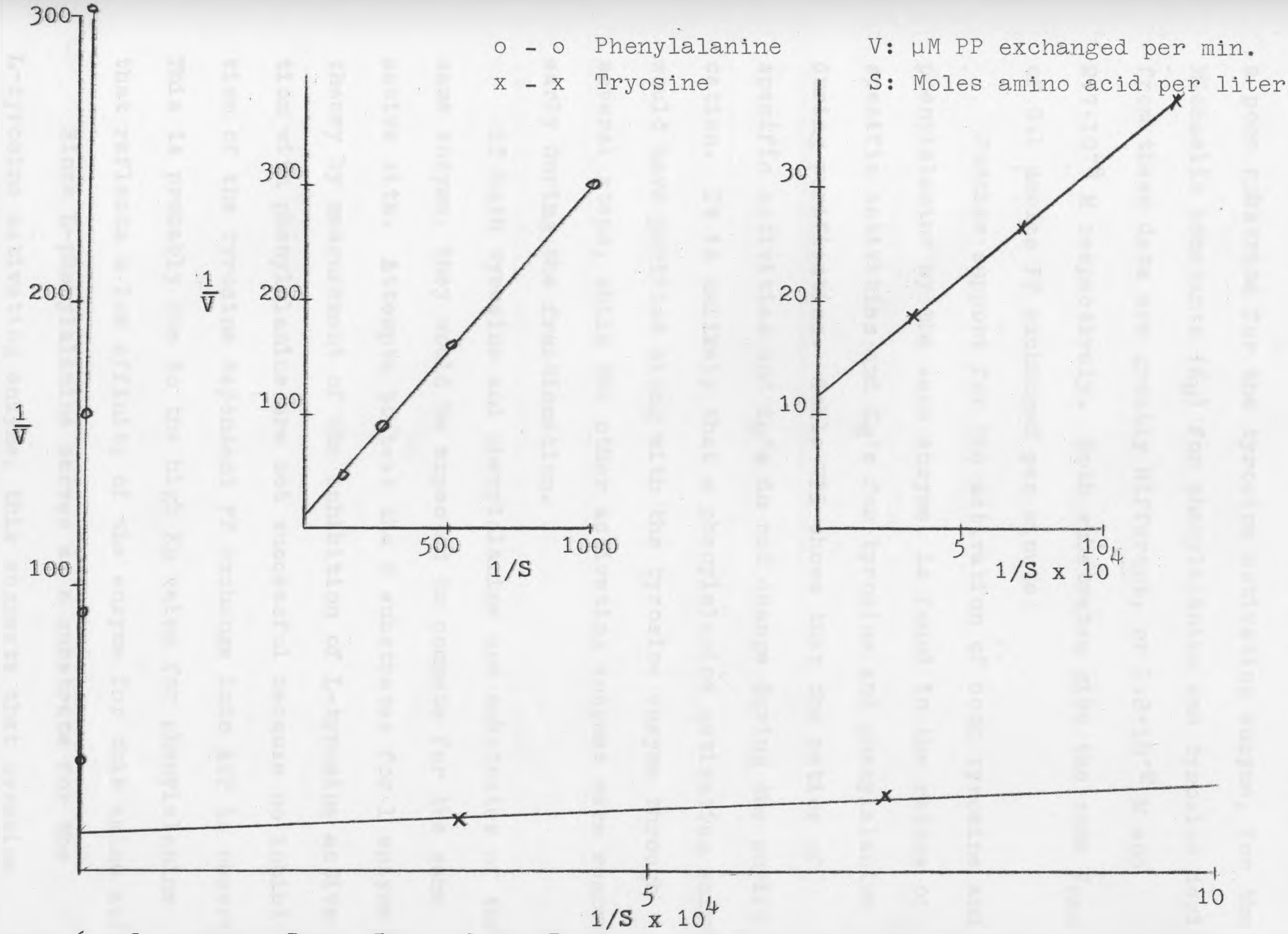


Figure 6. Lineweaver-Burk Plot of the Tyrosine and Phenylalanine Dependent PP Exchange.

Standard assay conditions (see Methods). 0.1 ml enzyme (hydroxamate specific activity 106, 0.055 mg/ml) used.

supports this hypothesis. As seen, phenylalanine is, at best, a poor substrate for the tyrosine activating enzyme, for the Michaelis constants (K_M) for phenylalanine and tyrosine derived from these data are greatly different, or $1.8 \cdot 10^{-2}$ M and $2.7 \cdot 10^{-5}$ M respectively. Both substrates give the same V_{max} of 0.1 μmoles PP exchanged per minute.

Further support for the activation of both tyrosine and phenylalanine by the same enzyme, is found in the ratios of specific activities and K_M's for tyrosine and phenylalanine during purification. Table VII shows that the ratios of specific activities and K_M's do not change during the purification. It is unlikely that a phenylalanine activating enzyme would have purified along with the tyrosine enzyme through several steps, while the other activating enzymes were removed early during the fractionation.

If both tyrosine and phenylalanine are substrates of the same enzyme, they would be expected to compete for the same active site. Attempts to test the 2 substrates for 1 enzyme theory by measurement of the inhibition of L-tyrosine activation with phenylalanine are not successful because no inhibition of the tyrosine dependent PP exchange into ATP is observed. This is probably due to the high K_M value for phenylalanine that reflects a low affinity of the enzyme for this amino acid.

Since L-phenylalanine serves as a substrate for the L-tyrosine activating enzyme, this suggests that tyrosine

Table VII

Relation Between the Specific Activities and K_M's of Tyrosine and Phenylalanine During Purification of the Pig Pancreas Tyrosine Activating Enzyme

Fraction	Specific Activity		Ratio	K_M	
	Tyrosine	Phenylalanine		Tyrosine	Phenylalanine
III	64.2	7.0	9.1	$1.7 \cdot 10^{-5}$M	$1.5 \cdot 10^{-2}$M
IV	80.4	16.2	5.0	--	--
V	321	96	3.3	--	--
VI	876	162	5.4	$2.7 \cdot 10^{-5}$M	$2.1 \cdot 10^{-2}$M

The enzymatic activity was measured using the PP exchange assay (see METHODS). Specific activity expressed in umoles PP exchanged per hour per mg protein.

analogs could be found that are substrates of the same enzyme. Test of this proposition, using various L-tyrosine analogs generally proved negative (Table VIII). The activity observed in the presence of chloroacetyl L tyrosine and tyrosine hydroxamate could be due to impurities. A 1% impurity of tyrosine (K_M of $2.7 \cdot 10^{-5}$ M) could account for the activity (from Table VIII, the concentration of a 1% impurity is 6×10^{-5} M). As a further test of the potential of various tyrosine analogs, some analogs were assayed for competitive inhibition of tyrosine dependent PP exchange (Table IX). Only tyramine shows considerable inhibitory power. These results indicate a high degree of specificity for the tyrosine activating enzyme.

7. Products of the incorporation reaction

In contrast to the less purified enzyme described by Schweet & Allen (106), the highly purified tyrosine activating enzyme obtained in this work is active in catalyzing the incorporation of tyrosine into SRNA. This incorporation activity requires the presence of a complete system (Table X) and is thus consistent with the mechanism of amino acid activation and transfer to SRNA.

Final characterization of the reaction requires identification of the products. As a result, several experiments were performed to identify these products. First, tyrosine C^{14} SRNA was treated with salt-free hydroxylamine at pH 7.2 and

Table VIII

Tyrosine Analogs as Substrates of the Tyrosine Activating Enzyme

Analog	μmoles PP Exchanged/Hr.	% Tyrosine Activity
D,L p-hydroxyphenylglycine*	0.030	0.25
D,L β-hydroxyphenylalanine**	0.030	0.25
D,L p-chlorophenylalanine***	0.069	0.57
D,L p-fluorophenylalanine*	0.080	0.67
p-hydroxyphenylacetic acid*	0.0035	0.03
L-thyroxine*	0.0040	0.03
chloroacetyl L tyrosine***	0.78	6.5
p-methoxy phenylacetic acid*	0	0
L-tyrosine hydroxamate*	2.13	17.8
L-tyrosine*	12	100

PP exchange assay as described in METHODS.

Concentration of tyrosine 4 μmoles/tube (4×10^{-3} M). At this concentration the exchange is independent of the tyrosine concentration.

Concentration of analogs: 6 μmoles/tube (6×10^{-3} M).

0.1 ml enzyme used per assay (specific activity 106 μmoles hydroxamate per hour per mg protein; 0.1 mg protein per ml).

*Obtained from California Corp. for Biochemical Research.

**Gift to Dr. John M. Clark.

***Obtained from Nutritional Biochemical Co., Cleveland, Ohio.

Table IX

Effect of Tyrosine Analogs as Inhibitors of the Tyrosine Dependent PP Exchange Catalyzed by the Purified Pig Pancreas Tyrosine Activating Enzyme

Expt.	Analog	µmoles PP Exchanged/Hr.	% Tyrosine Activity
A	D,L p-hydroxyphenylglycine	10.4	87
	β hydroxyphenylalanine	11.2	93.5
	D,L p-chlorophenylalanine	12.1	101
	D,L p-fluorophenylalanine	8.8	73
	p-hydroxyphenylacetic acid	10.6	88.5
	L-thyroxine	9.0	75
	chloracetyl L-tyrosine	10.9	91
	p-methoxyphenylacetic acid	11.4	95
	L-tyrosine hydroxamate	8.5	71
	L-tyrosine alone	12	100

In all tubes 2 µmoles of tyrosine and 6 µmoles of analog were added, 0.1 ml enzyme used (hydroxamate specific activity 106; 0.1 mg/ml protein).

B	3,4 dihydroxyphenylalanine (DOPA)	1.68	60
	tyramine	0.083	3.0
	tyrosine alone	2.8	100

In all tubes, 2 µmoles of tyrosine were used. 20 µmoles of DOPA and 2 µmoles of tyramine were added to their respective tube. 0.1 ml enzyme added (hydroxamate specific activity 126; 0.035 mg/ml).

PP exchange procedure as described in METHODS.

Table X

Requirements for L-tyrosine-C^{14} incorporation

Reactants	Counts/min/sample
1. Complete system (Enz, SRNA, ATP, Mg^{++}, L-tyrosine-C^{14})	401
2. Complete system minus enzyme	7
3. " " minus SRNA	2
4. " " minus ATP	8
5. " " minus Mg^{++}	37
6. " " using preboiled enzyme	7

Incorporation assay as described in METHODS. 0.1 ml enzyme used per tube (hydroxamate specific activity 140, 0.110 mg protein per ml). Reaction mixtures incubated for 10 minutes.

assayed by a modification of the method of Loftfield and Eigner (83) (see METHODS). Figure 7 shows that the addition of hydroxylamine to the tyrosine SRNA causes the formation of C^{14} tyrosine hydroxamate. The formation of this product is consistent with the existence of an ester bond between the amino acid and the SRNA.

Second, the products of RNAase treatment of tyrosine C^{14} SRNA were submitted to high voltage paper electrophoresis (see METHODS). The result of these runs (Figure 8), reveals a radioactive spot, containing U.V. absorbing material which moves towards the cathode, slightly ahead of adenosine. Tyrosine remains in the base line. This behaviour is what would be expected of a nucleoside amino acid, under the conditions described. No attempt was made to identify the nucleoside bound to tyrosine, but the results are in good agreement with those obtained by Zachau et al. (135), suggesting a terminal adenosine amino acid ester bond in SRNA.

8. Requirements for enzyme and SRNA in the transfer reaction

As seen in Table X, both activating enzyme and SRNA are essential in the incorporation reaction. Further, the time course of the incorporation reaction (Figure 9) follows the expected pattern; the rate of the reaction is directly dependent upon the enzyme concentration (Figure 10), and the extent of final equilibrium incorporation is independent of

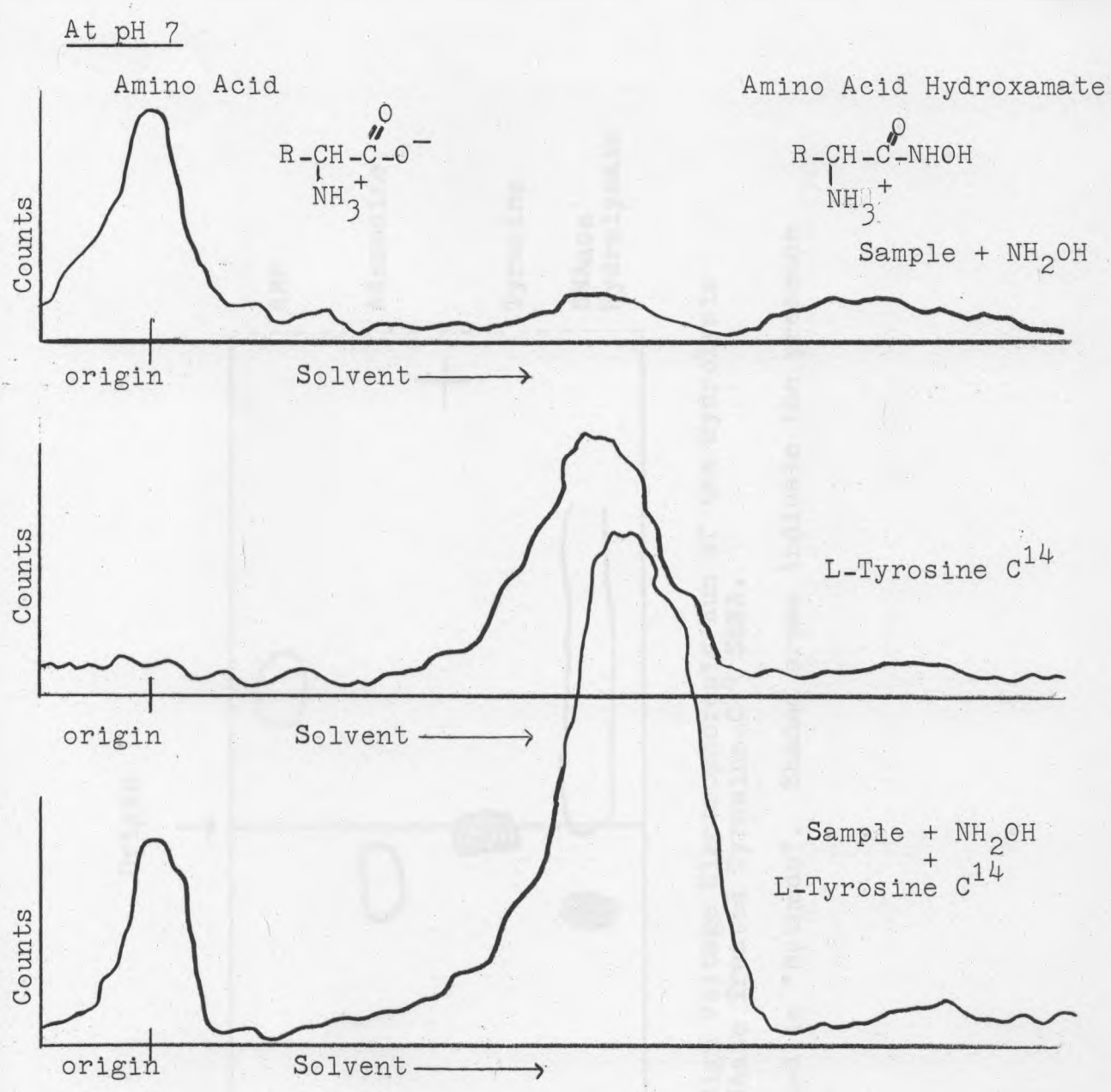


Figure 7. Scanning of Cation Exchange Paper Strips Developed in 0.05 M Phosphate Buffer pH 7.

Procedure as described in "Methods".

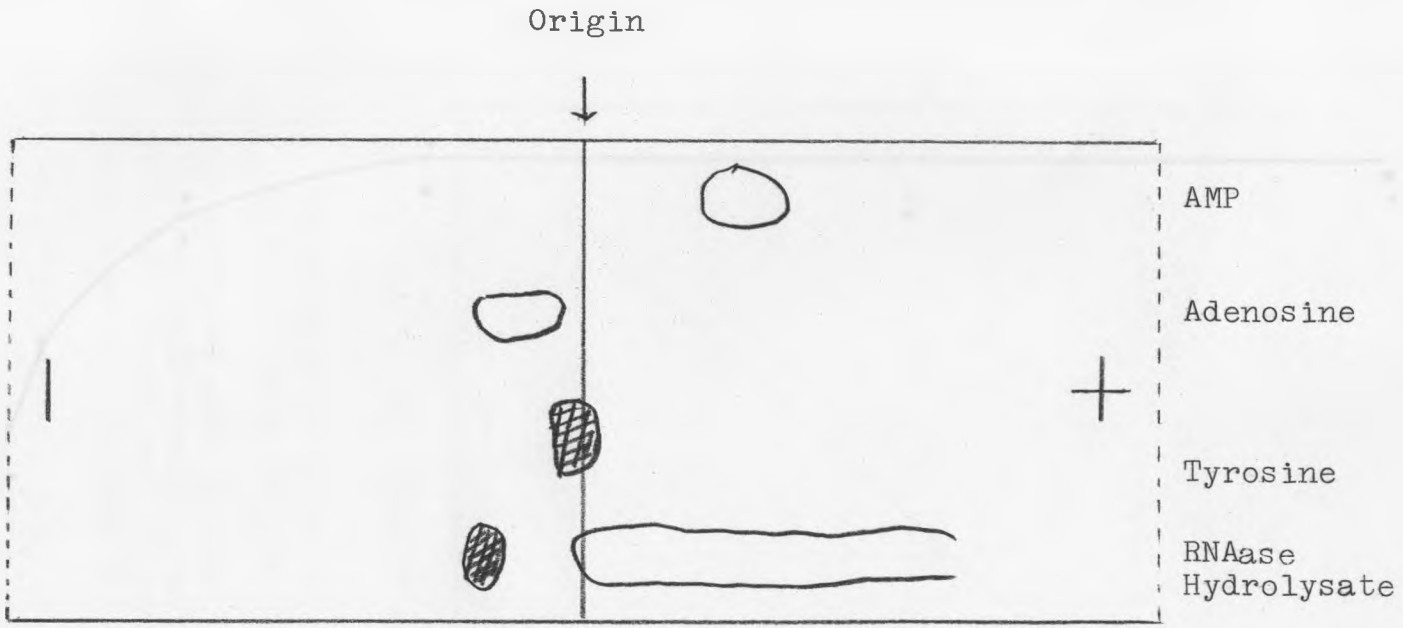


Figure 8. Tracing of a High Voltage Electrophoresis Run of the Hydrolysis Products of RNAase Treated Tyrosine-C^{14} SRNA.

Procedure as described in "Methods". Shaded areas indicate the presence of radioactivity.

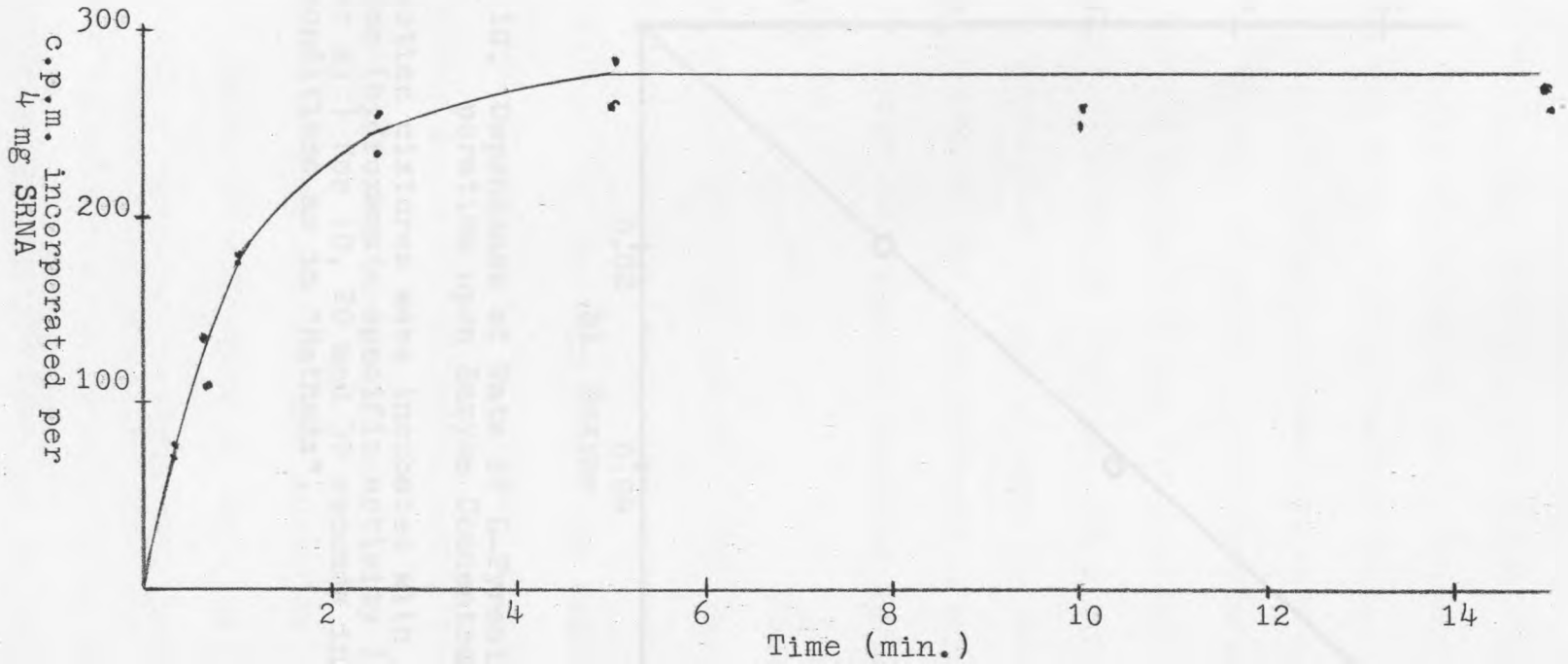


Figure 9. Time Course of the Incorporation of C^{14} Tyrosine into Yeast SRNA, Catalyzed by the Purified Pig Pancreas Tyrosine Activating Enzyme.

Procedure as described in "Methods", 0.1 ml of enzyme (hydroxamate specific activity 109, 0.03 mg of protein per ml used).

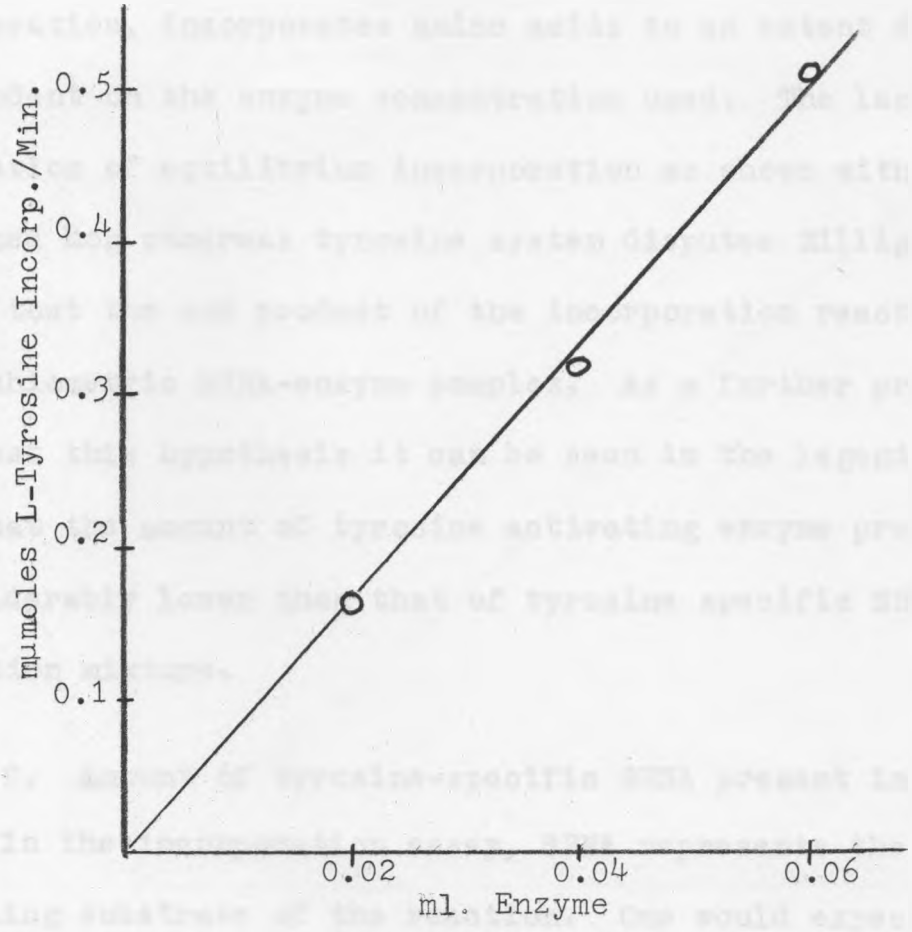


Figure 10. Dependence of Rate of L-Tyrosine C^{14} Incorporation upon Enzyme Concentration.

Reaction mixtures were incubated with increasing amounts of enzyme (hydroxamate specific activity 110, 0.08 mg. protein per ml) for 10, 20 and 30 seconds intervals. Other assay conditions as in "Methods".

the amount of enzyme used (Figure 11). These results are consistent with the known mechanism of amino acid activation and in contrast to the findings of Zillig et al. (139), who observed that an E. coli system, with a nucleotide-free enzyme preparation, incorporates amino acids to an extent directly dependent on the enzyme concentration used. The lack of variation of equilibrium incorporation as shown with this well defined hog pancreas tyrosine system disputes Zillig's hypothesis, that the end product of the incorporation reaction is a stoichiometric SRNA-enzyme complex. As a further proof against this hypothesis it can be seen in the legend of Figure 11 that the amount of tyrosine activating enzyme present is considerably lower than that of tyrosine specific SRNA in the reaction mixture.

c. Amount of tyrosine-specific SRNA present in yeast SRNA

In the incorporation assay, SRNA represents the ultimate limiting substrate of the reaction. One would expect the yeast SRNA used in this work to contain a heterogeneous population of molecules, i.e., different amino acid specific SRNA and an undetermined amount of "junk" RNA. Only a small fraction of this mixture are molecules specific for tyrosine and therefore substrate of the reaction under study.

The amount of tyrosine-specific SRNA present in the yeast SRNA of these experiments can be calculated by incubating known amount of SRNA with C^{14} tyrosine of known specific

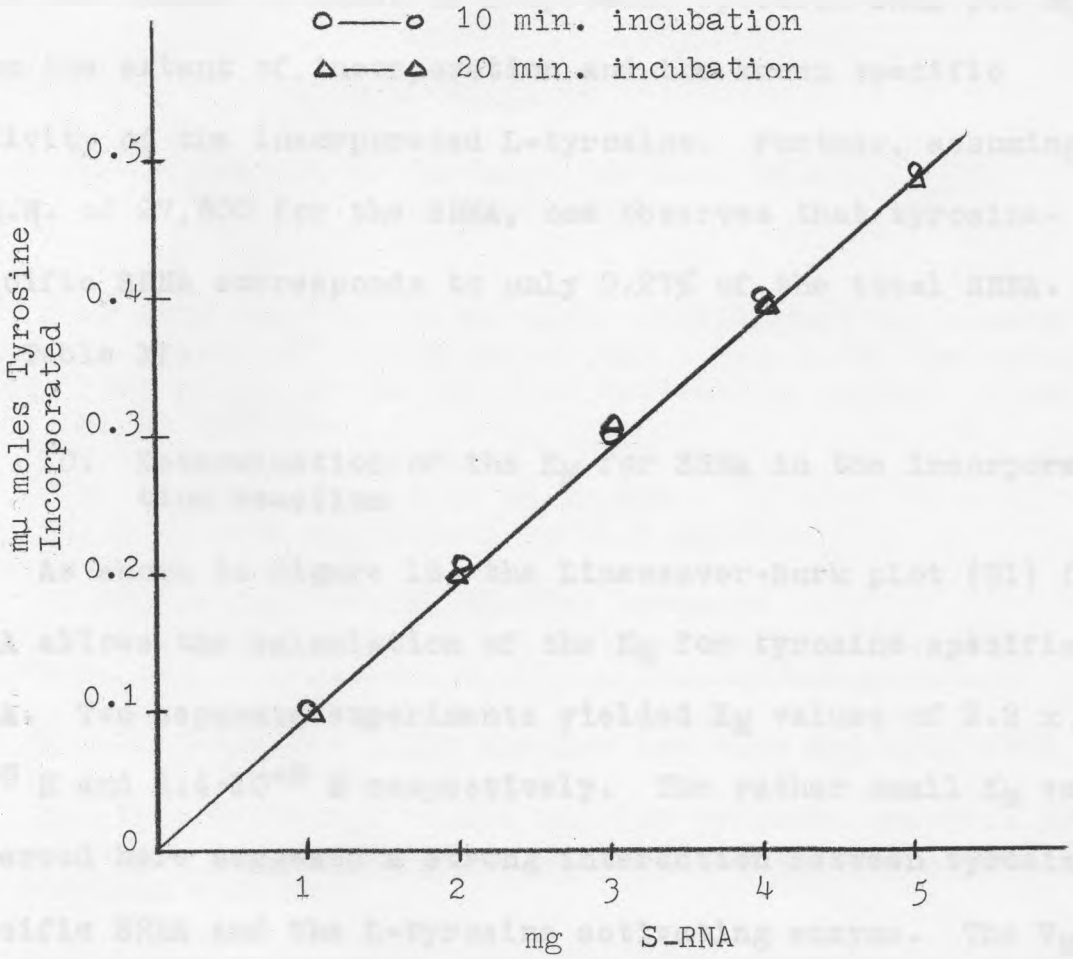


Figure 11. Dependence of L-Tyrosine-C^{14} Equilibrium Incorporation Upon the SRNA Concentration.

The reaction mixtures were incubated with 0.1 ml. of enzyme (hydroxamate specific activity 140, 0.078 mg. ml.) Procedure as described in "Methods".

With a M.W. of 118,000 there are 8.7 mμ moles of enzyme per mg. Therefore, in the assays above, 0.068 mμ moles of enzyme were added per tube (assuming that the enzyme is pure). 1 mg of yeast SRNA contains 0.1 mμ mole of tyrosine specific SRNA (see section 9, below).

activity and letting the reaction proceed to equilibrium. Assuming one L-tyrosine incorporated per molecule of L-tyrosine specific SRNA, a valid assumption, considering the excess ATP and tyrosine present in the reaction mixture, one can calculate the number of moles of L-tyrosine specific SRNA per mg from the extent of incorporation and the known specific activity of the incorporated L-tyrosine. Further, assuming a M.W. of 27,600 for the SRNA, one observes that tyrosine-specific SRNA corresponds to only 0.27% of the total SRNA. See Table XI.

10. Determination of the K_M for SRNA in the incorporation reaction

As shown in Figure 12, the Lineweaver-Burk plot (81) for SRNA allows the calculation of the K_M for tyrosine specific SRNA. Two separate experiments yielded K_M values of 2.2×10^{-8} M and $4.4 \cdot 10^{-8}$ M respectively. The rather small K_M value observed here suggests a strong interaction between tyrosine specific SRNA and the L-tyrosine activating enzyme. The V_{MAX} values obtained from these same experiments are 7 and 9.4 mμmoles incorporated per hour, respectively.

11. Homogeneity and sedimentation coefficient of yeast SRNA

A 1.6% solution of SRNA in 0.03 M tris Cl, 0.1 M NaCl pH 7.0, was centrifuged in the model E Spinco analytical ultracentrifuge, using rotor AN-D and cell 1387-1 at a speed

Table XI

Amount of Tyrosine Specific SRNA
in Yeast SRNA

2 mg SRNA were incubated until equilibrium was reached, with 0.05 µmoles C^{14} tyrosine (41,000 c.p.m.), in the presence of purified pig pancreas tyrosine activating enzyme. Procedure described in METHODS.

157.3 c.p.m. incorporated
M.W. of SRNA assumed to be 27,600
So, 2 mg SRNA correspond to 72.5 mµmoles

41,000 c.p.m. -- 50 mµmoles tyrosine
157.3 c.p.m. -- X mµmoles tyrosine specific SRNA

$$X = \frac{50 \times 157.3}{41,000} = 0.192 \text{ mµmoles tyrosine specific SRNA}$$

Specific activity: 0.096 mumoles tyrosine specific SRNA per mg SRNA. This corresponds to 0.27% of the total SRNA in the reaction mixture.

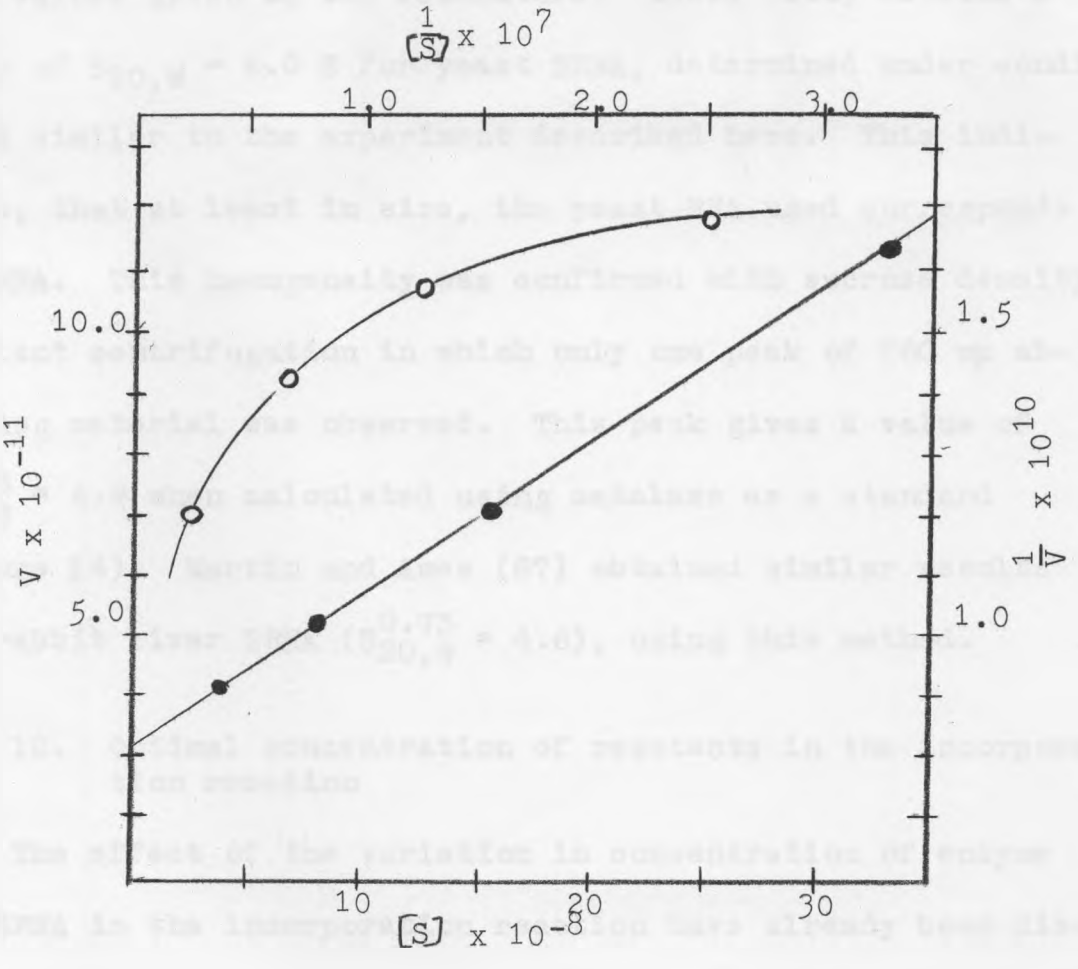


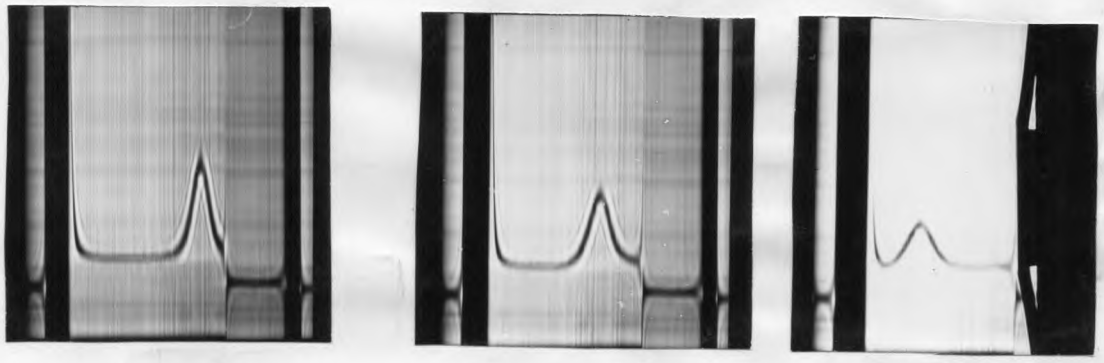
Figure 12. Influence of the SRNA Concentration on the Initial Velocity of Tryosine Incorporation (o - o) and Reciprocal Plot of the SRNA Concentration vs. Velocity (Lineweaver-Burk Analysis) (•—•).

Incorporation reactions performed as described in "Methods". For rate determination, samples were incubated for 10, 20 and 30 seconds. 0.1 ml. of enzyme used (hydroxamate specific activity 144; 0.1 mg. 1 ml.) with 0.5, 1, 2 and 4 mg. SRNA.

of 56,100 r.p.m. Under these conditions the SRNA behaves homogeneously with a sedimentation coefficient (uncorrected for infinite dilution) of 3.78 S (see Figure 13) in agreement with values given in the literature. Osawa (100) obtains a value of $S_{20,W}$ = 4.0 S for yeast SRNA, determined under conditions similar to the experiment described here. This indicates, that at least in size, the yeast RNA used corresponds to SRNA. This homogeneity was confirmed with sucrose density gradient centrifugation in which only one peak of 260 mμ absorbing material was observed. This peak gives a value of $S^{0.73}_{20,W}$ = 4.9 when calculated using catalase as a standard (Figure 14). Martin and Ames (87) obtained similar results for rabbit liver SRNA ($S^{0.73}_{20,W}$ = 4.6), using this method.

12. Optimal concentration of reactants in the incorporation reaction

The effect of the variation in concentration of enzyme and SRNA in the incorporation reaction have already been discussed. If the concentration of ATP is varied, keeping that of the other reagents constant, it is observed (Figure 15), that the reaction rate reaches a maximum when the concentration of ATP equals that of Mg^{++}. If the ratio of the concentrations of Mg^{++} and ATP is kept constant at 1:1, but the total concentration of both reagents is increased, the reaction rate reaches a maximum at 10 μmoles/ml of ATP and Mg^{++}



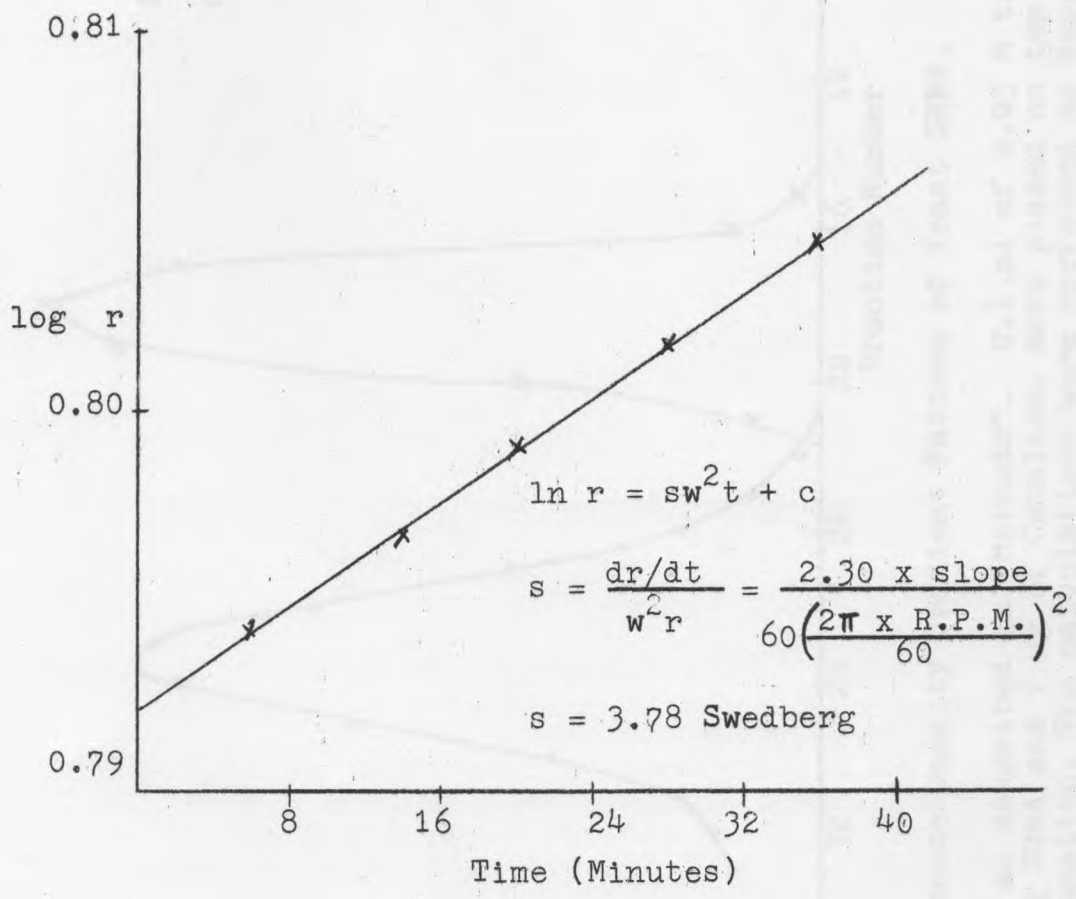


Figure 13. Determination of the Sedimentation Coefficient of Yeast SRNA.

The experimental conditions are described in the text.

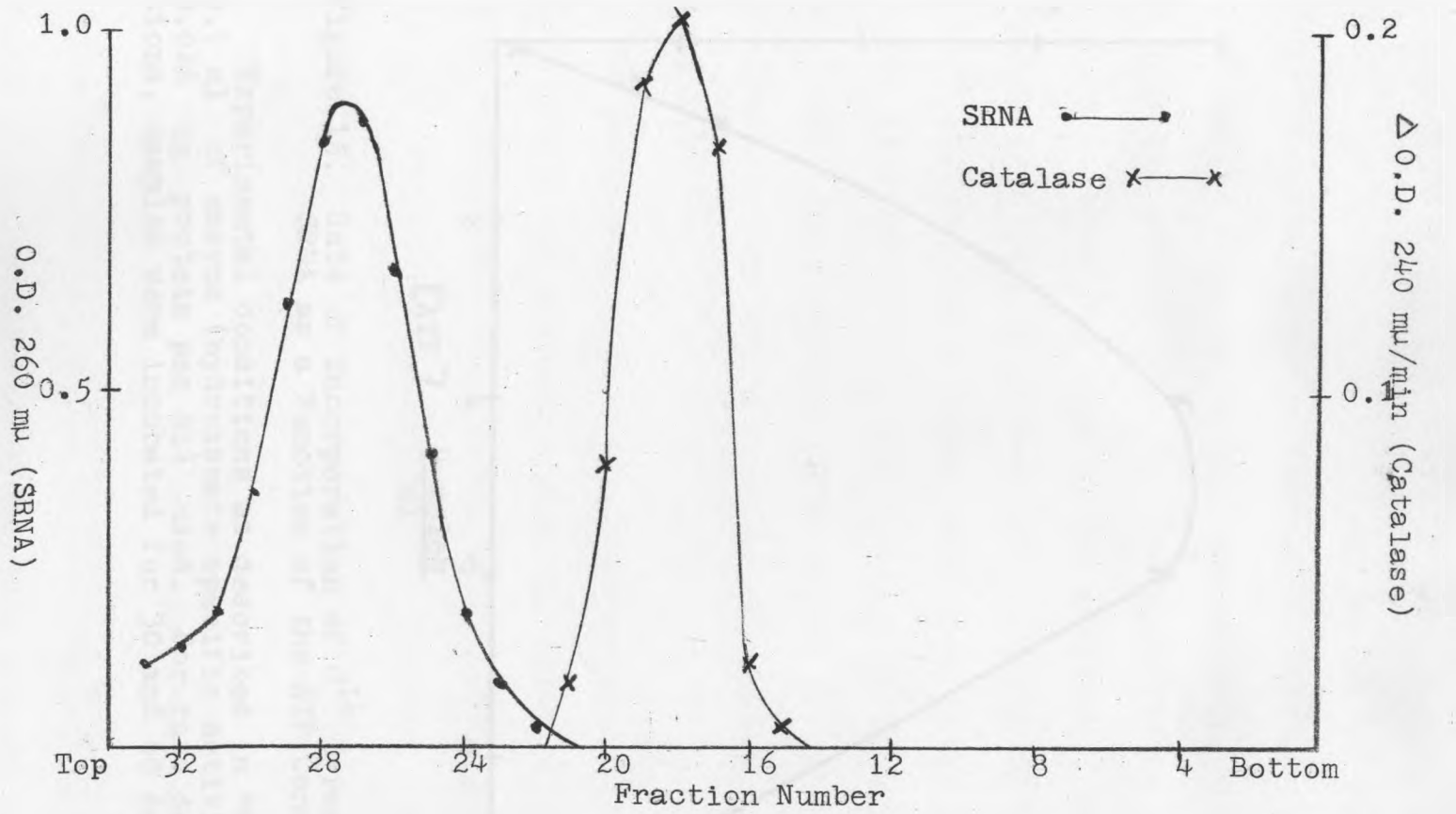


Figure 14. Sucrose Density Gradient Pattern of Yeast SRNA.

Procedure as described in "Methods". 0.1 ml of 0.05 M tris Cl pH 7.5 containing 1 mg SRNA and 7 μg of Catalase, were placed on top of the gradient before centrifugation. The calculations were performed as described in Table IV.

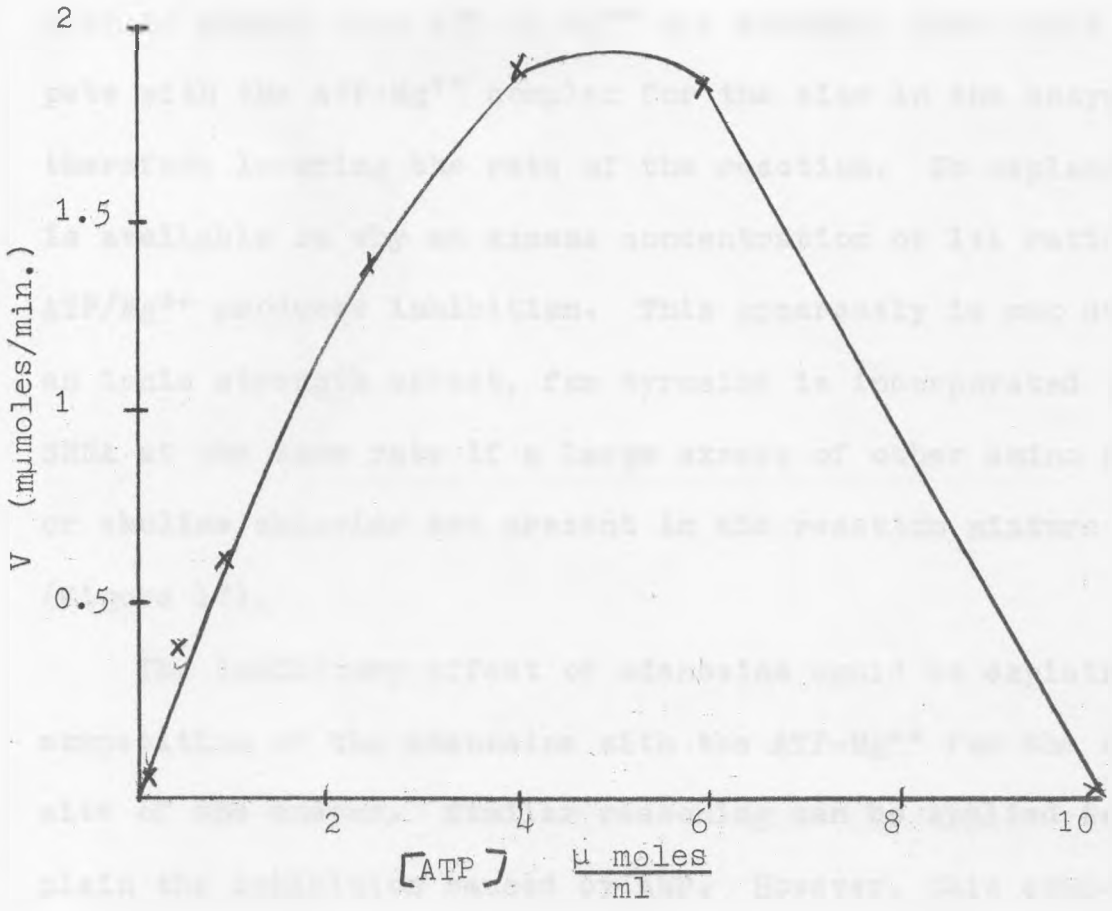


Figure 15. Rate of Incorporation of C^{14} Tyrosine into SRNA as a Function of the ATP Concentration.

Experimental conditions as described in "Methods". 0.1 ml of enzyme (hydroxamate specific activity 120, 0.026 mg. protein per ml) used. For rate determinations, samples were incubated for 30 and 60 seconds.

(Figure 16). The addition of AMP and adenosine also causes a decrease in the rate of incorporation (Table XII).

The fact that a 1:1 ratio of Mg^{++} and ATP produces an optimal rate of incorporation, may be due to the need for a Mg^{++}-ATP complex in the activation reaction. It is possible that if excess free ATP or Mg^{++} are present, they would compete with the ATP-Mg^{++} complex for the site in the enzyme, therefore lowering the rate of the reaction. No explanation is available on why an excess concentration of 1:1 ratio ATP/Mg^{++} produces inhibition. This apparently is not due to an ionic strength effect, for tyrosine is incorporated into SRNA at the same rate if a large excess of other amino acids or choline chloride are present in the reaction mixture (Figure 17).

The inhibitory effect of adenosine could be explained by competition of the adenosine with the ATP-Mg^{++} for the active site of the enzyme. Similar reasoning can be applied to explain the inhibition caused by AMP. However, this compound could also act reversing the incorporation reaction by simple mass action.

13. Reversal of the incorporation reaction by AMP, and AMP-ATP exchange

According to the mechanism accepted today, the final products of the incorporation reaction are AMP and SRNA-AA. Therefore, it would be expected that AMP, in the presence of

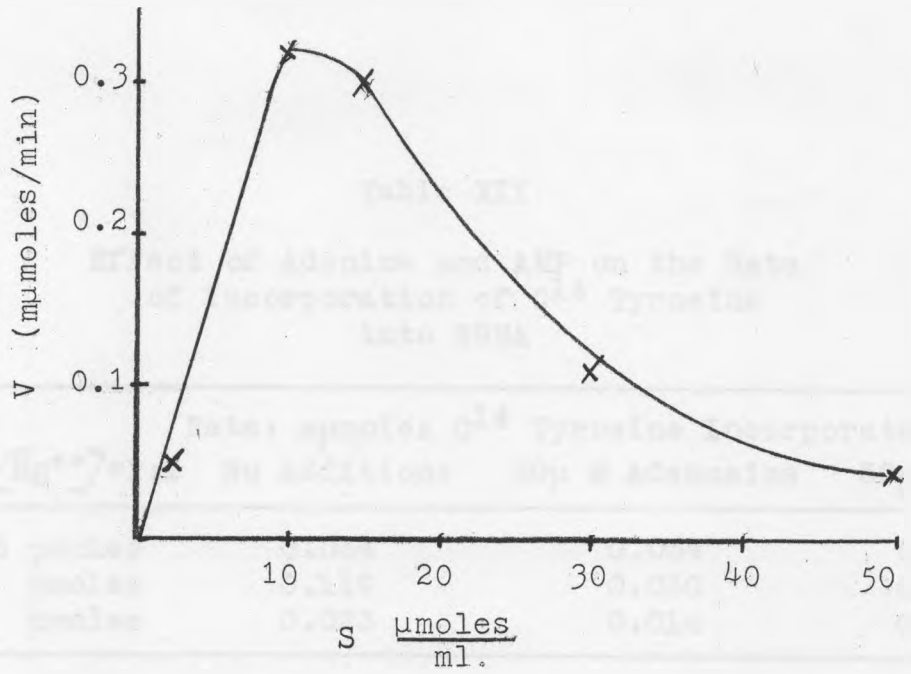


Figure 16. Effect of the Change in Concentration of ATP and Mg^{++} ([ATP] : $[Mg^{++}]$ = 1:1) on the Rate of Incorporation of C^{14} Tyrosine into SRNA.

Conditions as described in "Methods". 0.1 ml. of enzyme (hydroxamate specific activity 127, 0.016 mg. protein per ml. used per assay. Incubation times of 30 and 60 seconds were used to determine reaction rates.

Table XII

Effect of Adenine and AMP on the Rate of Incorporation of C^{14} Tyrosine into SRNA

[ATP]/[Mg^{++}]=1:1	Rate: mμmoles C^{14} Tyrosine Incorporated/Min. No Additions	30μ M Adenosine	30μ M AMP
2.5 μmoles	0.034	0.034	0.015
10 μmoles	0.119	0.030	0.015
30 μmoles	0.023	0.014	0.015

Procedure as described in METHODS. 0.1 ml enzyme used (hydroxamate specific activity 102, 0.03 mg/ml). Incubation times of 30 and 60 seconds were used to determine reaction rates.

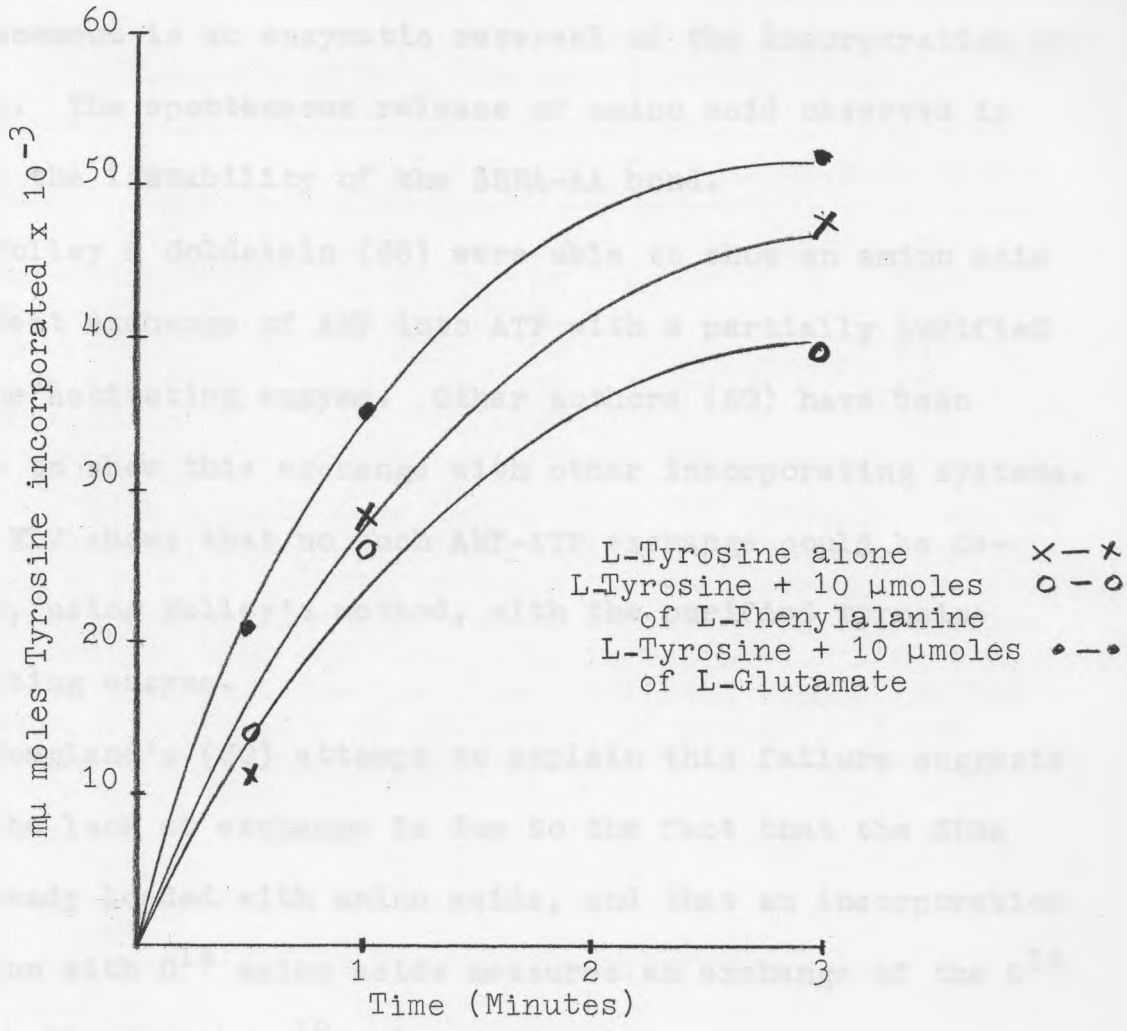


Figure 17. Effect of High Concentration of Several Amino Acids on the Rate of Incorporation of C^{14} Tyrosine into Yeast SRNA.

Assay procedure described in "Methods". 0.1 ml of enzyme of a hydroxamate specific activity of 126, 0.022 mg. protein per ml, was used. Incorporation in the presence of 10 μmoles of glycine, alamine or choline chloride gave rate between the extremes shown above. All amino acids and choline chloride were titrated to pH 7.5 before addition to the reaction mixture.

SRNA-AA, activating enzyme and PP, would displace the amino acid from its binding with SRNA. Table XIII shows that AMP in the presence of enzyme produces this effect. Adenosine, on the other hand, cannot produce a similar displacement, even in the presence of enzyme. This allows one to conclude that this displacement is an enzymatic reversal of the incorporation reaction. The spontaneous release of amino acid observed is due to the instability of the SRNA-AA bond.

Holley & Goldstein (65) were able to show an amino acid dependent exchange of AMP into ATP with a partially purified alanine activating enzyme. Other authors (60) have been unable to show this exchange with other incorporating systems. Table XIV shows that no such AMP-ATP exchange could be detected, using Holley's method, with the purified tyrosine activating enzyme.

Hoagland's (60) attempt to explain this failure suggests that the lack of exchange is due to the fact that the SRNA is already loaded with amino acids, and that an incorporation reaction with C^{14} amino acids measures an exchange of the C^{14} amino acids for the C^{12} amino acids already present:

$$AA^{*} + ATP + Enzyme \rightleftharpoons Enzyme < AMP\text{-}AA^{*} + PP$$
$$Enzyme < AMP\text{-}AA^{*} + SRNA\text{-}AA \rightleftharpoons SRNA\text{-}AA^{*} + Enzyme < AMP\text{-}AA$$

Thus, no free AMP is obtained, and therefore no AMP-ATP exchange can occur.

This mechanism can be checked by first stripping the SRNA free of amino acids by incubation with alkali. The product

Table XIII

Displacement of Tyrosine from Tyrosine-SRNA

	C.P.M. at 10'	C.P.M. at 30'
-enzyme	0	153
-enzyme - adenosine	50	156
-enzyme - AMP	91	150
+enzyme	76	144
+enzyme + adenosine	63	128
+enzyme - AMP	198	345

5 mg SRNA samples were loaded with C^{14} tyrosine (582,000 c.p.m. per tube) using 0.1 ml of enzyme (hydroxamate specific activity 102; 0.12 mg/ml), according to the procedure described in METHODS, without the addition of casein. The dry samples of C^{14}-tyrosine SRNA were suspended in 0.5 ml 0.2 M tris-Cl pH 7.5 and incubated for the times indicated with 10 umoles K-PP pH 7.5. 10 umoles of adenosine, 10 umoles of K-AMP pH 7.5 or 0.1 ml of enzyme were present in the tubes indicated.

Table XIV

AMP-ATP Exchange Catalyzed by the Purified Tyrosine Activating Enzyme from Pig Pancreas

Tube	SRNA	Enzyme	c.p.m. Present in ATP
complete system	pre-labeled	pig	1.6
complete system	stripped	pig	72.4
complete system	untreated	pig	79.7
complete system + KF	untreated	pig	76.9
complete system	untreated	--	52.7
complete system	untreated	yeast	6390
-tyrosine	untreated	yeast	5320
complete system	no addition	yeast	5320

Procedure as described by Holley & Goldstein (65).

Every assay tube contains 4 mg SRNA; 5 µmoles $MgCl_2$; 50 µmoles tris Cl, pH 7.5; 2.5 µmoles K-ATP, pH 7.2; 2.5 µmoles C^{14} AMP, pH 7.2, 66,000 c.p.m. (obtained from Schwarz Bio Research Inc., Mount Vernon, N. Y.); 2.5 µmoles K-PP, pH 7.5 and 1 µmole L-tyrosine, in 0.85 ml. To the tubes indicated, 0.1 ml pig pancreas enzyme (hydroxamate specific activity 102, 0.1 mg per ml), 0.1 ml yeast extract (prepared as described in METHODS) or 0.1 ml 0.1 M KF were added. All tubes were incubated for 20 minutes.

Pre-labeled SRNA: 4 mg SRNA labeled with non-radioactive tyrosine, using the procedure described in METHODS, without adding casein.

Stripped SRNA: 4 mg SRNA incubated for 30 minutes at 37°C in 0.5 ml 0.1 M tris-Cl pH 9.25 and titrated back to pH 7.5 with 1 N HCl.

of the incorporation reaction should then be free AMP, and an AMP-ATP exchange would proceed. Table XIV shows that even when "pre-stripped" SRNA is used in the AMP-ATP exchange system, no amino acid dependent AMP exchange occurs with ATP. No adequate explanation can be provided at the present time for the difference observed between the tyrosine and the alanine system with respect to AMP-ATP exchange. It would be interesting to study this exchange using other purified activating enzymes.

B. Studies on the Species Specificity of SRNAs and Activating Enzymes

As it was discussed in the introduction, the work of Berg et al. (12) and Benzen & Weisblum (5) has shown the existence of species specificity for the amino acid activating enzymes and the amino acid specific SRNAs.

It was therefore interesting to study the species specificity of the purified L-tyrosine activating enzyme of pig pancreas compared to tyrosine activating enzymes from other sources. Table XV shows the equilibrium incorporation values of several L-tyrosine activating preparations transferring to various SRNAs. It can be seen that there is complete incompatibility between yeast and hog on one side, and E. coli and Lactobacillus casei on the other. Rat liver SRNA is active with the yeast and pig enzymes, while SRNA from broccoli accepts small quantities of activated tyrosine with all the enzymes tested.

Table XV

Species Specificity in Terms of Millimicromoles of L-tyrosine Incorporated, at Equilibrium, Per mg of S-RNA

S-RNA Source	Enzyme Source		
	Hog Pancreas	Yeast	E. coli
hog liver	0.25	0.25	0
yeast	0.096	0.095	0
E. coli	0	0	0.156
rat liver	0.02	0.125	0.007
Lactobacillus casei	0	0	0.04
broccoli tips	0.01	0.03	0.01

Assay conditions as described in METHODS.

Although no detectable differences are observed between yeast and pig enzyme when equilibrium values were measured, it is possible that the enzyme from one species shows more affinity and therefore a higher rate of transfer towards its homologous SRNA. If identical amounts of hydroxamate activity are used, the pig enzyme usually shows a slightly faster rate of incorporation towards both pig and yeast SRNA when compared with the yeast enzyme. The results are not always reproducible and, therefore, of limited value. In addition, equal activity towards hydroxamate may not necessarily mean equal activity towards activation or incorporation. For example, a yeast extract containing noticeable hydroxamate activity for tyrosine, and negligible activity for valine and alanine, showed a considerable degree of incorporation activity towards all three amino acids.

C. Structural Requirements of Yeast SRNA for the Incorporation of Amino Acids

SRNA is a molecule of approximately 27,000 M.W. (19) and therefore a nucleotide chain of around 80 nucleotides per molecule. The nucleotide sequence at the amino acid acceptor end of all amino acid specific SRNAs is pCpCpA, (20,52) while the opposite ends terminate with a 5' guanylic acid (111). The amino acid specificity of SRNAs must then come from the internal nucleotide composition and/or sequence. It is therefore interesting to study what percentage of the internal

nucleotides of SRNA are required for SRNA to accept activated amino acids.

This problem can be approached by means of the enzymatic elimination of nucleotides from the non acceptor end of the SRNA chain, and a comparison of the remaining amino acid incorporation activity with the degree of hydrolysis of the chain. The enzyme spleen phosphodiesterase offers an effective tool with which to undertake this study. First, the enzyme is an exonuclease which sequentially hydrolyzes away mononucleotides from the 5' OH end of oligonucleotides (103). Second, spleen phosphodiesterase has been extensively purified by Hilmoe (54) and thus freed of other nucleases. However, the presence of 5' terminal phosphate groups renders oligonucleotides resistent to spleen phosphodiesterase (103). Although no studies involving large molecules such as SRNA have been published, one might expect some inhibition of spleen phosphodiesterase attack by the 5' terminal phosphate of SRNAs. Table XVI shows that the addition of bacterial phosphatase does not enhance the rate of hydrolysis of SRNA by spleen phosphodiesterase.

Figure 18 shows the effect of spleen phosphodiesterase treatment of yeast SRNA on the ability of the SRNA to incorporate tyrosine. The loss of activity during phosphodiesterase treatment is very marked and practically complete after 10% hydrolysis of the SRNA. Such results are not unlike those

Table XVI

Influence of the Presence of Bacterial Phosphatase on the Rate of Hydrolysis of SRNA by Spleen Phosphodiesterase

Incubation Time (minutes)	O. D. at 260 mμ		
	Phosphatase Alone	SPDE Alone	+SPDE Phosphatase
0	0	0	0
25	0	0.057	0.058
60	0	0.179	0.157

6 mg SRNA samples (purified through G-75 Sephadex) were dissolved in 0.05 M tris-Cl pH 7.5. Enzymes were added to start the reactions to the tubes indicated in the following quantities: bacterial phosphatase, 0.3 ml (0.025 mg/ml), (obtained from Worthington Biochemical Co.); spleen phosphodiesterase, 0.3 ml (about 2 units, 54). Total reaction volume was 9.5 ml. Samples of 0.5 ml were taken at the times indicated and added to tubes containing 0.5 ml 2.5% $HClO_4$, 0.25% uranium acetate. The precipitates were spun down at 10,000 x G for 10 minutes and the O.D. at 260 mμ of the supernatants determined against a blank of $HClO_4$ uranium acetate of the same concentration.

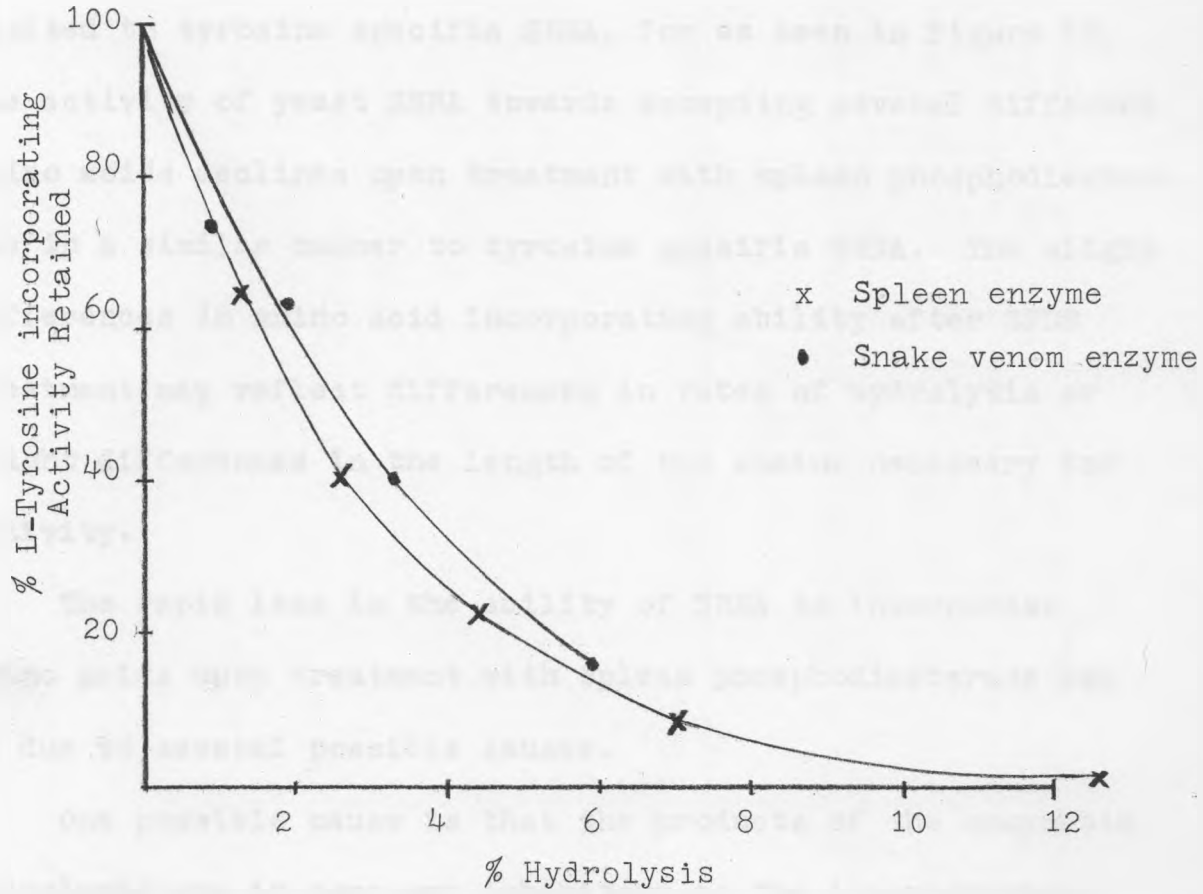


Figure 18. Effect of Spleen and Snake Venon Phosphodiesterase Treatment on the Ability of SRNA to Incorporate Tyrosine.

Procedure and calculations as described in "Methods". Separate samples were treated with about 5 units of spleen phosphodiesterase and 1.5 mg snake venom phosphodiesterase respectively. Aliquots for percent hydrolysis and percent activity determinations (containing about 2 mg. SRNA each) were taken at 0, 10 minutes, 20 minutes, 1 hour and 2 hours after the addition of the enzymes.

obtained when yeast SRNA is treated with the exonuclease snake venom phosphodiesterase, which sequentially removes nucleotides from the opposite or amino acid accepting end of the SRNA chain (103,132) (Figure 18).

The apparent need for most of the SRNA molecule is not limited to tyrosine specific SRNA, for as seen in Figure 19, the activity of yeast SRNA towards accepting several different amino acids declines upon treatment with spleen phosphodiesterase in a similar manner to tyrosine specific SRNA. The slight differences in amino acid incorporating ability after SPDE treatment may reflect differences in rates of hydrolysis or slight differences in the length of the chains necessary for activity.

The rapid loss in the ability of SRNA to incorporate amino acids upon treatment with spleen phosphodiesterase can be due to several possible causes.

One possible cause is that the products of the enzymatic hydrolysis are in some way inhibitory to the incorporation reaction. Figure 20 shows that this is not the case, for the addition of the products of diesterase hydrolysis does not inhibit the rate of incorporation of tyrosine into a non-hydrolyzed sample of yeast SRNA.

A second possible cause for the rapid drop in amino acid accepting activity when SRNA is treated with spleen phosphodiesterase is the presence of small amounts of endonuclease

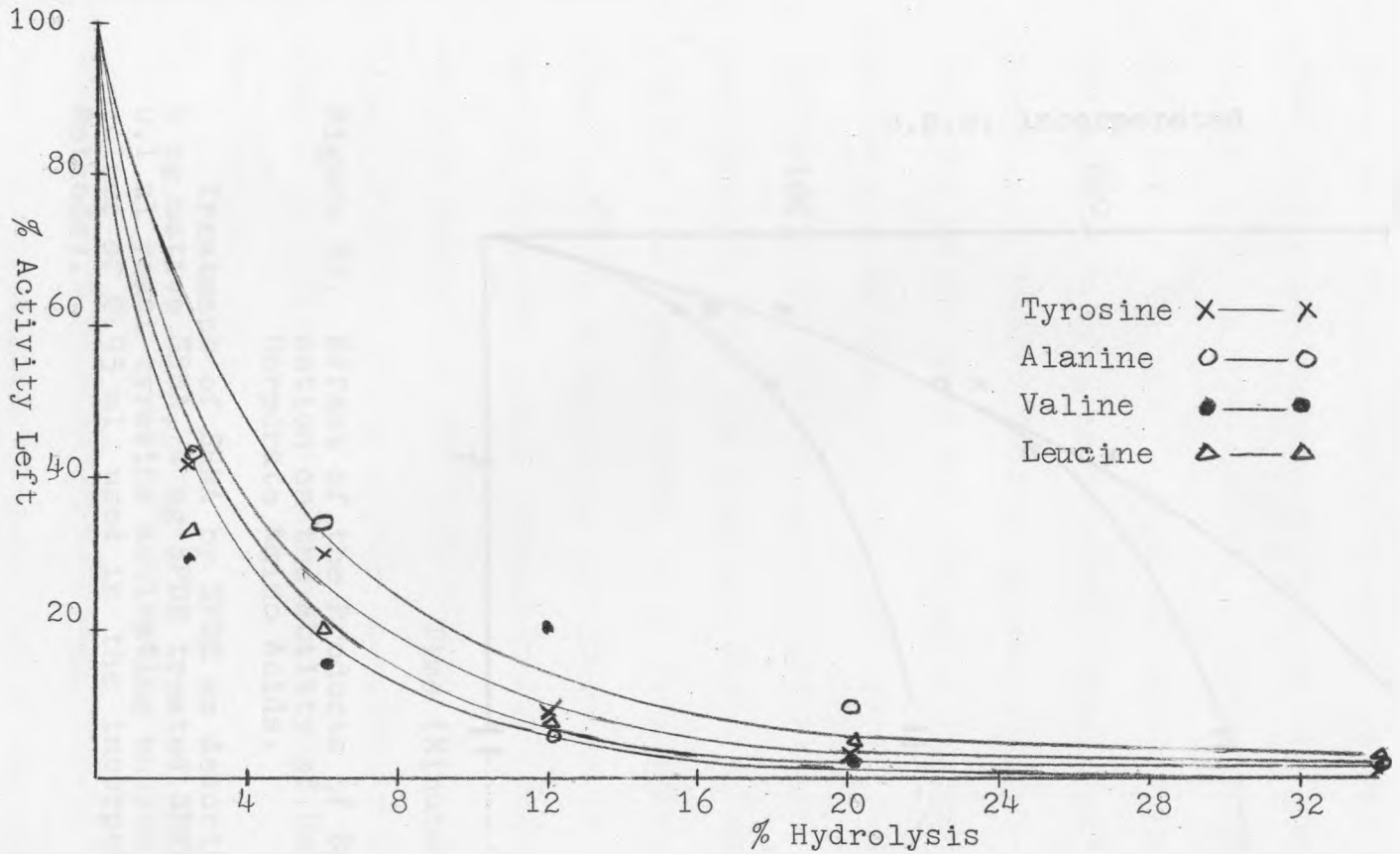


Figure 19. Comparison of the Ability of Tyrosine, Alanine, Valine and Leucine Specific SRNAS to Incorporate Amino Acid After Treatment With Spleen Phosphodiesterase

Procedure and calculations as described in "Methods". 67 mg SRNA were dissolved in 15.5 ml 0.1 M K succinate pH 6.7 and 2 ml SPDE (10 units) were added to start the reaction. Aliquots were taken for percent hydrolysis and percent activity determinations (containing approximately 2 mg SRNA) at 0, 10 minutes, 30 minutes, 1 hour and 2 hours. "Broad spectrum" yeast enzyme (see "Methods") was used for the incorporation assays.

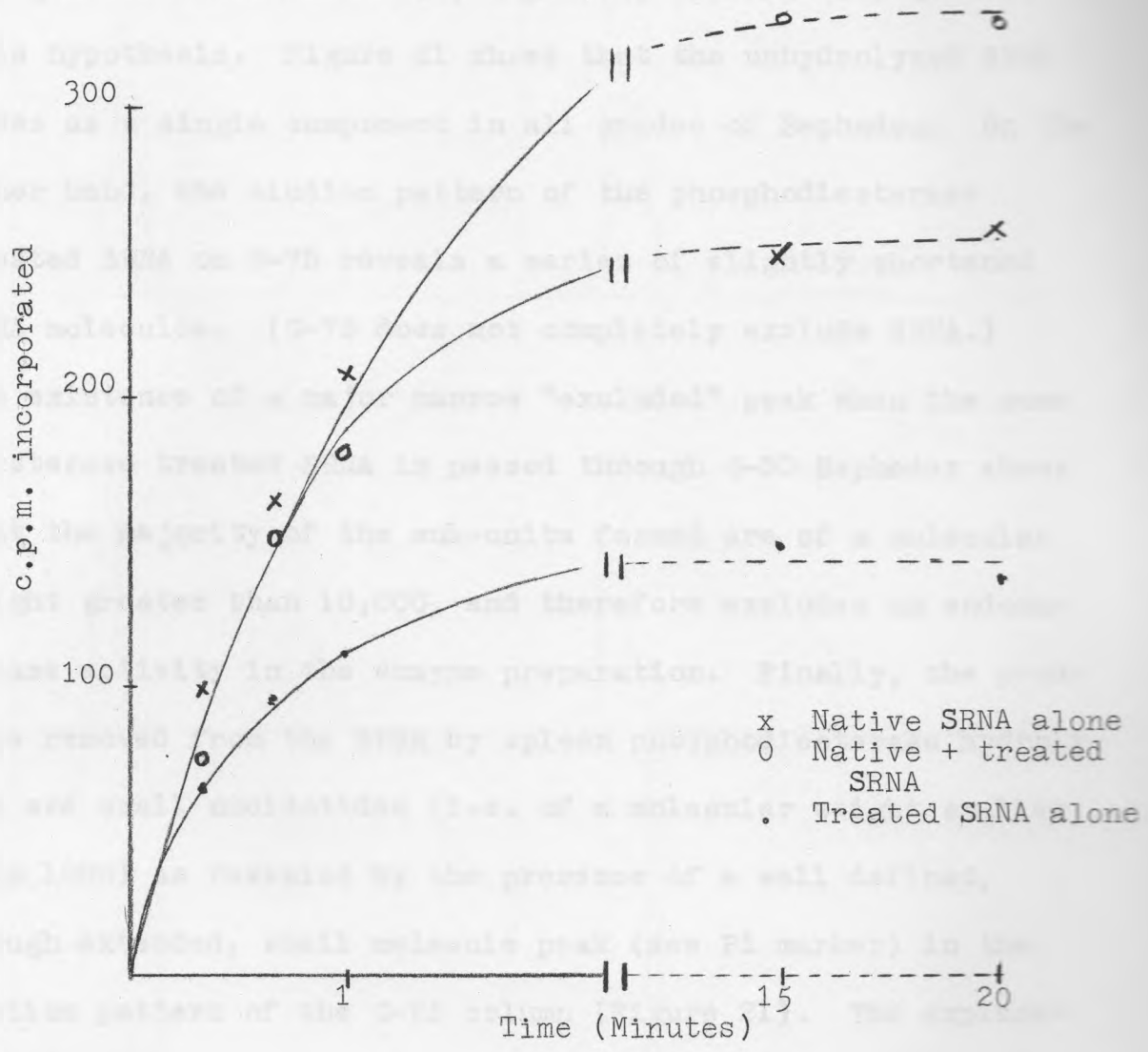


Figure 20. Effect of the Products of Spleen Phosphodiesterase Action on the Ability of Untreated SRNA to Incorporate Amino Acids.

Treatment of SRNA by SPDE as described in "Methods". 4 mg native SRNA, 4 mg SPDE treated SRNA (0.7% hydrolysis), 0.1 ml yeast tyrosine activating enzyme, with a total volume of 0.95 ml used in the incorporation assays (See Methods).

in the SPDE preparation, which results in a drastic change in the structural integrity of SRNA. The various forms of the gel filtration material, Sephadex, offer a tool to test this hypothesis. Figure 21 shows that the unhydrolyzed SRNA moves as a single component in all grades of Sephadex. On the other hand, the elution pattern of the phosphodiesterase treated SRNA on G-75 reveals a series of slightly shortened SRNA molecules. (G-75 does not completely exclude SRNA.) The existence of a major narrow "excluded" peak when the same diesterase treated SRNA is passed through G-50 Sephadex shows that the majority of the sub-units formed are of a molecular weight greater than 10,000, and therefore excludes an endonuclease activity in the enzyme preparation. Finally, the products removed from the SRNA by spleen phosphodiesterase hydrolysis are small nucleotides (i.e. of a molecular weight smaller than 1000) as revealed by the presence of a well defined, though extended, small molecule peak (see Pi marker) in the elution pattern of the G-25 column (Figure 21). The explanation for the trailing of the nucleotides in the small molecule region probably resides in certain adsorption properties of the Sephadex gels.

A third possible cause is the presence of exonuclease activity in the SPDE preparation, that hydrolyzes nucleotides from the amino acid acceptor end of the nucleotide chain. Our knowledge of the mechanism of action of spleen phosphodiesterase

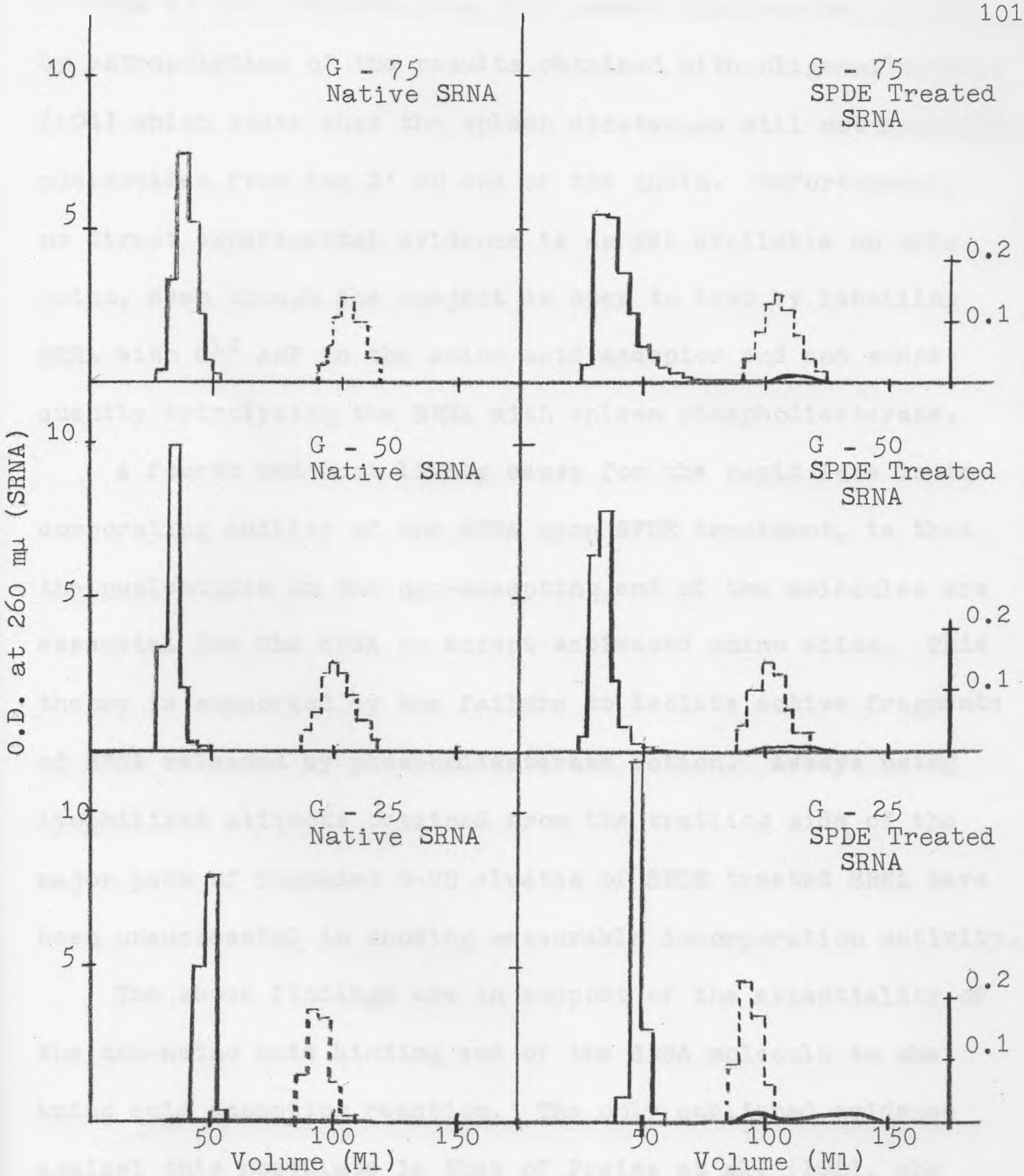


Figure 21. Elution Pattern of Native and SPDE Treated SRNA in Sephadex Columns.

G-25, G-50 and G-75 Sephadex columns of a total bed volume of 120 ml were used. 5 mg samples of native and SPDE treated (4% hydrolysis) SRNA were successively eluted from each column following the procedure described in "Methods". 6 μmoles of phosphate were used as a small molecule marker in each case.

on SRNA is not complete, but this possibility can be ruled out by extrapolation of the results obtained with oligonucleotides (104) which state that the spleen diesterase will not hydrolyze nucleotides from the 3' OH end of the chain. Unfortunately, no direct experimental evidence is as yet available on this point, even though the subject is open to test by labelling SRNA with C^{14} AMP in the amino acid acceptor end and subsequently hydrolyzing the SRNA with spleen phosphodiesterase.

A fourth and most likely cause for the rapid loss in incorporating ability of the SRNA upon SPDE treatment, is that the nucleotides on the non-accepting end of the molecules are essential for the SRNA to accept activated amino acids. This theory is supported by the failure to isolate active fragments of SRNA released by phosphodiesterase action. Assays using lyophilized aliquots obtained from the trailing side of the major peak of Sephadex G-75 eluates of SPDE treated SRNA have been unsuccessful in showing measurable incorporation activity.

The above findings are in support of the essentiality of the non-amino acid binding end of the SRNA molecule in the amino acid accepting reaction. The only published evidence against this postulate is that of Preiss et al. (101), who have reported that a 5% hydrolysis of E. coli SRNA by spleen phosphodiesterase results in a loss of only 20% of the activity to incorporate valine, while the same degree of hydrolysis with purified snake venom enzyme produces the expected complete

loss in incorporating ability. Owing to the lack of available data on the diesterase assay conditions and the purity of the SRNA used by Preiss et al., it is hard to make a full comparison of the two studies.

The action of spleen phosphodiesterase on SRNA was studied at two different temperatures using low and high salt concentrations. Figure 22 shows that the rate of hydrolysis of yeast SRNA by SPDE is lower at high ionic strength. As seen in Figure 23 and 24, either low temperature or high salt concentration requires a greater extent of hydrolysis of the SRNA to obtain loss in the incorporating ability.

SPDE is known to hydrolyze "core" RNA, ribooligonucleotides and deoxyribooligonucleotides, while it is inactive on DNA (54,104). This is possibly due to the presence of a well defined secondary structure in the DNA molecule. The studies of Brown et al. (18,114) show that SRNA, too, has a secondary structure, which is protected under conditions of low temperature and high ionic strength (119). This indicates that SPDE will hydrolyze more effectively under conditions that will weaken the secondary structure. Therefore, when this structure is protected, the enzyme will preferentially attack those nucleotide chains that have a less defined secondary structure, namely, the "junk" RNA present as a contaminant in the yeast SRNA preparations. The diesterase itself is not affected by the ionic strength, for as seen in Figure 25, the rate of

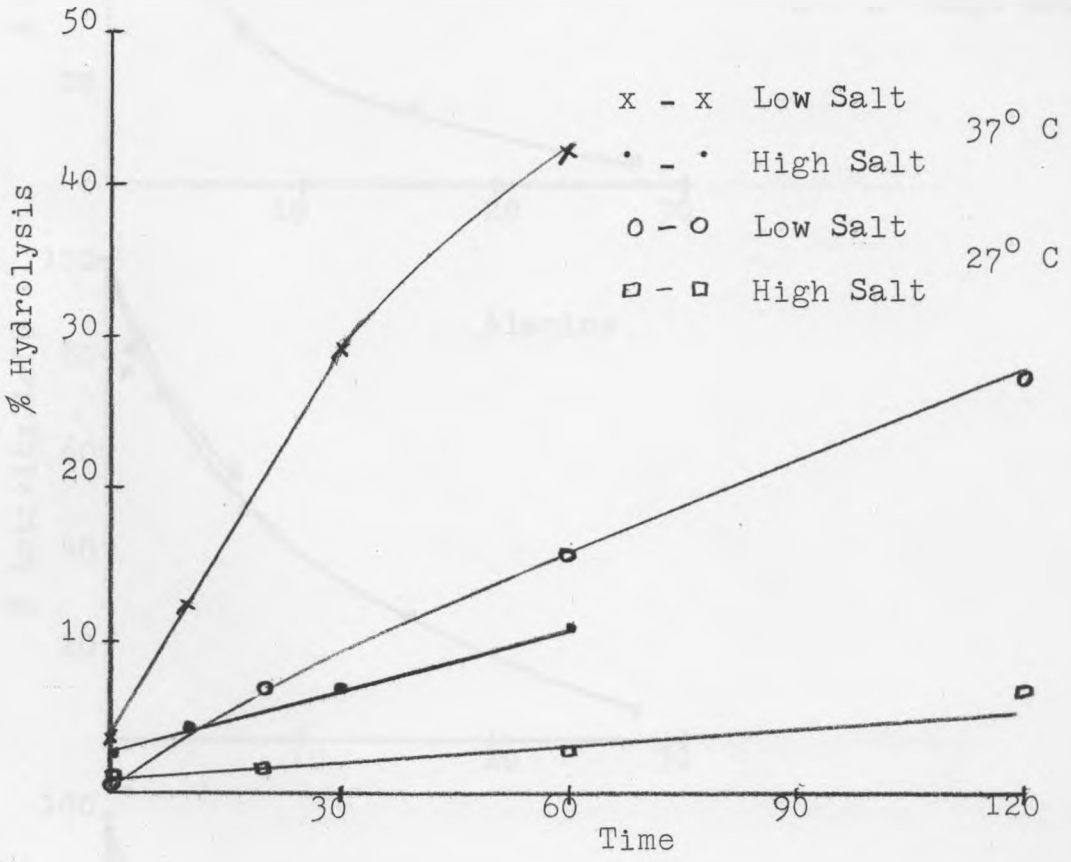


Figure 22. Influence of Salt Concentration and Temperature on the Rate of Hydrolysis of SRNA by Spleen Phosphodiesterase

Procedure and calculations as described in "Methods". High Salt: 0.1 M KCl, 0.01 M $MgCl_2$ in 0.01 M K-succinate pH 6.7. Low salt: 0.01 M K-succinate pH 6.7. 6 units of SPDE were added to start each reaction, with total volume of 8.2 ml. Samples for percent hydrolysis and percent activity determinations (see Fig. 23 and 24) were taken at the times indicated. "Broad spectrum" yeast enzyme (see Methods) was used for the incorporation assays, with aliquots of 1.6 mg of SRNA.

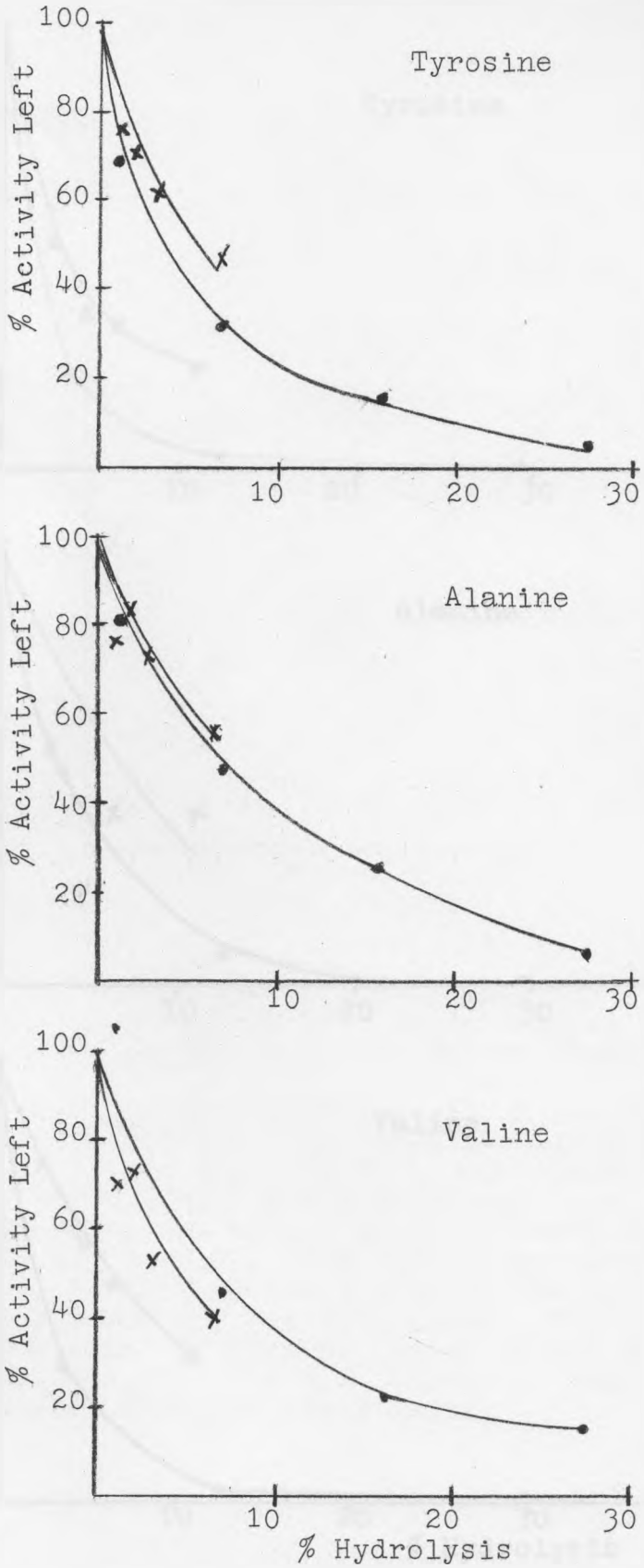


Figure 23. Per Cent Activity Left vs. Per Cent Hydrolysis as a Function of Salt Concentration.

For experimental conditions, see Figure 22.

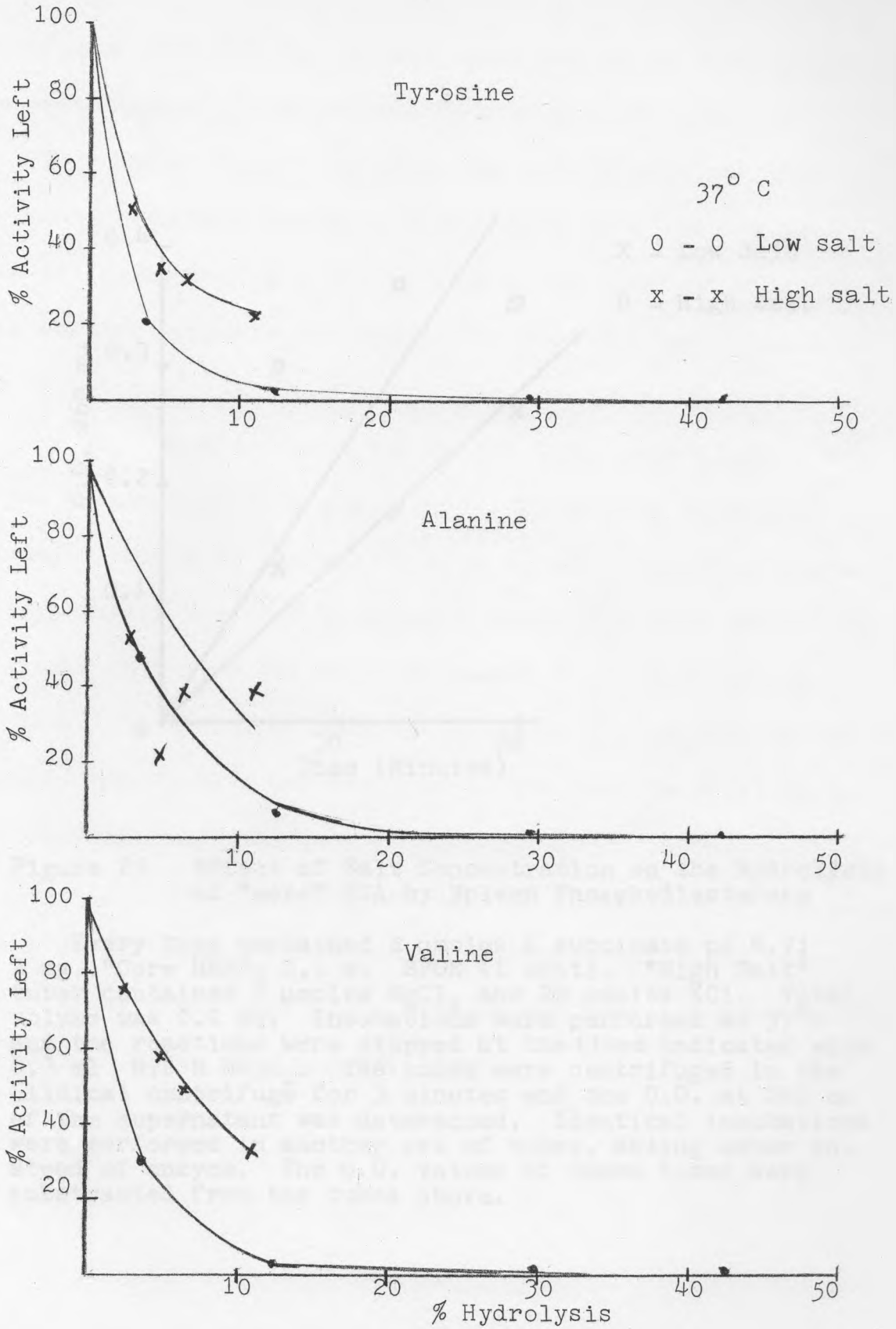


Figure 24. Per Cent Activity Left vs. Per Cent Hydrolysis as a Function of Salt Concentration.

For experimental conditions, see Figure 22.

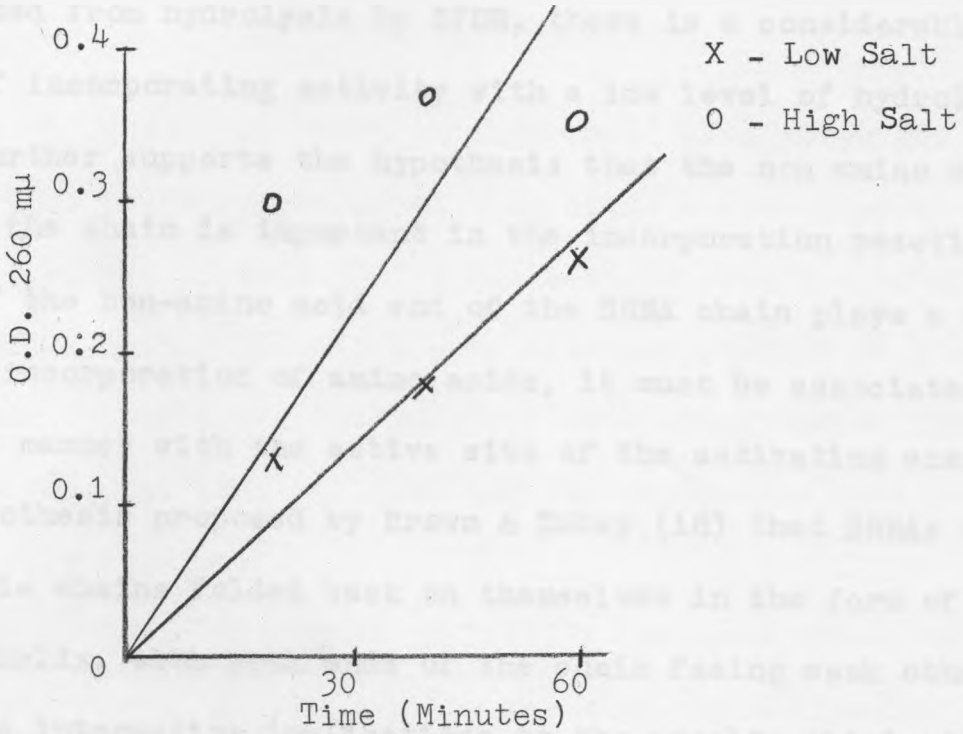


Figure 25. Effect of Salt Concentration on the Hydrolysis of "core" RNA by Spleen Phosphodiesterase

Every tube contained 2 μmoles K succinate pH 6.7; 2 mg "Core RNA"; 0.1 ml SPDE (1 unit). "High Salt" tubes contained 2 μmoles $MgCl_2$ and 20 μmoles KCl. Total volume was 0.2 ml. Incubations were performed at 37°C and the reactions were stopped at the times indicated with 0.3 ml 0.6 M $HClO_4$. The tubes were centrifuged in the clinical centrifuge for 3 minutes and the O.D. at 260 mμ of the supernatant was determined. Identical incubations were performed in another set of tubes, adding water instead of enzyme. The O.D. values of these tubes were substracted from the tubes above.

hydrolysis of "core" RNA is not inhibited by an increase in the salt concentration present in the reaction mixture.

Even under conditions where the SRNA chains are somewhat protected from hydrolysis by SPDE, there is a considerable loss of incorporating activity with a low level of hydrolysis. This further supports the hypothesis that the non amino acid end of the chain is important in the incorporation reaction.

If the non-amino acid end of the SRNA chain plays a role in the incorporation of amino acids, it must be associated in some manner with the active site of the activating enzyme. The hypothesis proposed by Brown & Zubay (18) that SRNAs exist as single chains folded back on themselves in the form of a double helix, with both ends of the chain facing each other, attaches interesting implications to the results obtained here (see GENERAL DISCUSSION).

Clearly, these results are only an indication of the size of the active SRNA involved in accepting an activated amino acid. Final proof must await similar and more detailed studies on purified amino acid specific SRNA.

CHAPTER IV

GENERAL DISCUSSION

In the past, most work on amino acid activation and transfer to SRNA has been performed with crude enzyme systems. One of the purposes of this thesis has been to obtain a purified preparation that would allow more detailed studies of these reactions. Therefore, the tyrosine activating enzyme from pig pancreas has been purified and its properties have been extensively studied.

The evidence presented in Table I indicates that the activating enzyme is responsible for both the activation of the amino acid and its subsequent transfer to SRNA. Thus, the active site of the enzyme must be rather complex in character, and therefore, most interesting and challenging to study. For example, the active site must partake in the following actions: (1) it must bind ATP, Mg^{++} and one, specific amino acid; (2) it must trigger the activation reaction through a proper arrangement of these substrates and the active groups of the amino acids comprising this active site; (3) it must stabilize the high energy amino acyl adenylate formed during the reaction; (4) the active site must have the ability to bind the correct specific SRNA and (5) it must catalyze the

transfer of the activated amino acid from the AMP to the 2' or 3' OH of the terminal adenosine of the acceptor SRNA. Hardly anything is known at present on the mechanism by which the activating enzyme performs these tasks.

Some information has been obtained in this thesis on the role of Mg^{++}. The studies on the requirements for Mg^{++} and ATP indicate that a Mg-ATP complex is essential for the activation to proceed. Perhaps the Mg^{++} by its complexing ability helps the enzyme stabilize the high energy AMP-AA.

The turnover number estimated for the tyrosine activating enzyme are, as indicated, relatively low. The fact that the turnover number for tyrosine incorporation is so much smaller than that of the PP exchange (18 vs. 1740), is no doubt a reflection of the thermodynamics of the overall reaction. The activation reaction is known to be endergonic towards the formation of AMP-AA, and therefore, readily reversible. This is particularly true if the AMP-AA is stabilized by the enzyme and does not hydrolyze spontaneously. On the other hand, the transfer reaction requires a successful collision of the enzyme $\leq$ AMP-AA with the specific amino acid binding site of a specific SRNA, something much less probable than a collision with a PP molecule.

The determined value for the K_M of the pig enzyme towards tyrosine specific SRNA of 3.3×10^{-8} M (average of two determinations) is a relatively low value when compared with other

enzymes, and implies a great affinity between the enzyme and its specific SRNA. This high affinity may account for the so called "pH5 enzymes" observed by several groups (59,63). It is likely that the relative insolubility of the SRNA caused by low pH, coupled with the high affinity of the enzyme for its specific SRNA results in coprecipitation. Further proof of this hypothesis is found in the purification of the pig pancreas tyrosine activating enzyme, in which a pH 5.4 fractionation is used. At this stage of the purification there is little polynucleotide material present (280/260 = 1, i.e., 3% nucleic acid) and as a result, the activating enzyme does not precipitate.

In light of this high affinity of the tyrosine activating enzyme for its specific SRNA, it appeared logical to use the enzyme as an aid in the purification of tyrosine specific SRNA. Thus, attempts were made to observe enzyme-SRNA complexes in sucrose density gradients. No change was observed in the shape of the SRNA peak, so as to overlap the activity peak of the added enzyme, perhaps due to the small amount of tyrosine specific SRNA present in the SRNA used (0.27%).

Comparative studies with yeast and pig tyrosine activating enzyme show that they can catalyze the incorporation of tyrosine into SRNA isolated from both species. Despite this similarity, these two enzymes present differences in properties. One of the possible reasons for the success in the purification

of the pig pancreas tyrosine activating enzyme, is the lack of "SH property" of the enzyme. In contrast, the yeast enzyme becomes very unstable after a low level of purification, and it is very sensitive to SH blocking reagents like p-chloromercuribenzoate (120). This suggests possible differences in the tertiary structure of both enzymes and perhaps in their active sites. More detailed studies with SH reagents and also with reagents specific for other active groups would provide valuable information on these differences.

An initial attempt has been made in this thesis to study the structural requirements for SRNA in the incorporation reaction. Although these results are of a preliminary nature, they open the door to more detailed studies, specially if a highly purified tyrosine specific SRNA becomes available. Since the information available in the literature indicates that this SRNA offers one of the best chances of being obtained in a high degree of purity (4,17,49,116), the prospects of this work are indeed promising.

The results obtained by Brown and collaborators (18,19, 114) on physical studies of SRNA have allowed them to propose a three dimensional structure for SRNA molecules. According to their hypothesis, the polynucleotide chain is folded back on itself, the two halves forming a double stranded helix and thus being equivalent to the antiparallel chains of DNA. Models of such a structure show that the fold at the

end of the molecule must consist of at least three nucleotides. The bases in these nucleotides have free hydrogen bonding sites, and it is possible that they represent the nucleotides which link to the messenger RNA and therefore specify the code for the specific amino acid.

The experimental results presented in this thesis support the hypothesis that both ends of the polynucleotide chain of SRNA are necessary for the incorporation of amino acids, as suggested by Brown's model that places both chain ends together in one extreme of the molecule.

If one accepts this model, it is likely that the species specificities observed in SRNA (see Table XV) reside in alterations of the nucleotide composition and sequence near the site of enzyme-SRNA association. This suggests that one may be able to correlate the nucleotide sequence near both ends of the polynucleotide chain of amino acid specific SRNAs with their biological source. Species specificity would then in no way affect the coding sequence in the fold point of the nucleotide chain. This would explain the observations of Nathans et al. (94) that ribosomes from rat liver can accept amino acids from E. coli SRNA as long as the transfer enzyme is from the same species as the ribosomes used, and the results presented in Table XV, which show that rat liver SRNA incorporates a negligible amount of C^{14} tyrosine in the presence of E. coli tyrosine activating enzyme.

The studies of degradation of SRNA with spleen phosphodiesterase suggest a further line of work, involving the study of the structural requirements of the SRNA molecule on the donation of amino acid from SRNA-AA to a polypeptide on the template. If the theory presented above is correct, short treatment of SRNA-AA with spleen phosphodiesterase should give an SRNA molecule that can transfer its amino acid to the polypeptide chain on the messenger, but becomes incapable of later accepting another activated amino acid from the activating enzyme.

Our sedimentation studies indicate that the yeast SRNA preparations used are quite homogeneous. In contrast, only a very small percentage of this SRNA was found to be tyrosine specific. Since both ends of the polynucleotide chain seem essential for the incorporation of amino acids, the removal of a few nucleotides from either end should cause a sharp loss in activity. Any such action during the preparation of the SRNA would produce considerable loss in activity without significantly altering the molecular weight of the chain, thus explaining the low levels of tyrosine specific SRNA found.

CHAPTER V

SUMMARY

This thesis is concerned with the mechanism of the reactions involved in the activation of amino acids and subsequent transfer to SRNA.

To this end, a highly purified preparation of the tyrosine activating enzyme from pig pancreas has been obtained. The purification procedure involves adsorption in $Ca_3(PO_4)_2$ gel, $(NH_4)_2SO_4$ fractionation, acid fractionation and chromatography in DEAE cellulose and $Ca_3(PO_4)_2$ gel cellulose columns.

The purified enzyme has a specific activity of 132 measured by the hydroxamate assay, and since it is free of nuclease activity, it catalyzes the incorporation of tyrosine into SRNA, unlike previously described preparations.

The enzyme has a very narrow substrate specificity. Of the natural amino acids, only tyrosine, and to a smaller extent, phenylalanine, are substrates of the PP exchange reaction. The K_M's for these amino acids are 2.7×10^{-5} M and 1.8×10^{-2} M respectively.

The molecular weight of the enzyme has been estimated by means of sucrose density gradient centrifugation, and a value of 115-118,000 has been obtained. The turnover numbers

calculated with this value are 1740 and 17.7 for the PP exchange reaction and incorporation of tyrosine into SRNA, respectively.

The enzyme catalyzed incorporation of tyrosine into SRNA requires the presence of ATP and Mg^{++}. An optimal rate of incorporation is observed if the ratio of concentrations of these two components is 1:1. The extent of equilibrium incorporation of tyrosine depends on the concentration of SRNA, while it is independent of the enzyme concentration. The K_M for SRNA in this reaction is very low (3×10^{-8}M) suggesting a strong interaction between the enzyme and its specific SRNA.

Species specificity studies show that the pig pancreas tyrosine activating enzyme will catalyze the transfer of tyrosine to SRNA from pig or yeast and is inactive towards E. coli SRNA, while the E. coli enzyme will only catalyze tyrosine transfer to its homologous SRNA.

Studies have been made to determine the structural requirements of the SRNA molecule for the incorporation of amino acids. Elimination of nucleotides from the 5' terminal end of the chain by hydrolysis with spleen phosphodiesterase shows a very sharp loss of incorporating activity after a low level of hydrolysis. The evidence obtained indicates that the diesterase does not possess endonuclease activity and that the products of hydrolysis do not inhibit the incorporation of amino acid into intact SRNA. It has been concluded that

the non-amino acid binding end of the SRNA chain is essential for the incorporation of amino acids.

The implications of these findings on the mechanism of amino acid activation and transfer are discussed.

CHAPTER VI

BIBLIOGRAPHY

1. Allen, E. H., E. Glassman & R. S. Schweet, J. Biol. Chem., 235, 1061, 1960.

2. Allen, E. H., E. Glassman, E. Cordes & R. S. Schweet, J. Biol. Chem., 235, 1068, 1960.

3. Allende, J. E., R. Monro, D. Nathans & F. Lipmann, Fed. Proc., 21, 414, 1962.

4. Apgar, J., R. W. Holley & S. H. Merril, J. Biol. Chem., 237, 796, 1962.

5. Benzer, S. & B. Weisblum, Proc. Natl. Acad. Sci., 47, 1149, 1961.

6. Berg, P., J. Am. Chem. Soc., 77, 3163, 1955.

7. Berg, P., J. Biol. Chem., 222, 991, 1956.

8. Berg, P., J. Biol. Chem., 222, 1025, 1956.

9. Berg, P., J. Biol. Chem., 233, 601, 1958.

10. Berg, P. & J. Ofengand, Proc. Natl. Acad. Sci., 44, 78, 1958.

11. Berg, P., Ann. Review of Biochem., 30, 293, 1961.

12. Berg, P., H. Bergmann, E. J. Ofengand & M. Dieckmann, J. Biol. Chem., 236, 1726, 1961.

13. Bergmann, F. H., P. Berg & M. Dieckmann, J. Biol. Chem., 236, 1735, 1961.

14. Bernlohr, R. W. & G. C. Webster, Arch. Bioch. & Bioph., 73, 276, 1958.

15. Bishop, J. O. & R. Schweet, Bioch & Bioph. Acta, 49, 235, 1961.

16. Brenner, S., Cold Spring Harbor Symp. in Quant. Biol., 26, 101, 1961.

17. Brown, G. L., A. V. Brown & J. Gordon, Brookhaven Symp. in Biology, 12, 47, 1959.

18. Brown, G. L. & G. Zubay, J. Mol. Biol., 2, 287, 1960.

19. Brown, G. L., L. Kosinski & C. Carr, Colloques Internationaux du Centre National de la Recherche Scientifique, 106, 183, 1962.

20. Canellakis, E. S. & E. Herbert, Proc. Natl. Acad. Sci., 46, 170, 1960.

21. Chapeville, F., Fed. Proc., 21, 414, 1962.

22. Chapeville, F., F. Lipmann, G. Von Ehrenstein, B. Weisblum, W. J. Ray, Jr., & S. Benzer, Proc. Natl. Acad. Sci., 48, 1088, 1962.

23. Clark, J. M., Ph. D. Thesis, California Institute of Technology, p. 120, 1958.

24. Clark, J. M., J. Biol. Chem., 233, 421, 1958.

25. Cole, R. D., J. Coote & T. S. Work, Nature, 179, 199, 1957.

26. Conway, T. W., E. M. Landsford, Jr. & W. Shive, J. Biol. Chem., 237, 2850, 1962.

27. Cormier, M. J. & D. Novelli, Bioch. & Bioph. Acta, 30, 135, 1958.

28. Cormier, M. J., M. P. Stulberg, & D. Novelli, Bioch. & Bioph. Acta, 33, 261, 1959.

29. Cox, R. A. & U. Z. Littaner, J. Mol. Biol., 2, 166, 1960.

30. Crick, F. H. C., Symp. Soc. Exptl. Biol., 12, 138, 1958.

31. Czok, R. & Th. Bucher, Adv. Protein Chem., 15, 315, 1960.

32. Davie, E. W., V. V. Koningsberger & F. Lipmann, Arch. Bioch. & Bioph., 65, 21, 1956.

33. Davis, J. W. & D. Novelli, Arch. Bioch & Bioph., 75, 299, 1958.

34. De Moss, J. A. & D. Novelli, Bioch. & Bioph. Acta, 18, 592, 1955.

35. De Moss, J. A., S. M. Genuth & D. Novelli, Proc. Natl. Acad. Sci, 42, 325, 1956.

36. De Moss, J. A. & D. Novelli, Bioch. & Bioph. Acta, 22, 49, 1956.

37. Dinzis, H. M., Proc. Natl. Acad. Sci., 47, 247, 1961.

38. Dixon, M. & E. C. Webb, "Enzymes," p. 17, Academic Press, N. Y., 1958.

39. Dixon, M. & E. C. Webb, "Enzymes," p. 479, Academic Press, N. Y., 1958.

40. Doctor, B. P., J. Apgar & R. W. Holley, J. Biol. Chem., 236, 1117, 1961.

41. Dunn, B. D., Bioch. & Bioph. Acta, 34, 286, 1959.

42. Fessenden, J. M. & K. Moldave, Biochemistry, 1, 485, 1962.

43. Fiske, C. H. & Y. Subbarow, J. Biol. Chem., 66, 377, 1925.

44. Fraser, M. J., Fed. Proc., 21, 415, 1962.

45. Glassman, E., E. H. Allen & R. S. Schweet, J. Am. Chem. Soc., 80, 4427, 1958.

46. Grossi, L. G. & K. Moldave, J. Biol. Chem., 235, 2370, 1960.

47. Groves, W., Personal communication.

48. Gunsalus, I. C., Editor, "Experimental Biochemistry," p. 29, Stipes Pub. Co., Champaign, Ill, 1959.

49. Gunther, J. K., Personal communication.

50. Hall, B. D. & S. Spiegelman, Proc. Natl. Acad. Sci., 47, 137, 1961.

51. Hecht, L. I., M. L. Stephenson & P. C. Zamecnik, Bioch. & Bioph. Acta, 29, 460, 1958.

52. Hecht, L. I., P. C. Zamecnik, M. L. Stephenson & J. F. Scott, J. Biol. Chem., 233, 954, 1958.

53. Hecht, L. I., M. L. Stephenson & P. C. Zamecnik, Proc. Natl. Acad. Sci., 45, 505, 1959.

54. Hilmoe, R. J., J. Biol. Chem., 235, 2117, 1960.

55. Hoagland, M. B., Bioch. & Bioph. Acta, 16, 288, 1955.

56. Hoagland, M. B., E. B. Keller & P. Zamecnik, J. Biol. Chem., 218, 345, 1956.

57. Hoagland, M. B., P. C. Zamecnik, N. Sharon, F. Lipmann, M. P. Stulberg & P. D. Boyer, Bioch. & Bioph. Acta, 26, 215, 1957.

58. Hoagland, M. B., P. C. Zamecnik & M. L. Stephenson, Bioch. & Bioph. Acta, 24, 215, 1957.

59. Hoagland, M. B., M. L. Stephenson, J. F. Scott, L. I. Hecht & P. C. Zamecnik, J. Biol. Chem., 231, 241, 1958.

60. Hoagland, M. B., Proc. IVth Inter. Congress Biochem., 8, 199, Pergamon Press, 1960.

61. Hoagland, M. B., "The Nucleic Acids," 3, 349, Academic Press Inc., N. Y., 1960.

62. Hoagland, M. B. & L. T. Comly, Proc. Natl. Acad. Sci., 46, 1554, 1960.

63. Holley, R. W., J. Am. Chem. Soc., 79, 658, 1957.

64. Holley, R. W., P. B. Doctor, S. H. Merrill, F. M. Saad, Bioch. & Bioph. Acta, 35, 272, 1959.

65. Holley, R. W. & J. Goldstein, J. Biol. Chem., 234, 1765, 1959.

66. Holley, R. W., J. Apgar & B. P. Doctor, Annals N. Y. Acad. Sci., 88, 745, 1960.

67. Holley, R. W., J. Apgar, B. P. Doctor, J. Farrow, M. A. Marini & S. H. Merrill, J. Biol. Chem., 236, 200, 1961.

68. Holley, R. W., E. F. Brunngraber, F. Saad & H. H. Williams, J. Biol. Chem., 236, 197, 1961.

69. Holley, R. W., J. Apgar, S. H. Merrill & P. L. Zubkoff, J. Am. Chem. Soc., 83, 4861, 1961.

70. Hultin, T., Bioch. & Bioph. Acta, 51, 219, 1961.

71. Jacob, F. & J. Monod, J. Mol. Biol., 3, 318, 1961.

72. Keller, E. B. & P. Zamecnik, J. Biol. Chem., 221, 45, 1956.

73. Kingdon, H. S., L. T. Webster, Jr. & E. W. Davie, Proc. Natl. Acad. Sci., 44, 757, 1958.

74. Klee, W. A. & G. L. Cantoni, Proc. Natl. Acad. Sci., 46, 322, 1960.

75. Koerner, J. F. and R. L. Sinsheimer, J. Biol. Chem., 228, 1049, 1957.

76. Krishnaswamy, P. R. & A. Meister, J. Biol. Chem., 235, 408, 1960.

77. Lagerkvist, U., P. Berg, M. Dieckmann & F. W. Platt, Fed. Proc., 20, 363, 1961.

78. Layne, E., "Methods in Enzymology," 3, 447, S. P. Colowick & N. O. Kaplan, Editors, Academic Press, Inc., N. Y., 1957.

79. Leahy, J., E. Glassman & R. S. Schweet, J. Biol. Chem., 235, 3209, 1960.

80. Lengyel, P., J. F. Speyer & S. Ochoa, Proc. Natl. Acad. Sci., 47, 1936, 1961.

81. Lineweaver, H. & D. Burk, J. Am. Chem. Soc., 56, 658, 1934.

82. Lipmann, F., W. C. Hulsmann, G. Hartmann, H. G. Boman & G. Acs, J. Cell. Comp. Phys., 54, Sup. 1, 75, 1959.

83. Loftfield, R. B., & E. A. Eigner, J. Am. Chem. Soc., 81, 4753, 1959.

84. Maas, W. K. & G. D. Novelli, Arch. Biochem. & Bioph., 43, 236, 1953.

85. Marcus, A., J. Biol. Chem., 234, 1238, 1959.

86. Markham, R. & J. D. Smith, Biochem. J., 52, 552, 1952.

87. Martin, R. G. & B. N. Ames, J. Biol. Chem., 236, 1372, 1961.

88. Massey, V., Bioch. & Bioph. Acta, 37, 310, 1960.

89. Matthaei, J. H., O. W. Jones, R. G. Martin & M. W. Nirenberg, Proc. Natl. Acad. Sci., 48, 666, 1962.

90. McCully, K. S. & G. L. Cantoni, J. Mol. Biol., 5, 80, 1962.

91. Monier, R., M. L. Stephenson & P. C. Zamecnik, Bioch. & Bioph. Acta, 43, 1, 1960.

92. Morris, A. J. & R. S. Schweet, Bioch & Bioph. Acta, 47, 415, 1961.

93. Nathans, D. & F. Lipmann, Proc. Natl. Acad. Sci., 47, 497, 1961.

94. Nathans, D., G. Von Ehrenstein, R. Monro & F. Lipmann, Fed. Proc., 21, 127, 1962.

95. Nirenberg, M. W., & H. Matthaei, Proc. Natl. Acad. Sci., 47, 1588, 1961.

96. Nismann, B., F. H. Bergmann & P. Berg, Bioch. & Bioph. Acta, 26, 639, 1957.

97. Ofengand, E. J., M. Dieckmann & P. Berg, J. Biol. Chem., 236, 1741, 1961.

98. Ogata, K. & H. Nohara, Bioch. & Bioph. Acta, 25, 660, 1957.

99. Ogata, K., H. Nohara, K. Ishikawa, T. Morita & H. Asaoka, "Protein Biosynthesis," p. 163. R. J. C. Harris, Editor, Academic Press, N. Y., 1961.

100. Osawa, S., Bioch. & Bioph. Acta, 43, 110, 1960.

101. Preiss, J., P. Berg, E. J. Ofengand, F. H. Bergmann & M. Dieckmann, Proc. Natl. Acad. Sci., 45, 319, 1957.

102. Preiss, J., M. Dieckmann & P. Berg, J. Biol. Chem., 236, 1748, 1961.

103. Razzell, W. E. & H. G. Khorana, J. Am. Chem. Soc., 80, 1770, 1958.

104. Razzell, W. E. & H. G. Khorana, J. Biol. Chem., 236, 1144, 1961.

105. Schweet, R. S., Bioch. & Bioph. Acta, 18, 566, 1955.

106. Schweet, R. S. & E. H. Allen, J. Biol. Chem., 233, 1104, 1958.

107. Schweet, R. S., F. C. Bovard, E. Allen & E. Glassman, Proc. Natl. Acad. Sci., 44, 173, 1958.

108. Schweet, R. J. Bishop & A. Morris, Lab. Inv., 10, 992, 1961.

109. Sharon, N. & F. Lipmann, Arch. Bioch. & Bioph., 69, 219, 1957.

110. Simkin, J. L., Biochem. J., 70, 305, 1958.

111. Singer, M. F. & G. L. Cantoni, Bioch. & Bioph. Acta, 39, 182, 1960.

112. Singer, T. P. & E. B. Kearney, Arch. Bioch., 29, 190, 1950.

113. Smith, K. C., E. Cordes & R. S. Schweet, Bioch. & Bioph. Acta, 33, 286, 1959.

114. Spencer, M., W. Fuller, M. H. F. Wilkins & G. L. Brown, Nature, 194, 1014, 1962.

115. Stephenson, M. L. & P. C. Zamecnik, Proc. Natl. Acad. Sci., 47, 1627, 1961.

116. Sueoka, N. & T. Yamane, Proc. Natl. Acad. Sci., 48, 1454, 1962.

117. Swingle, S. M. & A. Tiselius, Biochem. J., 48, 171, 1951.

118. Tanford, C., "Physical Chemistry of Macromolecules," p. 381, John Wiley & Sons, Inc., N. Y., 1961.

119. Tissières, A., J. Mol. Biol., 1, 365, 1959.

120. Van de Ven, A. M., V. V. Koningsberger & J. Th. G. Overbeck, Bioch. & Bioph. Acta, 28, 134, 1958.

121. Volkin, E., Fed. Proc., 21, 112, 1962.

122. Von der Decken, A. & T. Hultin, Bioch. & Bioph. Acta, 45, 139, 1960.

123. Von Ehrenstein, G. & F. Lipmann, Proc. Natl. Acad. Sci., 47, 941, 1961.

124. Wahl, A. C. & N. A. Bonner, "Radioactivity Applied to Chemistry," p. 7, John Wiley & Sons, N. Y., 1951.

125. Warburg, O. & W. Christian, Bioch. Z., 310, 384, 1941.

126. Webster, G., Ann. Rev. Plant Physiol., 12, 113, 1961.

127. Webster, G. C., Bioch. & Bioph. Acta, 49, 141, 1961.

128. Webster, G. & J. B. Lingrel, "Protein Biosynthesis," p. 301, R. J. C. Harris, Editor, Academic Press, Inc., N. Y., 1961.

129. Webster, L. T., & E. W. Davie, J. Biol. Chem., 236, 479, 1961.

130. Weisblum, B., S. Benzer & R. W. Holley, Proc. Natl. Acad. Sci., 48, 1449, 1962.

131. Wieland, Th. & G. Pfleiderer, Ann. N. Y. Acad. Sci., 94, 655, 1961.

132. Williams, E. J., S. Sung & M. Laskowski, Sr., J. Biol. Chem., 236, 1130, 1961.

133. Wong, K. K. & K. Moldave, J. Biol. Chem., 235, 694, 1960.

134. Ycas, M. & W. S. Vincent, Proc. Natl. Acad. Sci., 46, 804, 1960.

135. Zachau, H. G., G. Acs, & F. Lipmann, Proc. Natl. Acad. Sci., 44, 885, 1958.

136. Zamecnik, P. C. & E. Keller, J. Biol. Chem., 209, 337, 1954.

137. Zamecnik, P. C. & M. L. Stephenson, Ann. N. Y. Acad. Sci., 88, 708, 1960.

138. Zamecnik, P. C., M. L. Stephenson & J. F. Scott, Proc. Natl. Acad. Sci., 46, 811, 1960.

139. Zillig, W., D. Schachtschabel & W. Krone, Hoppe Zeyler's Z. fur Phys. Chem., 318, 100, 1960.

140. Zubay, G., J. Mol. Biol., 4, 347, 1962.

VITA

Jaime Eyzaguirre was born on October 16, 1935, in Santiago, Chile. He attended elementary and secondary school in the same city, and graduated in 1952. In 1953 he entered the Catholic University of Chile Medical School, and in June of 1958 he obtained the degree of Bachelor in Biological Sciences.

In September 1958 he entered the Graduate College of the University of Illinois in the Department of Chemistry and Chemical Engineering. He held an International Educational Exchange Service fellowship during the academic years 1958-1959 and 1959-1960, a University of Illinois fellowship in the year 1960-1961, a National Institute of Health traineeship for 1961-1962, and a research assistantship in the summer and fall of 1962. The author is also a recipient of a Fulbright travel grant.