BIOSYNTHESIS OF PANCREATIC RIBONUCLEASE

by

Allan J. Morris

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by

Allan J. Morris

has been approved by

_____ Supervisory Committee

_____ Super ory Committee

Reader, Supervisory _____

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Reader, Supervisory Committee

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Head, Major Department

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INTRODUCTION

Concepts of the formation and breakdown of proteins in the living animal
have undergone a number of major changes since 1922 when Mitchell et al. (1)
proposed that, under normal conditions, tissue proteins were inert to
metabolism. Soon after (1924), Folin (2) stated that metabolism of proteins
is limited to that which is necessary to replace proteins lost through "wear
and tear" on the tissues. Borsook and Keighley (3) (1935), however, found
neither of these ideas adequately described their experimental findings.
They were able to show that fifty to sixty per cent of the urinary nitrogen
of humans is derived from endogenous sources rather than the direct metabolism
of dietary substances. They termed the endogenous metabolism "continuing
metabolism" and challenged the idea of metabolically inert proteins.

It remained for Schoenheimer (4) (1939) to demonstrate, with the use
of N^{15} labeled amino acids, that the proteins of the body are undergoing
rapid and continual breakdown and resynthesis. These studies established
that the proteins of the body tissues are in a dynamic rather than a static
state of metabolism.

Since that time the increasing availability and use of isotopes of the
common elements found in biological compounds has made possible a number of
significant advances in the knowledge and understanding of fundamental
processes of protein synthesis. The following pages will present a review
of selected aspects of the investigations conducted to date of the synthesis

of proteins in the cells of plants and animals. In particular, it will be
the purpose of this review to summarize the studies of the locus of protein
synthesis in various types of cells. Consideration will also be given to
the criteria which must be fulfilled in these studies if one is to interpret
the data with a minimum of ambiguity.

The later pages of this thesis will be devoted to a description of the
methods developed by the author for the study of the rate and intracellular
location of the biosynthesis of the pancreatic enzyme, ribonuclease. The
data obtained through the use of a radioisotopic tracer will be presented in
detail as well as a consideration of the significance of the information
gained.

HISTORY

Following the early studies by Hevesy (5) (1923) of the uptake of
radioactive lead by plants, and the general adaptation of isotopic methods
to biological investigations by Schoenheimer (4) (1939), a wide usage of
isotopic tracers has developed. A number of workers have employed labeled
amino acids to trace their incorporation into general protein fractions
of tissue slices, homogenates and cell fractions. Melchoir and Tarver (6)
(1947) reported a small amount of incorporation of S^{35} labeled methionine
into the proteins of rat liver slices. The same year Winnick, Friedberg,
and Greenberg (7) reported the incorporation of C^{14} labeled glycine into
the proteins of intestinal tissue sections. The addition of small amounts
of sodium azide to the incubation was found to completely inhibit the
incorporation of amino acid. In view of the known effects of azide in
biological systems, these workers proposed that the incorporation of an
amino acid requires an oxidative process. Frantz, Loftfield, and Miller
(8) (1947) also noted that the incorporation of labeled alanine into the
proteins of rat liver slices is greatly reduced in the presence of a nitrogen
atmosphere. Radioactive carbon dioxide has also been used to label the
proteins of rat liver slices. Anfinsen et al. (9) (1947) found that upon
incubation the label is incorporated into the dicarboxylic amino acid
residues.

The first successful incorporation of a labeled amino acid into proteins

of a homogenate was achieved by Friedberg, Winnick, and Greenberg (10) (1947).
In the presence of a complex incubation medium containing metal salts, gly-
colysis intermediates, enzyme cofactors and buffers, a small amount of incor-
poration of labeled glycine into the proteins of either a spleen or liver
homogenate was obtained. A study of the requirements of the system showed
that incorporation was inhibited by heating the homogenate, by omission
of the nutrient medium, or by anaerobiosis. Addition of adenosine triphosphate
to the complete system had no effect on the uptake of amino acids.

Several groups of investigators have studied the distribution of injected
amino acid throughout the body of the animal. Friedberg et al. (11) (1948)
found that after intravenous injection of S^{35} labeled methionine into the rat,
the proteins of the intestinal mucosa possess the highest concentration of
radioactive label. The amount of labeling of the proteins of other tissues
was found to be (in decreasing order) kidney, blood plasma, liver, testes,
heart, brain and muscle. Although the pancreas of the rat proved to be too
diffuse for study, these workers showed that the pancreas of the dog incorpo-
rated almost as much amino acid into protein as the intestinal mucosa and
incorporated more than any of the other organs. Winnick et al. (12) (1948),
as well as Borsook and coworkers (13) (1950), found a similar distribution
using other amino acids and other experimental animals. The latter group
subjected guinea pig liver homogenates to differential centrifugation and
demonstrated that the microsomal fractions of the cells possessed the highest
degree of incorporation of radioactive label. Similar results had been found
after incubation of liver homogenates in the presence of radioactive amino
acids (14). Studies of the incorporation of labeled glycine, leucine and

lysine into three particulate fractions and a soluble fraction of guinea pig liver homogenates revealed that each fraction is capable of incorporating amino acid into proteins independently of the others. However, the combination of two particulate fractions produces a synergistic effect upon the incorporation process. Also, differences were noticed in the optimal conditions for lysine incorporation into supernatant proteins from those required for incorporation into the particulate fractions. Too, Peterson et al. (15) have reported synergistic effects on the incorporation process by combining soluble and particulate fractions from rat liver.

Siekevitz and Zamecnik (16) (1951) showed the dependence of in vitro incorporation into liver cell fragments on an oxidizable substrate such as α-ketoglutarate.

Following these early papers on protein synthesis in vivo and in vitro, considerable effort has been directed toward studying the role of the respective cell parts in the overall scheme of protein synthesis. From these works has emerged a wealth of knowledge which, at the time of this writing, leaves one with the concept that the formation of proteins in the intact cell is not a single process but rather a number of processes leading to a similar end. Each cell part studied so far possesses peculiarities with respect to the synthetic process carried out there. It will be useful in evaluating the experimental findings to be presented later in this thesis to consider what is known concerning the synthesis of proteins by isolated cell parts. First, let us consider the synthesis in isolated nuclei.

In 1954 Allfrey (17) described a cell free system consisting of calf thymus nuclei, α-ketoglutarate and phosphate buffer which is capable of

incorporating labeled amino acids into the nuclear proteins. Incorporation of these amino acids is dependent upon the presence of an oxidizable substrate and of the L isomers of the amino acid. Once bound to the nuclear protein, the radioactivity cannot be removed by dilute alkali, by treatment with ninhydrin or the addition of unlabeled "carrier" amino acid (18). Because of these properties the investigator assumed that the incorporated amino acid was present in peptide linkage. It is noteworthy, however, that a net synthesis of protein has not been demonstrated during the period of incubation nor has it been possible to refine the system in order that incorporation into a specific protein could be studied. The desirability of fulfilling both of these experimental conditions will be discussed later in this section.

It is significant that preincubation of the nuclei with bovine ribonuclease is without effect on the incorporation process, since such treatment will be seen to inhibit the microsomal system. However, preincubation with deoxyribonuclease greatly impairs the incorporation process. During a study of the effects of deoxyribonuclease it was observed that addition of supplementary calf thymus deoxyribonucleic acid will restore activity to the system. Allfrey et al. (19) have since investigated a number of polynucleotide substances for their capacity to restore the incorporation process. It was found that deoxyribonucleic acid from many sources, as well as deoxyribonucleic acid in varying states of chemical and enzymatic degradation, can restore the activity loss due to deoxyribonuclease. Of the synthetic molecules tested, only polyadenylic acid shows any restoration activity. These investigators have been able to correlate the effects of

the substances studied with the adenosine triphosphate content of the nucleus. They have concluded that deoxyribonuclease interrupts the oxidative phosphorylation of nucleotide monophosphates which is known to occur in isolated nuclei (20) and results in an insufficient supply of adenosine triphosphate to support protein synthesis. The exact role of deoxyribonucleic acid in this process remains obscure.

Although the studies conducted with isolated nuclei have revealed that an apparent synthesis of proteins can be carried out in that cell part, it is clear from other studies that all cellular proteins do not originate in the nucleus. After removing the nucleus from the unicellular algae _Acetabularia_ _mediterranea,_ Brachet, Chantrenne and Vanderhaeghe (21) (1955) found that the cell fragment still possessed the capacity to grow and to differentiate with a concomitant increase in protein. The cytoplasmic fraction of the cell retained the ability to incorporate labeled amino acids into its proteins at a substantial rate for several weeks following enucleation. In contrast to the isolated nuclei system, however, incubation of the cytoplasmic fraction in the presence of a dilute solution of pancreatic ribonuclease was shown by Stich (22) (1958) to inhibit completely net synthesis of protein. Incubation of the complete algae cell with ribonuclease similarly produces an inhibition of growth, but, unlike the enucleated portion, cell growth is resumed when the ribonuclease is removed. Other instances of an influence upon protein synthesis in one cell part by the presence of another portion will be mentioned later.

It can be seen, then, from studies with cytoplasmic fraction of algae, that the cytoplasm also plays a significant role in the formation of proteins.

A critical examination of cytoplasmic protein synthesis in animals was begun by Zamecnik (23) (1953) using cell fractions from rat liver. The addition of hexose diphosphate to the cytoplasmic fraction of rat liver homogenates increased anaerobic incorporation of labeled amino acids ten fold. A comparison of the in vivo and in vitro uptake of labeled amino acids into the proteins of liver cell fractions showed the microsomal protein to accumulate radioactivity more rapidly in both instances. Subfractionation of the microsomes into the ribonucleoprotein and endoplasmic reticulum portions with deoxycholic acid revealed the ribonucleoprotein to be the most rapidly labeled site in the cell (24). It was later observed that when phosphoenol pyruvate or phosphocreatine was added to the incubation media as substrate the presence of the mitochondrial fraction of the cell was no longer required for incorporation to occur. Under these conditions the microsomal and soluble portions of the cell, when combined, carried out incorporation of amino acids into the general mixture of proteins of the incubation system. Although the microsomal proteins constitute only 28 per cent of the proteins present, they have been found to be labeled eighteen times more rapidly than the supernatant proteins (25). The incorporation mechanism is specific for the L isomers of amino acids. Partial hydrolysis studies of labeled microsomal proteins indicate that the labeled amino acid residues are present in peptide linkage. As in the case of cytoplasmic fraction from algae, incorporation into the rat microsomal system is completely inhibited by the presence of small amounts of pancreatic ribonuclease.

After dialysis of the supernatant fraction, it has been possible to demonstrate a direct requirement for adenosine triphosphate in the microsomal

plus supernatant system. While investigating certain aspects of this require-
ment, Hoagland (26) found an enzyme system in the soluble portion of the
liver homogenate which in the presence of adenosine triphosphate, magnesium
chloride, and a pyrophosphatase inhibitor (potassium fluoride) can carry out
an exchange of P^{32} labeled pyrophosphate with adenosine triphosphate.
Addition of L amino acids to the enzyme system increased the rate of exchange
two to three fold. The rate of exchange depends not only on the concentration
of the amino acids in the media but also on the number of amino acids, which
suggests that each amino acid is acting independently of the others.

In the presence of potassium fluoride, the pyrophosphate exchange
reaction does not lead to an accumulation of pyrophosphate in the reaction
media. However, when fluoride was omitted and salt free hydroxylamine was
put into the system to trap "high energy" compounds, amino acid hydroxamates
of the amino acids present were formed rapidly with a concomitant production
of two equivalents of inorganic phosphate. Under the latter conditions the
adenosine triphosphate content of the incubation media is diminished rapidly.
The formation of amino acid hydroxamates by the liver supernatant possesses
characteristics similar to the amino acid dependent pyrophosphate exchange
reactions. The reaction is specific for L amino acids, and the reaction
rates are dependent not only upon the amino acid concentration but upon the
number of amino acids added to the reaction media.

Hoagland has proposed that the pyrophosphate exchange and hydroxamate
reaction are manifestations of the same set of reactions which lead to the
activation of the amino acid prior to its incorporation into peptide linkage.
The amino acid activation reactions can be explained as follows:

In the presence of KF and absence of NH_2OH acceptor

    1) Enzyme + ATP* $\rightleftharpoons$ Enzyme-AMP-PP

    2) Amino Acid + Enzyme-AMP-PP $\rightleftharpoons$ Enzyme-AMP-Amino Acid + P-P
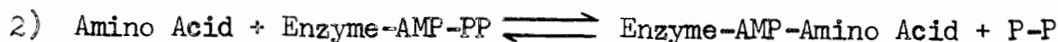
In the presence of NH_2OH acceptor

    3) Enzyme-AMP-Amino Acid + $NH_2OH \longrightarrow$ Amino Acid hydroxamate +

                                             AMP + Enzyme
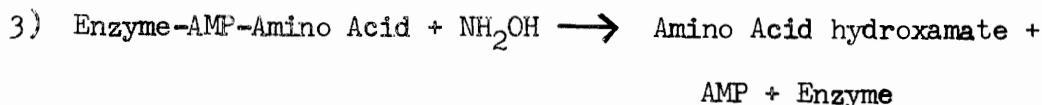
Fractionation of the supernatant of rat liver by isoelectric precipitation at pH 5.1 to 5.3 produced an insoluble fraction possessing all the pyrophosphate exchange and hydroxamate forming activities of the original supernatant fraction but only one-fifth as much total protein. When this "pH 5 enzyme" fraction was incubated with microsomes, adenosine triphosphate, and amino acids, incorporation of the amino acids into microsomal proteins showed a strict requirement for the addition of guanosine triphosphate or diphosphate. The guanosine triphosphate has no effect on the exchange reaction or the hydroxamate formation but seems to be required for some later step in the overall reaction.

An unknown number of activating enzymes are present in the "pH 5 enzyme" fractions. Activating enzymes which are specific for tryptophan (27) and tyrosine (28) have now been prepared in a highly purified form. Evidence for the existence of other activating enzymes specific for the other amino acids has been found (26,29,30). The wide occurrence of activating enzymes throughout biological systems of plant and animal origin implies a role of

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*Abbreviations used in these equations are: ATP (Adenosine triphosphate), P-P (pyrophosphate) and AMP (Adenosine monophosphate).

central importance (27,31,32,33,34).

Using the purified tryptophan activating enzyme, Sharon and Lipmann (35) have been able to correlate the activation of tryptophan analogues with incorporation of these substances into proteins. Their findings imply that the natural amino acid must undergo activation by this enzyme prior to incorporation. In the presence of the purified tryptophan activating enzyme, tryptophan labeled in the carboxyl group with a heavy oxygen atom has been shown to transfer its O^{18} to the phosphate group of adenosine monophosphate during the formation of tryptophan hydroxyamate (36). The transfer is consistent with an intermediate formation of a carbon-oxygen-phosphate bond and lends support to the proposed mechanism of reaction (see equation 2). Synthetic amino acid adenylates have been synthesized and found to serve as a source of adenosine triphosphate indicating that reactions 1 and 2 are reversible processes (34,37).

A critical examination of the "pH 5 enzyme" fraction has revealed that 5 per cent (by weight) of the material present is ribonucleic acid (38). In the presence of adenosine triphosphate and activating enzymes this ribonucleic acid has the unique ability of incorporating amino acids (39). The carboxyl group of the amino acid is now known to be linked by a covalent bond to the 2' or 3' hydroxyl group of the terminal adenylic acid residue on the soluble ribonucleic acid ($_s$RNA) molecule (40). The presence of ribonuclease inhibits formation of this linkage. In ascites tumor cells Hoagland et al. (39) have demonstrated that the $_s$RNA fraction is labeled more rapidly than the ribonucleo protein portion of microsomes. Incorporation into the microsomal protein of a cell free system has also been shown to

depend upon the amount of $_s$RNA present (41). It appears from the most recent evidence that a specific $_s$RNA molecule is required for the binding of each amino acid. The separation of an alanine acceptor $_s$RNA from a tyrosine acceptor $_s$RNA by counter current distribution has been reported by Holley et al. (42). Schweet and coworkers have attained a partial separation of leucine acceptor $_s$RNA from tyrosine acceptor $_s$RNA (43).

Protein synthesis in the microsomal fraction of rat liver has been envisioned by Zamecnik et al. (41) to proceed through at least four distinct steps:

1) Amino acid activation

2) Bonding of the amino acid with soluble ribonucleic acid

3) Peptide formation in the ribonucleoprotein particles of the microsomes

4) Crosslinking and patternization to complete the 3^0 structure.

Although the studies with the microsomal system above have afforded considerable information about the incorporation of amino acids into proteins, the system has not been adaptable to a demonstration of net synthesis during the period of incubation. Since incorporation of amino acids has not been equated to the synthesis of proteins, reservations must be kept as to whether the observed reactions are those of true protein synthesis (44). While most of the work to date with the microsomal fraction has dealt with the radio-activity of the mixed protein fraction, recent immunological assays indicate that the fraction precipitated from the microsomes by rat serum albumin antibody has at least five times as much radioactivity per milligram of protein as the general protein fraction (45). In a similar system of rabbit

reticulocyte origin, Schweet et al. (46) showed the incorporation of labeled amino acids into the hemoglobin molecule.

On the basis of these investigations it would be tempting to speculate that all cytoplasmic proteins are synthesized by the microsomes. Recent studies of the role of the mitochondria of various tissues in the synthesis of protein indicate that the synthetic process is occurring in that fraction as well. Simpson and McLean (47) (1955) found the in vivo distribution of injected amino acid is very different in rat muscle from that in rat liver. While the uptake of labeled leucine by liver mitochondrial protein was less than one-fourth of that taken up by the microsomal protein, the mitochondrial and microsomal fractions of muscle were labeled at approximately equal rates. Cell free systems prepared from rat muscle showed a similar relationship between mitochondrial and microsomal incorporation.

It is now known that isolated rat liver mitochondria will incorporate labeled amino acids into proteins without the presence of either the supernatant or microsomal fractions of the cell (48). The incorporation has been shown to require the presence of a "high energy" compound such as adenosine triphosphate, and, in contrast to the microsomal system, incubation in the presence of pancreatic ribonuclease stimulates rather than inhibits the incorporation of amino acids. Other marked differences between the microsomal and mitochondrial incorporation of amino acids into proteins are the apparent absence of the amino acid activating enzymes and the lack of a requirement for guanosine triphosphate in the mitochondrial system.

The in vitro mitochondrial system from calf heart has now been shown to be capable of carrying out a net synthesis of the specific protein cytochrome C (49,50).

Although the nuclear, microsomal and mitochondrial systems have been seen to possess markedly different properties and requirements for the synthesis of protein, recent evidence suggests that the processes are not entirely independent of one another. Campbell and Greengard (51) have reported that the addition of the nuclei fraction of rat liver to the microsomal plus supernatant system results in an almost complete inhibition of amino acid incorporation. Similarly, addition of the mitochondrial portion of the cell to the microsomal system decreased the amino acid incorporation into microsomal proteins. On the other hand, the presence of the microsomes increases the amount of incorporation of amino acids into the mitochondrial proteins. These findings suggest that a particular cell part may exert a controlling influence upon the synthetic processes occurring elsewhere in the cell. In addition, this work casts considerable doubt upon conclusions, dealing with the rate of synthesis in cell parts, which have been deduced from studies of an isolated cell fraction.

Brookes et al. (52), as well as Butler and coworkers (53), have suggested that other, as yet undefined, pathways of protein synthesis may occur in bacteria. They have observed that the isolated cytoplasmic membrane is capable of incorporating amino acids into bacterial proteins. In addition, Hendler (54) has reported the apparent participation of a lipid fraction in the incorporation of amino acids into ovalbumin by hen oviduct minces.

Most of the investigations described to date have used the radioactivity found in the trichloroacetic acid precipitable protein as the measure of the incorporation of amino acids into proteins. Studies of the labeling of a

specific protein, however, have yielded important information regarding the rate and possible mechanisms of protein synthesis.

Askonas, Simpkin and Work (55) have studied the formation of labeled antiovalbumin by sensitized guinea pig lymph glands. Following the addition of radioglycine to the incubation media, labeled antiovalbumin appears after a short lag period. The rate of appearance of label into the antibody is approximately three times greater than incorporation into the remainder of the gamma globulin fraction. In addition, mild cell damage has been found to prevent incorporation into antiovalbumin, yet the incorporation of label into the gamma globulin fraction, as well as other cell proteins, is unaltered. One must conclude that if incorporation rates are to be used as a measure of synthetic processes then it becomes necessary to relate that incorporation to a specific protein.

Peters and Anfinsen (56) (1950) developed an immunological method for the analysis of chicken serum albumin and showed that after incubation of chicken liver slices with labeled carbon dioxide the serum albumin present was labeled forty-seven times more than the corresponding albumin fraction. Rate studies have indicated the microsomal serum albumin to be labeled some-what earlier than that present in the mitochondrial portion of the cell.

Egg ovalbumin has been labeled by Steinberg and Anfinsen (57) by means of radioactive bicarbonate injection into a laying hen. Enzymatic degradation of the purified ovalbumin revealed that the aspartic acid residues in different portions of the molecule possessed significantly different amounts of C^{14}. Ovalbumin prepared by an _in vitro_ technique using labeled alanine gave similar results. Incorporation of amino acids into insulin and ribonuclease by

slices in vitro during short term incubations was also found to result in uneven distribution of the label. As the period of incubation is increased the distribution of labeled amino acid throughout the protein molecule becomes more uniform. Although these studies were not attended by a net increase of the particular protein under investigation, the recent report of the net synthesis of cytochrome C by isolated calf heart mitochondria has described a non-uniform labeling of that protein (58).

While silk fibroin has been reported to possess non-uniform distribution of labeled glycine after injection of that amino acid into the silk worm Bombyx mori (59), most of the attempts to demonstrate uneven labeling in vivo have been unsuccessful (60). It has been suggested that the differences obtained in vivo and in vitro may be due to differences in the rate of equilibrium of protein precursors under these respective conditions. In order that a non-uniform distribution of label can occur, Steinberg et al. (61) propose that some intermediate peptides or amino acid pools must be present between the free amino acids and the completed peptide chain.

The possibility that a direct replacement of a portion of the peptide chain in an intact protein might occur was suggested by Schoenheimer (62) as early as 1939. Craddock and Dalgliesh (63) have proposed that uneven distribution of labeled amino acid in a protein may be the result of such an exchange phenomenon. If, in the absence of net synthesis, the finished protein were to collide with the template from which it originated, a portion of that protein might be labilized and exchange several of its residues with other amino acids or peptides. In the presence of net synthesis these investigators speculate that the template would be inaccessible to the free

protein since it would be covered by amino acids and peptides in the process of polypeptide formation. They have observed no lag period during the labeling of ribonuclease in rat pancreas slices when no net synthesis of that protein occurred. Others have found as much as fifty minutes lag before the appearance of labeled proteins in pancreatic secretions in vivo (64). Craddock and Dalgliesh have proposed that labeling occurred by exchange in the first instance and by de novo synthesis in the latter.

Other evidence which indicates that an exchange reaction may occur independently of de novo synthesis of protein comes from inhibition studies with amino acid analogues. Growth and net synthesis of protein in Staphlococcus aureus are completely blocked by the addition of p chlorophenylalanine to the incubation media. The incorporation of labeled glutamic acid into bacterial proteins has been found to continue unabated under these conditions (65). Similar incorporation has been noted during the inhibition of growth by antibiotics. Rabinovitz et al. (66) reported that the presence of o fluoro-phenylalanine in an ascites tumor cell suspension completely inhibited the incorporation of phenylalanine into cellular proteins. The other amino acids in the reaction media mixture were incorporated at a normal rate. An exchange reaction which is not necessarily a synthetic mechanism has been offered as one possible explanation for incorporation of an amino acid in the absence of the availability of a complete complement of amino acids.

In human He La cell cultures Eagle and coworkers (67) found than an exchange reaction occurred at the rate of one per cent per hour in the presence or absence of net synthesis. Their studies, however, do not

differentiate between intracellular breakdown and reutilization of proteins and a direct exchange with intact proteins of the type proposed above. The distribution of heavy oxygen (O^{18}) in the peptide chains of E. coli proteins following incubation in the presence of H_2O^{18} indicates that the peptide bonds are continually being opened and closed in the absence of net synthesis (68).

Because of the number of processes leading to protein formation which seem to take place in living cells, as well as the marked differences of labeling which have been observed from one protein to another in the same system, it is highly desirable that any study of the pathway or rate of protein formation be conducted with respect to a specific protein. Since previous studies strongly suggest the presence of a direct exchange of amino acids in intact proteins, a demonstration of net synthesis of that specific protein is required if the experimental findings are to be interpreted with respect to the synthetic process.

In the pages which are to follow will be found a detailed account of studies, carried out by the author, of the in vivo site of formation of ribonuclease within mouse pancreas. Preliminary investigations were directed toward the development of a set of conditions wherein a net synthesis of pancreatic ribonuclease would occur. Injection of a labeled amino acid, L valine, during a period of rapid synthesis of ribonuclease permitted introduction of sufficient radioactivity into that enzyme to allow a determination of its specific activity. Differential centrifugation of the pancreatic homogenates into four cell fractions, followed by a determination of the specific activity of ribonuclease in each fraction, has established the

microsomes as the site of the _in vivo_ synthesis of ribonuclease. A study of the distribution of labeled ribonuclease in the cell fractions as a function of time indicates that the enzyme is transferred to the zymogen granule fraction by way of the soluble portion of the cell.

MATERIALS AND METHODS

Animals

General procedures.

All of the animal studies reported here were performed with adult mice
of the CBA strain. An equal number of males and females was used in order
to minimize any sex difference which might exist. After fasting the mice
for three hours each animal was given an intraperitoneal injection of 0.1
ml. of aqueous pilocarpine hydrochloride solution (10 mg/ml). The animals
were then placed in open screen cages and allowed free access to water.
Following one additional hour of fasting, the treatment of the animals differed
as described below.

Fasted mice.

Following the administration of pilocarpine hydrochloride the mice were
allowed free access to water but were maintained without food for the remainder
of the experiment. At specific intervals the mice were killed by snapping
the spinal cord. The pancreatic tissue was rapidly removed, weighed on a
Roller-Smith torsion balance, and homogenized as described in "Preparation
of pancreatic homogenates".

Fed mice.

After the injection of pilocarpine, the mice were fasted for one hour,
then offered Rockland Mouse Pellets and water for the remainder of the
experiment. At specific intervals the mice were killed and the pancreas

removed and processed as described in the above section.

Cell fractionation studies.

In order to obtain sufficient pancreatic tissue to allow fractionation of the homogenate into cell fractions, it became necessary to develop a procedure which would allow thirty mice to be treated and sacrificed in a uniform manner. Fifteen mice from each sex, three months of age, were selected and fasted for three hours. Pilocarpine was then injected as described above except that the animals were treated in an "assembly line" fashion and injected at regular intervals over a period of thirty minutes. The mice were fasted for one hour after injection of the drug and then allowed Rockland Mouse Pellets and water for the remainder of the experiment.

Exactly eighteen hours after the pilocarpine had been injected, each mouse was given an intraperitoneal injection of 2 μc. of uniformly labeled $\underline{L}$ valine in 0.1 ml. of distilled water. At an exact period of time after introduction of the labeled amino acid the mice were sacrificed and the pancreatic tissue rapidly removed. Pancreatic tissue was pooled in a tared beaker containing approximately 10 ml. of cold $(0°)$ 0.25 $\underline{M}$ sucrose. By processing the animals in the same sequence at each step in the preparation, it was possible to keep the time period between drug administration and isotope injection as well as isotope injection and sacrifice the same for each of the thirty mice.[*] The beaker containing the sucrose solution and pancreatic tissue was then weighed again on an analytical balance, and the tissue was homogenized as described under "Cell fractionation procedure".

Reagents

Pilocarpine hydrochloride (U.S.P. grade) was purchased from the Merck, Sharp and Dohme Company. A fresh solution was prepared for each series of injections.

Starch used as a substrate in the amylase assays was Merck Soluble Starch, suitable for iodometry. Starch (600 mg.) was suspended in 50 ml. of deionized water, 10 ml. of 0.5 $\underline{M}$ sodium chloride and 30 ml. of 0.2 $\underline{M}$ phosphate buffer at pH 7.2. The slurry was heated to boiling and a clear solution was obtained. The mixture was then cooled to 37° and the volume was adjusted to 90 ml. with water, if necessary. A fresh starch substrate solution was prepared for each set of amylase assays.

Iodine reagent. Fifteen grams of reagent grade potassium iodide, obtained from the Baker Company, and 1.5 gms. of Merck crystalline iodine were dissolved in deionized water to a final volume of 500 ml.

Yeast ribonucleic acid substrate was prepared from Schwarz sodium yeast nucleate by the method of Vischer and Chargaff (69). The purified ribonucleic acid was stored in a dry form at -20° until needed. A 0.3 per cent aqueous ribonucleic acid solution was then prepared and adjusted to pH 5.0 with dilute sodium hydroxide. After filtration the substrate solution was placed in 10 ml. lustroid centrifuge tubes and frozen until used.

Standard ribonuclease solutions were prepared from recrystallized bovine pancreatic ribonuclease furnished by Armour and Company. The crystalline enzyme was weighed on a Mettler automatic balance and diluted with 0.2 $\underline{M}$, pH 5.0 acetate buffer to a final concentration of 1 μg. per ml. The diluted solutions were frozen until used.

<u>Resin for chromatography</u>. The carboxylated polystyrene XE-64 was purchased from the Rohm and Haas Company and purified and sized as described by Hirs (70) for analytical chromatography. It was found that a better resolution of materials could be obtained if the resin was regenerated periodically as described by Hirs and stored in the cold in a dry state until used. The resin was prepared for chromatography by suspending it in a small volume of water and adjusting the pH of the stirred suspension to approximately 6.5 with concentrated sodium hydroxide or hydrochloric acid. The wet resin was then autoclaved for forty-five minutes at 15 p. s. i. pressure to remove bacterial contamination. The suspension was allowed to settle and the aqueous phase decanted. Several changes of 0.2 $\underline{M}$, pH 6.47 phosphate buffer were then added, and the mixture was stirred magnetically in the cold room (5°) overnight. The resin was then poured into a chromatography column as described later.

<u>Phosphate buffer</u>, 0.2 $\underline{M}$, pH 6.47, used in chromatographic studies was prepared by weight from analytical reagent grade mono- and di- basic sodium phosphate (70). Deionized or glass distilled water was used in all instances. A few small crystals of thymol was added as a preservative, and the solution was autoclaved as described above. Buffer solution was prepared for each chromatographic analysis and stored in the cold room during the preparation and equilibration of the column.

<u>Acetate buffer</u>.

The acetate buffers used in the analysis of ribonuclease activity were prepared by mixing solutions of 0.2 $\underline{M}$ acetic acid and 0.2 $\underline{M}$ sodium acetate and observing the final pH of the solution on a Leeds and Northrup or

Cambridge pH meter. Gelatin, which had been dissolved in a small volume of boiling buffer, was added to a final concentration of 0.001 per cent.

For analysis of crude homogenates, cell fractions and other unbuffered solutions an acetate buffer of pH 5.00 was prepared. Analysis of column eluates and other solutions which contained $0.2 \underline{M}$, pH 6.47 phosphate were carried out with $0.2 \underline{M}$ acetate buffer of pH 4.88 containing 0.001 per cent gelatin in order that the pH of the incubation media would be 5.0. All acetate buffer solutions were stored at 5° until used.

Orcinol was obtained from the Matheson Company and further purified according to the procedure of Schneider (71).

Fresh orcinol reagent was prepared each day by dissolving 100 mg. of orcinol in 10 ml. of a mixture of 0.5 per cent ferric chloride in concentrated hydrochloric acid.

Solutions for phosphate analyses were prepared according to the procedure of Gomori (72).

Standard phosphate solution.

A sample of analytical reagent grade KH_2PO_4 obtained from the Mallincrodt Chemical Works was dried at 120° overnight. A portion of the dried reagent was weighed on an analytical balance and diluted to a final concentration of 0.1 mg. of phosphorus per ml. with deionized water. The standard solution was kept frozen until used.

Standard deoxyribonucleic acid solution.

Twenty-five mg. of highly polymerized deoxyribonucleic acid obtained from the Worthington Biochemical Corporation was dissolved over a 12 hr. period in 25 ml. of cold deionized water and dialyzed against 20 l. of distilled

water. The solution was then standardized by total phosphate analysis as described under "Analytical Methods". The standard solution was kept frozen when not in use.

Standard ribonucleic acid solution.

Twenty-five mg. of purified yeast ribonucleic acid was dissolved in 75 ml. of deionized water. The solution was standardized by total phosphate analysis as described under "Analytical Methods". The standard solution was stored in a frozen state.

Micro-Kjeldahl reagents.

Boric acid solution.

Ten grams of boric acid was dissolved in 500 ml. of boiling distilled water according to the procedure of Ma et al. (73). Sufficient methyl red-bromcresol green indicator was added to provide a distinct color change during titration.

Catalyst.

Ten grams of HgO (red oxide) was dissolved in a solution of 12 ml. of dilute sulfuric acid (12 ml. H_2SO_4 + 88 ml. H_2O).

Basic thiosulfate reagent.

To 500 ml. of 40 per cent sodium hydroxide (w/v) was added sodium thiosulfate to a final concentration of five per cent.

Standard sulfuric acid.

The sulfuric acid solution used to titrate the analytical sample was 0.00985 N as determined with a dilute solution of sodium hydroxide which in turn had been standardized with potassium acid phthalate.

Standard urea solution.

A solution of 213 mg. of urea in 100 ml. of water was prepared. The solution thus contained 1 mg. of nitrogen per ml. of standard solution.

Radioisotope.

Uniformly labeled $\underline{L}$ valine of a specific activity of 8.0 mc. per $\mu\underline{M}$ was purchased from the Nuclear Instrument and Chemical Corporation. The amino acid was dissolved in water to a final concentration of 2 μc. per 0.1 ml. and frozen until used.

Sucrose solutions.

To 100 ml. of deionized or glass distilled water was added 8.56 gms. of analytical reagent grade sucrose obtained from the Mallinckrodt Chemical Works. A fresh solution was prepared for each usage and kept in an ice bath during the course of the experiment.

Bromsulfalein reagent.

One ml. of 5 per cent bromsulfalein solution obtained from Hynson, Westcott and Dunning, Inc. was diluted with 100 ml. of 1 $\underline{N}$ hydrochloric acid, 50 ml. of 1 $\underline{M}$ citric acid and deionized water to a final volume of 250 ml.

Trichloroacetic acid reagent.

All trichloroacetic acid solutions were prepared from analytical reagent grade material obtained from the Mallinckrodt Chemical Works.

Standard serum albumin solution.

As a protein standard for the colorimetric analysis of protein concentration, 40 mg. of four times recrystallized bovine serum albumin was weighed on an analytical balance and dissolved in 10 ml. of water. The protein

standard was diluted further with water as necessary.

Cysteine hydrochloride solution.

A fresh 1 per cent aqueous solution of cysteine hydrochloride (Nutritional Biochemicals Corp.) was prepared each day for the deoxyribonucleic acid analyses.

Hydrogen peroxide.

The peroxide solution used in phosphorus analysis digestions was Mallinckrodt analytical reagent grade, 30 per cent hydrogen peroxide containing 0.0005 per cent or less analyzed phosphorus.

Analytical Procedures

Preparation of pancreatic homogenates.

Upon removal from the mouse the pancreatic tissue was weighed rapidly on a Roller-Smith torsion balance and placed in 2.0 ml. of cold 0.25 $\underline{M}$ sucrose solution. A homogenate was prepared using a Potter-Elvehjem homogenizer with a Teflon pestle. The tissue was homogenized for two minutes in the cold and then frozen. When samples were to be removed for analysis, the homogenate was thawed in an ice bath.

Ribonuclease assays.

Ribonuclease activity of pancreatic homogenates.

A portion of the pancreatic homogenate was diluted with cold 0.9 per cent sodium chloride solution and assayed by the procedure of Dickman et al. (74). Ribonuclease activity at pH 5.0 is expressed as the A_{260} reading per mg. wet weight of pancreatic tissue. An inactive control was included with each sample analyzed.

Ribonuclease activity of cell fractions.

All cell fractions were diluted with 0.9 per cent sodium chloride solution and analyzed as above. In order that ribonuclease pool sizes could be measured the microsomal and soluble fractions were treated with 1 $\underline{N}$ H_2SO_4 to a final concentration of 0.25 $\underline{N}$ unless otherwise stated. The solution was then adjusted to pH 5.0 and analyzed using pH 5.0 acetate buffer. Enzymatic activity is expressed as ribonuclease units. One unit is defined as that quantity of enzyme resulting in an A_{260} reading of 1.0.

Ribonuclease activity of column eluates.

Enzymatic activity was determined as above except that the pH 4.88 acetate buffer was used in order to compensate for the phosphate buffer present. When necessary, the eluate samples were diluted with 0.2 $\underline{M}$ phosphate buffer of pH 6.47. An inactive control was routinely included with the analysis of every fourth eluate tube. It was observed that A_{260} absorbing materials are eluted near the column front. The two areas of ribonuclease were reassayed in duplicate with an inactive control for each fraction.

Amylase activity of pancreatic homogenates.

A portion of the pancreatic homogenates was diluted with cold 0.067 $\underline{M}$ phosphate buffer at pH 7.2 and analyzed for amylytic activity by the Hokin modification of the Smith and Roe assay (75). A reagent blank was included with each set of analyses performed. The homogenate from each pancreas was analyzed individually. Contrary to the information available (76), the starch-iodine color was found to be markedly influenced by variations in temperature. For this reason, the distilled water used to dilute the starch-iodine color was stored in a separate bottle and maintained at 26 $\pm$ 1°.

All values have been expressed as Smith and Roe units of amylase activity per mg. wet weight of pancreas. A Smith and Roe unit has been defined as the amount of enzyme that will bring about the degradation of 10 mg. of starch in thirty minutes under the conditions of the assay (76).

Pooling of pancreatic homogenate samples.

A pooling of homogenates was necessary in order to obtain sufficient quantities of the homogenates to analyze for nucleic acids and proteins. An equivalent amount of homogenate from each animal was combined with the other homogenates of that time period, and analyses were performed on the pooled homogenates.

Protein analyses.

Bromosulfalein method.

A modification of the colorimetric assay for protein described by Nayyar and Glick (77) was developed and used to analyze the protein content of the pancreatic homogenates. A 0.5 ml. portion of the diluted homogenate was placed in a conical centrifuge tube, and 0.45 ml. of 1 $\underline{N}$ sodium hydroxide was added. The mixture was stirred with a glass rod, and 1.10 ml. of the bromsulfalein reagent was added, mixed, and allowed to stand for twenty minutes at room temperature. The solution was centrifuged in a clinical centrifuge for fifteen minutes at maximum speed. A 0.5 ml. aliquot of the supernatant solution was transferred to a tube containing 5.0 ml. of 0.1 $\underline{N}$ NaOH. The purple solution obtained was placed in a 16 x 150 mm. cuvette, and the absorbancy at 580 mμ. was determined in the Coleman Jr. Spectrophotometer.

The serum albumin standard solution was used to establish a protein concentration curve for the method. As can be seen in the curve shown

below (Graph I), the values reach a maximum at a concentration of 550 μg. of protein and then decline. Thus, when using this analytical method it was necessary to employ several concentrations of the same sample to ascertain that the absorbancy values obtained were those for the ascending side of the curve in the range of from 0 to 350 μg. of protein.

ΔA_{580}

$$\Delta A_{580} = \text{Control } A_{580} - \text{Observed } A_{580}$$

μg. protein per 0.5 ml.
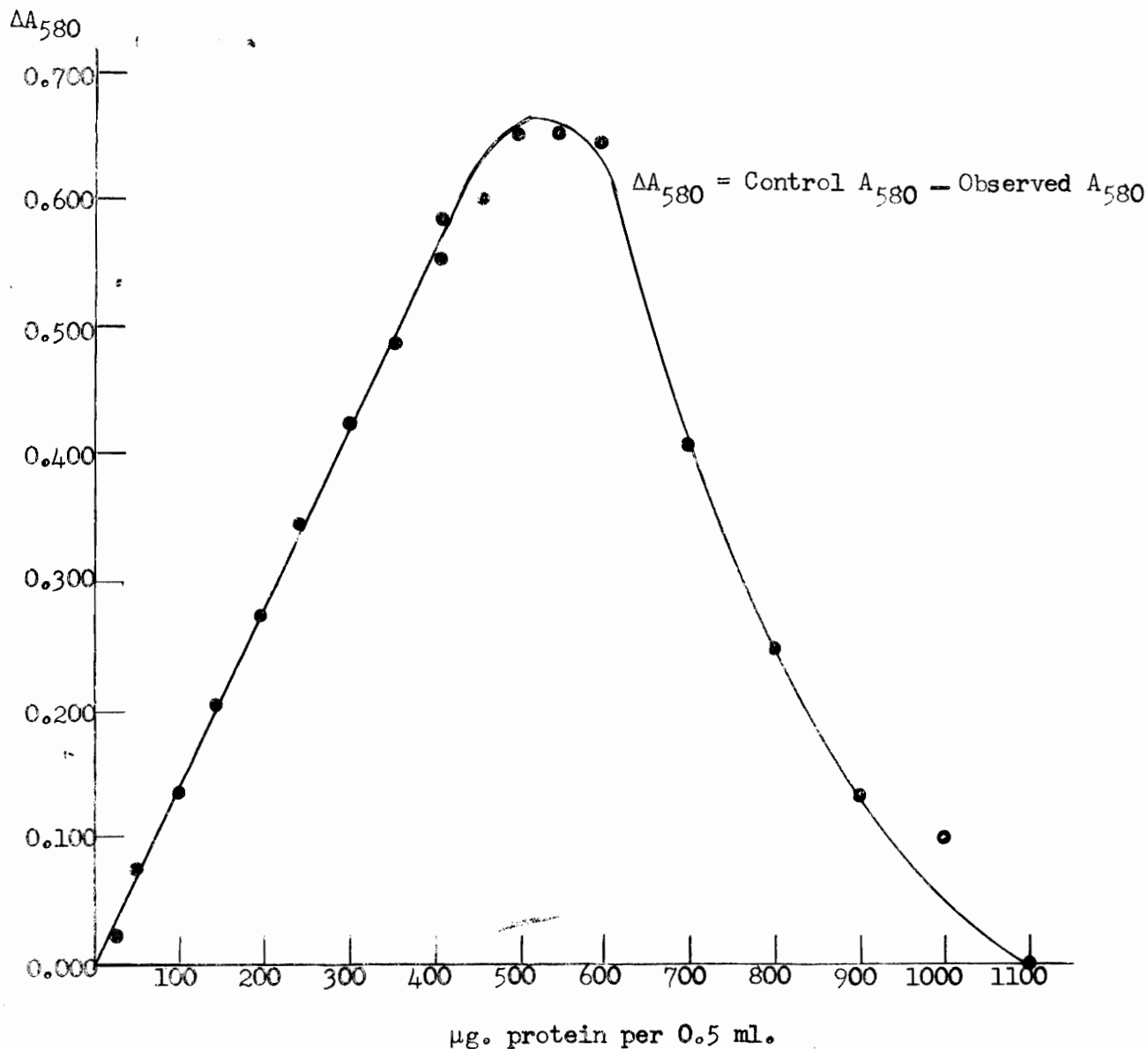
Graph I. Protein Concentration Curve - Bromsulfalein Assay

Micro-Kjeldahl analysis of pancreatic homogenates.

A 0.4 ml. portion of the pooled homogenate described in an earlier section was pipetted into the bottom of a clear micro-Kjeldahl flask. One ml. of concentrated sulfuric acid was then added followed by approximately 0.5 gm. of crystalline K_2SO_4 and 0.5 ml. of the catalyst reagent. The neck of the flask was washed down with 2.0 ml. of distilled water and several glass beads were added to control bumping. The sample was heated on a Kjeldahl digestion rack for at least one hour and until the sample remaining was clear and colorless. The flask was then cooled and transferred to a distillation apparatus. Following the addition of 5.0 ml. of the basic thiosulfate reagent and an appropriate quantity of distilled water, the flask was heated to boiling, and the ammonia was distilled into 10 ml. of the boric acid reagent. The distilled sample was then titrated with the standard sulfuric acid reagent. Six samples could be digested and distilled at one time with the equipment available. A urea standard and a reagent blank were included in each set of analyses.

The protein concentration of the pancreatic tissue has been computed on the assumption that homogenate protein is sixteen per cent nitrogen. The nitrogen content of the nucleic acids was calculated from the ribonucleic acid and deoxyribonucleic acid analyses and subtracted from the nitrogen values for the crude homogenate. Protein concentration has been expressed as the per cent protein per wet weight of pancreas.

Micro-Kjeldahl analysis of trichloroacetic acid precipitable proteins.

The protein fraction remaining after the extraction of nucleic acids (see section on nucleic acid extraction) was dissolved in 10.0 ml. of 1 $\underline{N}$

sodium hydroxide. A 2.0 ml. portion of the solution was analyzed for nitrogen as described above. Protein concentration has been expressed as per cent protein per wet weight of pancreas.

Extraction of nucleic acids.

A 3.0 ml. portion of each pooled homogenate was fractionated for both nucleic acids and proteins by the Schneider procedure (71). A 7.5 ml. portion of cold 10 per cent trichloroacetic acid was added to the homogenate sample, and the mixture was centrifuged in a clinical centrifuge at 5° for fifteen minutes. The sediment was extracted twice with 5 ml. of 95 per cent ethanol at room temperature and centrifuged after each extraction. The residue was then suspended in a mixture of three parts ethanol to one part diethyl ether and heated for five minutes at 50°. After centrifugation the precipitate was suspended in 2.5 ml. of 5 per cent trichloroacetic acid and heated for fifteen minutes at 90° in a water bath. The solutions were cooled, centrifuged, and the supernatant drawn off with a capillary pipette. The precipitate was washed twice with 1.0 ml. portions of 5 per cent trichloroacetic acid. Centrifugation was performed after each wash. The combined supernatant and washes were analyzed for nucleic acid content. The precipitate was dissolved in 1 $\underline{N}$ sodium hydroxide and analyzed for protein nitrogen content as described in the preceding section.

Phosphorus analyses.

Colorimetric phosphate analyses were performed according to the procedure of Gomori (72). A concentration curve was determined using the standard phosphate reagent. The standard deoxyribonucleic acid and ribonucleic acid solutions were digested with 2 ml. of concentrated sulfuric acid at 110°.

After the water had evaporated, three drops of the peroxide reagent were added
to each tube, and the temperature was elevated to 200° for one hour. The tubes
were cooled, and 2.0 ml. of water were added to each. After heating again for
a few minutes in a boiling water bath, the solutions were analyzed colorimetri-
cally for phosphorus content. All subsequent uses of deoxyribonucleic acid
and ribonucleic acid standard reagents are based upon their concentrations
as determined by phosphorus analyses.

Analyses of ribonucleic acid.

Ribonucleic acid concentrations of the trichloroacetic acid extracts were
determined by the orcinol method (71). A concentration curve was established
with the standard ribonucleic acid reagent; the experimental concentrations
were determined from that curve. A sample of the trichloroacetic acid extract
and water of 1.5 ml. total volume was combined with 1.5 ml. of the orcinol
reagent and heated on a boiling water bath for twenty minutes. The solution
was cooled rapidly, and the absorbancy at 660 mμ. was determined in the
Coleman Jr. Spectrophotometer using 9 x 75 mm. calibrated culture tubes as
cuvettes.

Ribonucleic acid content of the pancreatic tissue was calculated on the
assumption that the molecule contains 9.14 per cent phosphorus. All values
were expressed as per cent ribonucleic acid per wet weight of pancreas.

Analyses of deoxyribonucleic acid.

Deoxyribonucleic acid content of the trichloroacetic acid extracts was
determined by the cysteine-sulfuric acid method of Brady (78). A 0.5 ml.
sample to be analyzed was placed in a thin-walled glass test tube, and 0.05
ml. of the one per cent cysteine hydrochloride reagent was added with a micro-

pipette. The tube was then chilled in a sodium chloride-ice-water mixture, and a 5.0 ml. portion of very cold (-15°) concentrated sulfuric acid was added down the side of the tube so that a layering was obtained. While in the ice bath the solutions were mixed with a glass stirring rod, and the tube was placed in a 25° water bath for twenty minutes. The reaction mixture was then transferred to a 16 x 150 mm. calibrated test tube, and the absorbancy at 474 mµ. was read immediately in the Coleman Jr. Spectrophotometer. A concentration curve was determined using the standard deoxyribonucleic acid reagent, and all experimental values were related to that curve. A reagent blank and a standard solution were included with each set of analyses carried out on the trichloroacetic acid extracts. The concentrations determined experimentally are expressed as per cent deoxyribonucleic acid per wet weight of pancreas and are calculated on the assumption that deoxyribonucleic acid contains 9.14 per cent phosphorus.

Extraction of ribonuclease.

A modification of the extraction procedure of Hirs, Stein and Moore (79) was developed in order to prepare the ribonuclease of homogenates and cell fractions for chromatography. The homogenate or cell fraction was mixed with cold dilute H_2SO_4 to a final concentration of 0.25 $\underline{N}$. After standing for a few minutes at 0° the mixture was centrifuged in an International clinical centrifuge, and the supernatant was separated by decantation. The soluble fraction was then adjusted to pH 5.7 with dilute sodium hydroxide and allowed to stand for ten minutes. After centrifugation the supernatant was decanted and used in the chromatographic procedures. For certain of the cell fractions, especially nuclei and supernatant fractions, further purifi-

cation was necessary due to the large mass of materials present. The pH 5.7 supernatant was passed through a 1.2 x 10 cm. column containing XE-64 resin at pH 6.47. The extract was allowed to seep into the resin bed and was eluted with 60 ml. of 0.2 $\underline{M}$ phosphate buffer at pH 6.47. A large portion of the contaminating substances remained in the resin bed during this procedure while ribonuclease was eluted. The eluate was then dialyzed against 0.001 $\underline{M}$ versene at pH 7.0 in a constant flow, rocking dialyzer for approximately seven hours, and the dialyzed extract was lyophilized. The remaining solids were then dissolved in a small volume of water and used in the chromatographic procedures.

Incubation of microsomal and soluble fractions of pancreas with 0.01 $\underline{M}$ versene at pH 9.0 has been reported by Dickman et al. (80) to result in a significant increase in ribonuclease activity. Thus, this versene treatment was included for most of the microsomal and supernatant fractions. The mixture was then brought to pH 1.2 with cold, dilute H_2SO_4 and extracted as described above. Recent work, however, indicates the H_2SO_4 treatment alone is sufficient to provide maximal activation of ribonuclease (81).

Chromatographic procedures.

Preparation of chromatography columns.

A slurry of the equilibrated XE-64 resin was poured into a glass column to produce a resin bed of 0.9 x 30 cm. The resin was then washed rapidly with 0.2 $\underline{M}$ phosphate buffer at pH 6.47 for at least twenty-four hours. All preliminary preparation of the chromatography columns was carried out at 5°.

When the eluate solution became exactly pH 6.47 the column was transferred

to a Technicon drop-counting fraction collector. After allowing the buffer to drain to the level of the resin bed, the enzyme extract was added in a volume of 2 to 4 ml. The sample was allowed to drain into the resin, and 1 ml. of buffer solution was added to wash the sample into the resin. Buffer solution was then added slowly to fill the column and tubes leading to the reservoir. The rate of eluate flow was adjusted to 2-3 ml. per hour. Elution was carried out at room temperature.

Collection of column eluate.

Collection of the eluate solution was begun at the instant the ribonuclease extract was first placed on the top of the column. The fraction collector was usually adjusted to collect twenty-two drops or 1.6 ml. per tube. As elution progressed the collected tubes were covered with a Parafilm seal and frozen until enzyme analyses could be carried out.

Cell fractionation procedure.

The pooled pancreatic tissue from thirty mice (3.5 to 4.0 gm.) was homo-genized at 0° for two minutes in 26 to 29 ml. of 0.25 $\underline{M}$ sucrose. Differential centrifugation of the pancreatic homogenates into the respective cell fractions was performed by a modification of the procedure of Van Lancker et al. (82) as indicated in Table I. Cell fractions were washed by resuspension. All operations were conducted at 0°, and the cell fractions were frozen immediately after preparation.

Determination of radioactivity.

Chromatography column eluates were counted by plating 0.5 ml. of the eluate solution. Homogenates and cell fractions were diluted with 0.2 $\underline{M}$ phosphate buffer before being plated. The solution to be counted was placed

Table I. Differential Centrifugation of Pancreatic Homogenates

Cell fraction	R.P.M.	g.	Time mins.	Head No.	Centri-fuge	No. of washes	Vol. of washes ml.
Nuclei	1,500	600	10	250	International No. 2	3	5-7
Zymogen granule and mitochondria	13,000	11,125	16	40	Spinco Model L	2	3-5
Microsome	40,000	105,000	30	"	"	2	9
Supernatant $_I$	40,000	105,000	30	"	"	--	--
Postmicrosome $_I$	40,000	105,000	60	"	"	0	--
Postmicrosome $_{II}$	40,000	105,000	240	"	"	0	--
Supernatant $_{II}$	40,000	105,000	240	"	"	--	--

on a tared aluminum phanchet with a pipette and was spread over an inscribed

area of 381 mm^2. The solution was then dried under an infrared lamp, and

the planchets were weighed again on the Mettler analytical balance. The dried

sample weighed from 13 to 14 mg. Radioactivity of the dried samples was deter-

mined with a Nuclear Chicago, automatic, windowless, gas-flow counter con-

sisting of a model 161 A scaler, model C-110B automatic sample changer, model

C 1118 interval timer, model D47 gas flow counter and a model T 3 time delay.

Each sample was counted for the period of at least 2,560 counts. Background

radiation was subtracted from the observed radioactivity. The self-absorption

of radioactivity by the phosphate present on the planchet was determined, and

the correction curve illustrated below was computed (Graph II). All values of
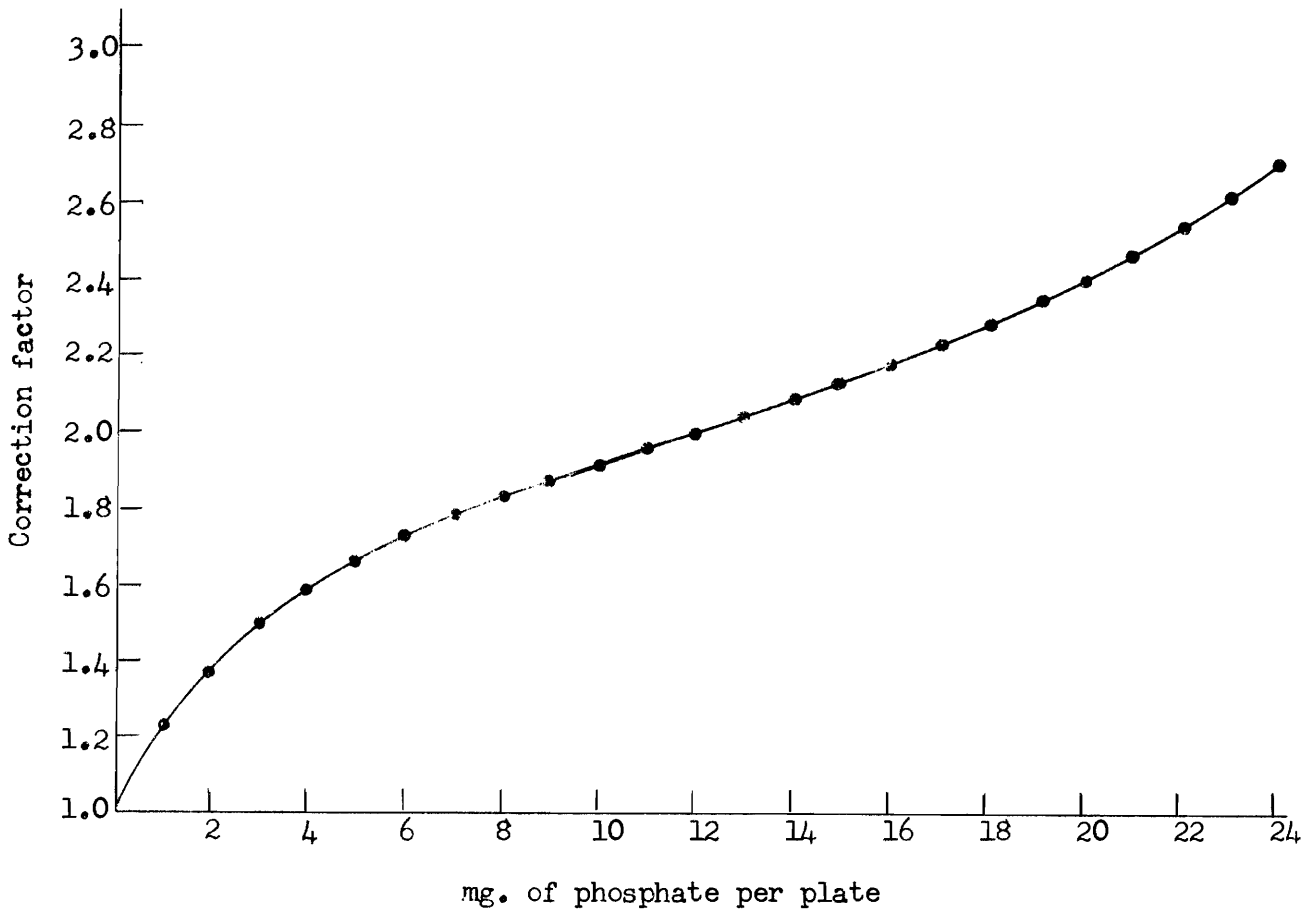
Graph II. Correction Curve for Phosphate Self-absorption

radioactivity reported have been corrected for self-absorption. Excepting the zymogen granule and nuclei fraction chromatograms (five minutes after isotope injection), eluate fractions containing the maximum enzymatic activity possessed more than fifty counts per minute per 0.5 ml.

Calculations.

Radioactivity. The radioactivity of each sample was calculated as follows:

$(\text{CPM}_{observed} - \text{CPM}_{background})$ X Self Absorption Correction Factor equal $\text{CPM}_{corrected}$.

Specific activity.

The specific activity of radioactive ribonuclease has been defined as the counts per minute per 0.5 ml. of column eluate divided by the enzymatic activity (A_{260} value) obtained with 0.1 ml. of eluate multiplied times one hundred. Only those tubes containing sufficient enzymatic activity to give an A_{260} value of 0.100 or more per 0.1 ml. were used for the determination of specific activity.

Standard deviations.

In order to compute the standard deviation in the determination of the specific activity of ribonuclease, the eluate tubes were analyzed separately for radioactivity and enzymatic activity. The specific activity values obtained for the major elution peak of ribonuclease were then averaged, and the standard deviation was computed by the method of Youden (83).

RESULTS AND DISCUSSION

Pilocarpine Studies

Objectives.

The preliminary studies described in this thesis have been performed in order to establish a set of experimental conditions which would allow an investigation of the role of the various components of the pancreas cells during net synthesis of a specific protein in vivo.

The rapid incorporation of labeled amino acids into pancreatic proteins was mentioned in an earlier section (see History). Studies of the formation of amylase in mouse pancreas by Allfrey and coworkers (84) led to the conclusion that protein synthesis in the pancreas, when compared on a weight basis, is more than ten times greater than that in the liver or the kidney.

Pancreatic tissue is capable of rapid protein synthesis without concomitant changes in cell size or cell population (85). Therefore, changes in the amount of protein present due to tissue growth or increased cell structure do not occur. The acinar cells of the pancreas appear to be highly specialized for the formation, storage, and secretion of digestive enzymes (85). Correlation of the number of secretory granules (zymogen granules) with the enzymatic activity of the pancreas dates back well into the last century (1875) (86). Recently Hokin (87) isolated zymogen granules from dog pancreas homogenates and demonstrated the concentration of amylase and lipase there to be greater than in any other portion of the cell. The zymogen granules are now generally

accepted to be the storage place of the secretory enzymes prior to their secretion into the duct system of the pancreas (85).

Harper and Mackay (88) have shown that the secretions of the pancreas are controlled by both hormonal and neural factors. While the secretion of the alkaline fluid from the pancreas is stimulated by the hormone secretin, the release of the enzyme-rich zymogen granules can be induced by either the hormone pancreozymin or by stimulation of the vagus nerve. Consequently, wide use has been made of parasympathomimetic agents such as pilocarpine and carbamylcholine to deplete the acinar tissue of zymogen granules and secretory enzymes (89,90,91,85,92,93). The depletion of zymogen granules is maximal one hour after pilocarpine injection into mice or rats (85). There exists, however, a considerable lack of agreement in the literature concerning the rate of restoration of the zymogen granules and secretory enzymes of the acinar cells. Hirsch (94) described the regeneration of zymogen granules in mouse pancreas as early as four hours after pilocarpine injection. Reis (95) reported regeneration occurred between twelve and twenty hours after injection of the drug. Daly and Mirsky (96) studied the restoration of enzymatic activity in mouse pancreas following pilocarpine treatment and have reported the contents of the cell are completely replenished six hours after injection. Farber and Sidransky (93) found that amylase activity was generally increasing in rat pancreas by four to eight hours after stimulation but much variation in the recovery rates was observed. It was apparent from these reports that, in order to use pilocarpine depletion of pancreatic enzyme stores as a means of inducing synthesis and storage of proteins, it would be necessary to investigate the rate of

regeneration of those proteins more thoroughly than had been done previously.

Several investigators have found pancreatic tissue adaptable to the study of protein synthesis in vitro. Hokin (75) used pigeon pancreas slices to investigate the amino acid requirements of amylase formation. Younathan et al. (97) employed a similar system to study the influence of amino acid analogues on the synthesis of amylase. Schucher and Hokin (91) later adapted the in vitro system to a demonstration of a net increase of lipase and ribonuclease activities.

During studies, described in this thesis, of the effects of pilocarpine upon the enzymatic content of mouse pancreas, amylase and ribonuclease activities have been determined. Convenient, quantitative, and sensitive assays are available for both enzymes (see Materials and Methods). Ribonuclease was chosen for the studies of in vivo incorporation of amino acids into a specific protein since it is a relatively stable enzyme and can be purified by established procedures of chromatography (79).

Pilocarpine studies with fasted mice.

Adult mice were fasted and injected with pilocarpine as described in "Methods and Materials". The animals were sacrificed at specific intervals, and the pancreatic tissue was analyzed for total amylase and ribonuclease activities. The data, shown in Table II, indicate amylase activity decreased fifty-five per cent during the first hour following pilocarpine injection. Ribonuclease activity decreased thirty-three per cent during that interval. By twenty-four hours after pilocarpine injection the amylase activity was almost that of the control value. Ribonuclease activity declined steadily during the period of study and showed no signs of significant recovery. The

Table II. Effect of Pilocarpine Injection on the

Activities of Pancreatic Enzymes – Fasted Mice

Hours after pilocarpine	Amylase Smith & Roe units/mg.*	Ribonuclease A_{260}/ mg.*	Number of animals
0	48.8	0.332	10
1	22.3	0.221	10
4	28.0	0.221	5
7	30.5	0.175	5
12	24.4	0.131	5
24	47.0	0.178	5

*
 mg. wet weight of pancreatic tissue

data indicate that recovery of the various digestive enzymes following pilo-
carpine injection does not necessarily occur at similar rates or time inter-
vals.

Pilocarpine studies with fed mice.

Analyses for amylase and ribonuclease activities of the pancreatic

tissue from pilocarpine treated, fed mice, revealed a markedly different

enzyme recovery from that of fasted mice. The enzyme levels, represented

by the values shown in Table III, showed that amylase activity had almost

returned to that of the control seven hours after pilocarpine injection.

Ribonuclease activity remained low during the first twelve hours at which

time a rapid increase was initiated. The period between fifteen and

twenty-one hours after injection was characterized by a rapid increase in

Table III. Effect of Pilocarpine Injection on the

Activities of Pancreatic Enzymes - Fed Mice

Hours after pilocarpine	Amylase Smith & Roe units/mg.[*]	Ribonuclease A_{260}/mg.[*]	Number of animals
0	48.8	0.332	10
1	22.3	0.221	10
4	27.1	0.213	4
7	43.1	0.179	10
12	53.3	0.180	10
15	54.8	0.222	24
18	71.1	0.277	25
21	99.0	0.341	25
24	72.9	0.300	10

[*] mg. wet weight of pancreatic tissue

the activities of both enzymes. In each instance the value at twenty-one

hours was greater than the control value. The enzyme levels observed at

twenty-four hours indicate some decrease is again taking place.

All of the studies performed during the remainder of the investigations

described have been carried out using pilocarpine treated, fed mice. While

the data presented here strongly suggests that net synthesis and storage of

pancreatic enzymes are taking place during the fifteen to twenty-one hour

interval, further work has been performed in order to define other variables

more clearly.

Protein content of mouse pancreas following pilocarpine injection.

As latent ribonuclease activity has been reported by Elson (98) to occur in E. coli and by Dickman et al. (80) to occur in mouse pancreas, it was conceivable that an increase in enzyme activity could be due to the release of an enzyme from an inactive form rather than de novo synthesis. Therefore, protein analyses were performed on the pancreatic homogenates in order to determine whether an increase in protein content could be correlated with the observed increases in enzyme activity. Protein nitrogen was analyzed by the Kjeldahl method using both the pooled pancreatic homogenates and the trichloroacetic acid precipitable protein fractions. In addition, colorimetric analyses for the homogenate proteins were performed (see "Materials and Methods").

Table IV. Effect of Pilocarpine Injection on the

Total Protein Content of Mouse Pancreas

Hours after pilocarpine	Method		
	Colorimetric % wet weight	Kjeldahl trichloroacetic precipitate % wet weight	Kjeldahl homogenate % wet weight
0	15.7	11.4	17.3
1	13.7	10.7	17.1
4	13.4	11.0	14.0
7	13.2	11.5	15.4
12	14.0	11.7	16.0
15	14.9	12.5	16.5
18	15.0	13.0	16.5
21	16.0	14.1	18.0
24	15.2	13.6	17.0

Although it can be seen in Table IV that different absolute values are
obtained with each of the three analytical methods used, the changes of
protein concentration observed in each case bear a marked similarity to one
another. A decrease of protein content of the gland during the first few
hours after stimulation is apparent by all three methods of analysis. It
will be noted that the per cent decrease of total protein concentration is
not as great as the per cent decrease of the activity of either of the
secretory enzymes, indicating that amylase and ribonuclease participate in
the secretory process to a greater extent than the cellular proteins in
general. In addition, it can be seen that during the period of from fifteen
to twenty-one hours after pilocarpine injection the total protein content of
the pancreas increases. The amount of protein present, like the enzymatic
activity, is greater at twenty-one hours than the control value and decreases
somewhat by twenty-four hours. These relationships are shown in Graph III.

While the most rapid increase in enzyme and protein content of the tissue
occurs between fifteen and twenty-one hours after pilocarpine injection, it
cannot be ascertained from these data that protein synthesis begins at that
time. The effect of pilocarpine upon the secretory processes of the pancreas
has already been mentioned. Schucher et al. (91) have suggested that the
so-called cycle of secretion and resynthesis of pancreatic enzymes, based
on the fall and subsequent rise in the enzyme and zymogen granule content
of the pancreas following cholinergic stimulation, may merely reflect a
constant rate of enzyme synthesis with superimposed variations in the secretory
cycle. It is possible that the changes in enzymatic and protein contents of
the pancreas reported here are due to a prolonged effect of pilocarpine, after
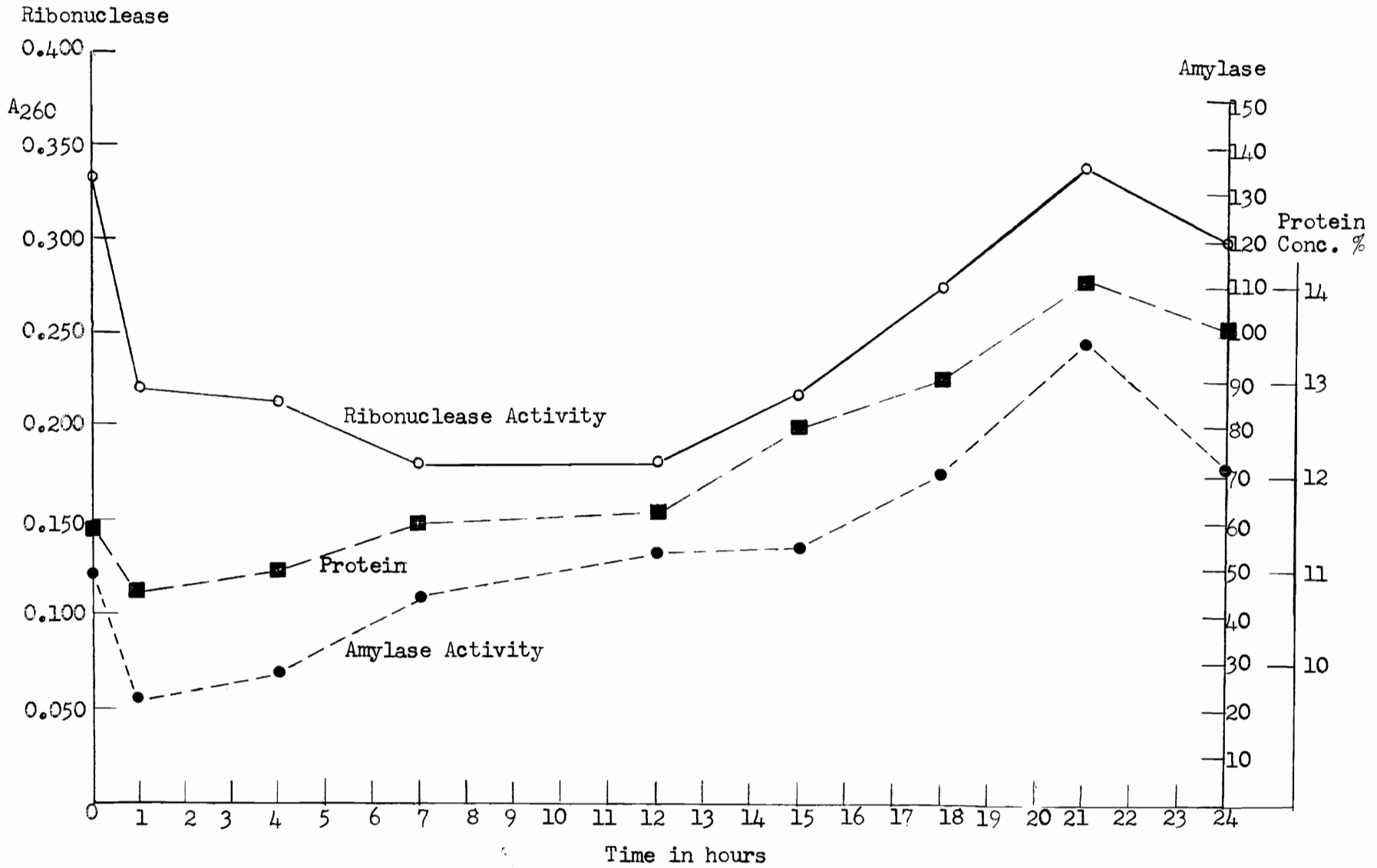
Graph III. Correlation of Enzymatic Activity and Protein
Concentration Following Pilocarpine Stimulation

which the secretory components of the cells can once more accumulate.

Deoxyribonucleic acid content of mouse pancreas following pilocarpine injection.

In view of the report by Faber and Sidransky (93) of the loss of water from the rat pancreas after pilocarpine injection, it was necessary to determine whether or not such changes in water content could account for the observed changes in enzymatic activity and protein concentration. The constancy of the cell population in the pancreas following pilocarpine administration was mentioned earlier. Since the average amount of deoxyribonucleic acid per nucleus of any animal tissue is constant (99), analyses of the deoxyribonucleic acid content of pancreatic tissue were performed as a measure of cell volume. Major changes in the water content of the pancreatic cells after pilocarpine treatment would be reflected in the concentration of deoxyribonucleic acid in the tissue.

Although some water loss from the pancreas may occur during the first few hours after stimulation, the data presented in Table V show that during the period when enzymatic activity and total protein are increasing rapidly, the cell volume remains constant.

It has been concluded from the data presented in these sections that net synthesis of both amylase and ribonuclease is occurring in the pancreatic tissue of fed mice during the period of from fifteen to twenty-one hours following pilocarpine injection.

Ribonucleic acid content of mouse pancreas during rapid protein formation.

On the basis of cytochemical studies Brachet (100) and Caspersson (101) have stated that a correlation exists between the magnitude of the protein synthetic activities and the ribonucleic acid content of a number of tissues

Table V. Deoxyribonucleic Acid Content of Mouse Pancreas

Following Pilocarpine Stimulation

Hours after pilocarpine	Deoxyribonucleic acid % of wet weight
0	0.234
1	0.234
4	0.274
7	0.257
12	0.257
15	0.250
18	0.252
21	0.250
24	0.250

and organisms. Caspersson et al. (102) have reported changes in the ribo-
nucleic acid content of pancreatic cells during different phases of the
secretory cycle. Although several investigators have been unable to confirm
this finding (96,103), it was of interest to determine if changes in the
pentose nucleic acid content of pancreatic cells could be detected under
the present experimental conditions.

Table VI contains the analytical values obtained for the ribonucleic
acid content of pancreatic tissue following pilocarpine injection. It can
be seen that during the period of rapid synthesis and storage of proteins
which occurs between fifteen and twenty-one hours after pilocarpine injection

there is no detectable increase in the concentration of that nucleic acid.

Table VI. Ribonucleic Acid Concentration of Mouse Pancreas

Following Pilocarpine Stimulation

Hours after pilocarpine	Ribonucleic acid % of wet weight
0	2.12
1	2.28
4	2.06
7	2.16
12	2.00
15	2.17
18	2.02
21	2.02
24	1.99

Radioactivity Studies

Labeling of pancreatic ribonuclease.

Introduction of $3\text{-}C^{14}$-phenylalanine into the peptide chain of pancreatic ribonuclease by calf pancreas slices has been reported by Vaughn and Anfinsen (104). Craddock and Dalgliesh (63) obtained incorporation of a mixture of labeled amino acids into the ribonuclease of rat pancreas slices. Studies were undertaken by the author to determine if radioactive labeling of ribonuclease could be achieved in vivo during the period of net synthesis of that

enzyme described above. The amino acid valine was used in these experiments since analyses of bovine ribonuclease indicate a high concentration of that amino acid (105).

Eighteen hours after pilocarpine treatment, nine mice were each given an intraperitoneal injection of 2 μc. of C^{14}-$\underline{L}$-valine. Two hours later the mice were sacrificed, the pancreatic tissue was removed and homogenized, and the ribonuclease was extracted as described in "Materials and Methods". The extract was subjected to the chromatographic procedures described previously. The eluate fractions obtained were analyzed for both ribonuclease activity and radioactivity.

Graph IV shows that two areas of ribonuclease activity are present in the elution pattern. While the first to be eluted is grossly contaminated by other radioactive materials, the second and major component of ribonuclease activity bears a close correlation to the radioactivity in that region. The two elution patterns may be virtually superimposed in the latter region of ribonuclease activity. Thus, by a procedure of extraction and chromatographic separation it became possible to resolve ribonuclease from the other materials present and to demonstrate the incorporation of a labeled amino acid into that enzyme under conditions of net synthesis. The ratio of the radioactivity and enzymatic activity of ribonuclease has been used to define a specific activity of radioactive ribonuclease (see calculations).

Cell fractionation studies of mouse pancreas.

Fractionation of pancreatic homogenates by differential centrifugation was carried out in conjunction with radioactive labeling of ribonuclease in order to determine the site of ribonuclease synthesis in the cells. In
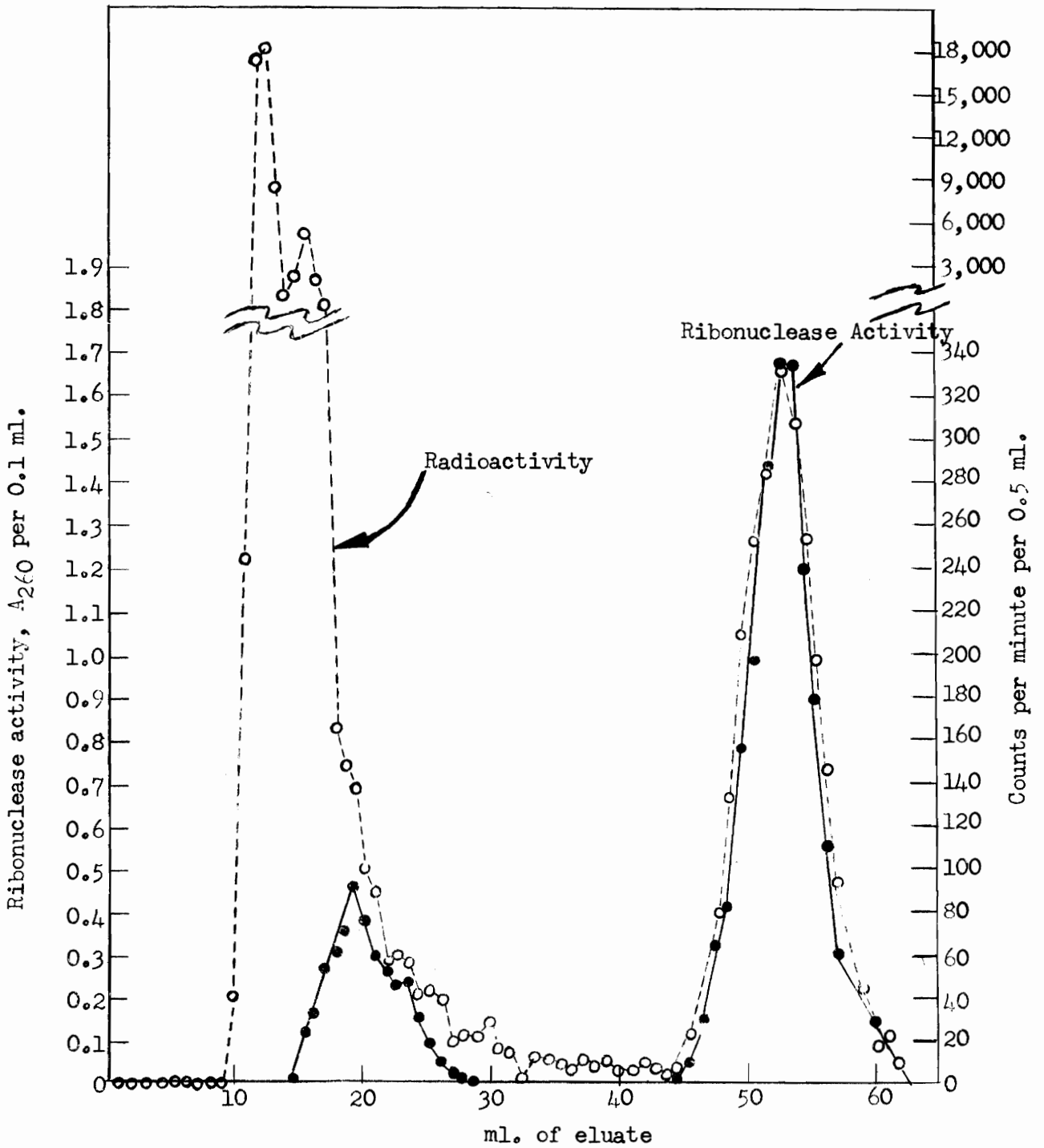
Graph IV. Elution Patterns of Ribonuclease
Activity and Radioactivity

addition, these studies have furnished information concerning the passage of newly synthesized enzyme to various parts of the secretory cell.

The pancreatic tissue from thirty mice was homogenized and fractionated into cell components as described in "Materials and Methods". The specific activity of the labeled ribonuclease in the various cell fractions has been determined at five time periods after labeled amino acid injections. Pancreatic homogenates were prepared five, ten, fifteen, thirty and one hundred twenty minutes after C^{14} valine injection. Analyses for total radioactivity and total ribonuclease content were carried out on each homogenate and cell fraction.

Cell fractions corresponding to a nuclear fraction, a combined zymogen granule and mitochondrial fraction, a microsomal fraction, and a supernatant $_I$ fraction were prepared from each homogenate (106). Since the mitochondrial portion of the cell has been shown to contain only minimal amounts of ribonuclease, that portion was combined with the zymogen granules of the cell (107).

Ribonuclease content of cell fractions and homogenates.

The magnitude of the ribonuclease "pool" in each cell fraction has been determined by analysis of the total ribonuclease activity (Table VII). Values have been expressed as total ribonuclease units (see "Materials and Methods").

Although some variation is apparent in the total amount of ribonuclease present in any one particular cell fraction at different time periods, the data indicate that no major changes in pool sizes are occurring during the period of investigation.

Table VII. Total Ribonuclease Activity of Cell Fractions

Cell fraction	Minutes after C^{14}-valine Injection				
	5	10	15	30	120
Nuclei	123 units	100 units	117 units	104 units	111 units
Zymogen	68	46	104	103	95
Microsome	368	267	275	264	308
Supernatant $_I$	330	361	481	383	285*
Tissue weight (mg.)	3520 mg.	3548 mg.	3730 mg.	3537 mg.	3966 mg.
Total homogenate*	957 units	730 units	855 units	1012 units	1104 units
A_{260}/mg. of tissue	0.272	0.206	0.229	0.286	0.278

* not treated with H_2SO_4

The total wet weight of pancreatic tissue obtained at each time interval and the total ribonuclease content of each pancreatic homogenate were determined. The analytical results are shown in the lower portion of Table VII.

Total radioactivity of the pancreatic tissue.

A portion of each homogenate was diluted, and the radioactivity analyzed in order to determine the rate of appearance of radioactivity in the pancreatic tissue. Graph V shows the total radioactivity content of the pancreatic tissue at specific intervals after isotope injection.

An appreciable quantity of radioactivity is seen to reach the pancreas during the first five minute interval. Maximum radioactivity of the tissue is observed ten minutes after injection, and the C^{14} content remains fairly
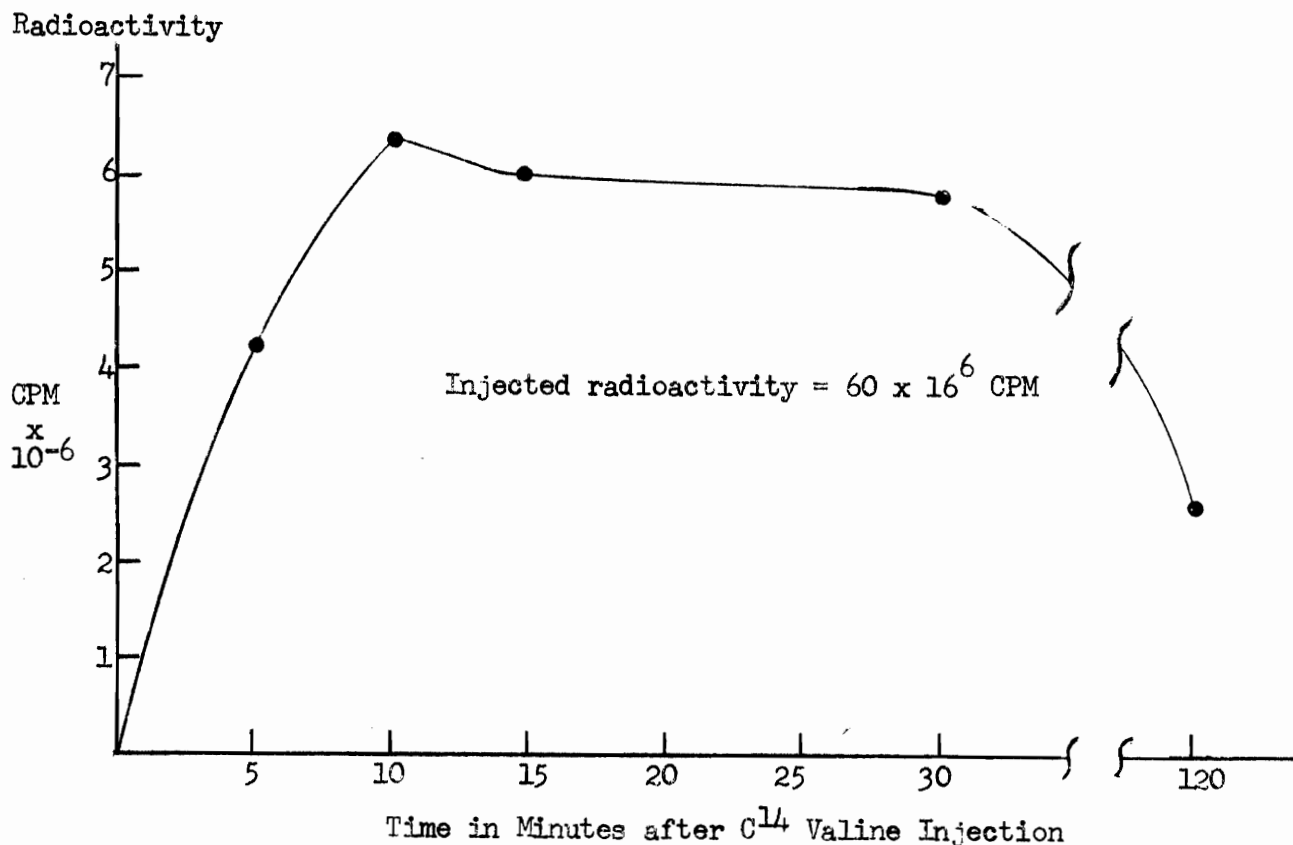
Radioactivity

Graph V. Total Radioactivity of Pancreatic Homogenates

constant during the next twenty minutes. By two hours after injection total radioactivity in the pancreas is reduced to less than one-half of the maximum value.

It is interesting to note that approximately ten per cent of the injected dose of radioactivity is found in the pancreas after ten minutes. Considering that the pancreatic tissue of a mouse weighs approximately 125 mg. and the total body weight of an adult mouse is 20 to 25 gms., it can be calculated that the pancreatic tissue has concentrated the radioactivity twenty fold. Farber and Sidransky (93) have observed that _in vivo_ uptake of C^{14}-L-valine by

rat pancreas is doubled by pilocarpine treatment.

Total radioactivity of cell fractions of mouse pancreas.

The general distribution of total radioactivity in the pancreatic cells was studied as a function of time. A portion of each cell fraction was diluted and counted for radioactivity. As can be seen in Graph VI, most of the radioactivity incorporated into the pancreas during the first ten minutes is present in the microsomal fraction of the cell. At fifteen minutes after injection, as well as the later periods, the supernatant fraction contains a greater proportion of the radioactivity than any other cell fraction. The nuclei and zymogen granule fractions are seen to increase slowly in total radioactivity content during the first thirty minutes.
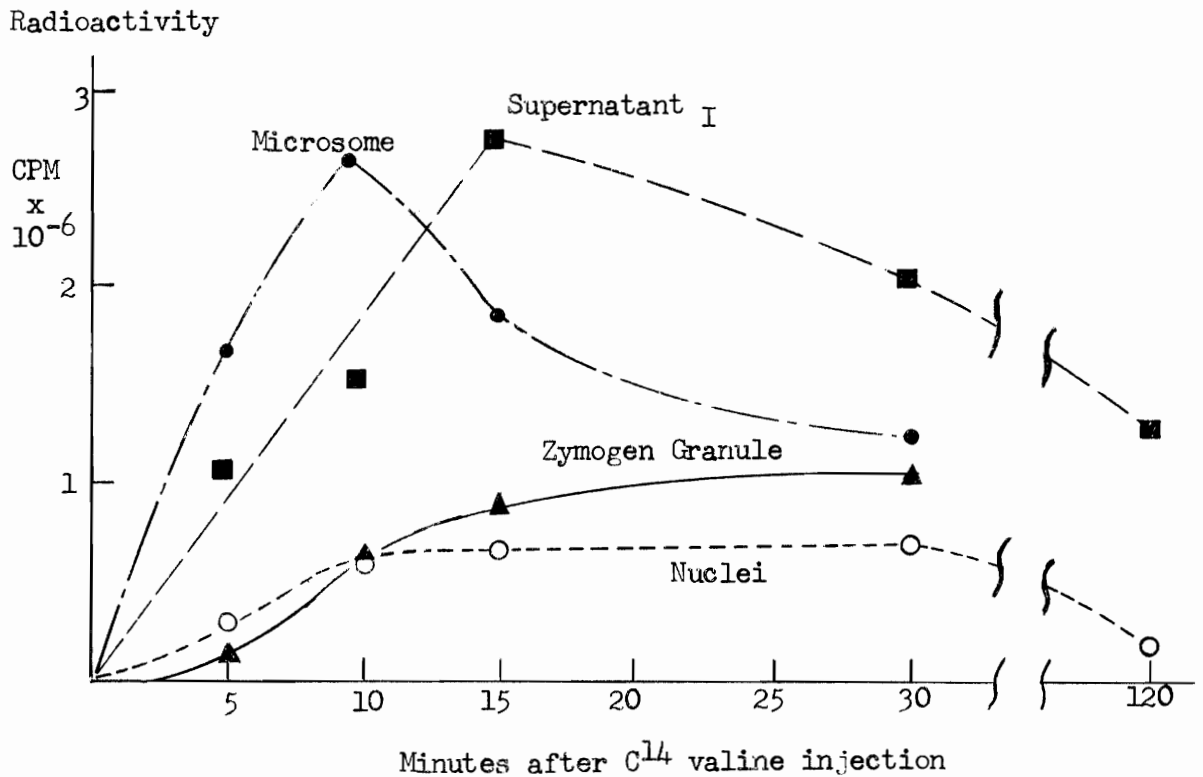
Graph VI. Distribution of Radioactivity in Cell Fractions from Mouse Pancreas

Although determination of total radioactivity is a gross measurement, the data show that the microsomal portion of the cell is labeled more rapidly than the supernatant fraction. This information seems incompatible with the findings of Zamecnik et al. (108) that the soluble ribonucleic acid fraction binds amino acids prior to their incorporation into microsomal proteins in vitro. The binding of labeled amino acid to the soluble ribonucleic acid portion of the pancreas may constitute such a small proportion of the total radioactivity present that it is not apparent during measurement of total radioactivity.

The specific activities of ribonuclease in cell fractions of mouse pancreas.

Specific activities of the ribonuclease in each cell fraction have been studied as a function of time. Ribonuclease was extracted from each cell fraction and resolved by the chromatographic procedure mentioned previously. In all cases two areas of ribonuclease activity were detected in the eluate fractions. All specific activity data have been obtained from analyses of the major area of ribonuclease activity, which is eluted last from the column. A standard deviation has been calculated for each specific activity (see "Materials and Methods") in order to obtain an estimation of the significance of the experimental results.

The specific activity values summarized in Table VIII indicate the degree of incorporation of labeled amino acid into the ribonuclease molecule. The most striking feature indicated by the data is the rapid labeling of the microsomal ribonuclease. The radioactivity found in the microsomal ribonuclease

Table VIII. The Specific Activities of Mouse Pancreas Ribonuclease

Following C^{14} Valine Injection

Cell fraction	Time in minutes				
	5	10	15	30	120
Nuclei	0.40 ± 0.08	--	1.67 ± 0.17	1.18 ± 0.25	2.83 ± 0.8
Zymogen granule	0.45 ± 0.15	1.23 ± 0.39	1.57 ± 0.18	3.53 ± 0.32	3.53 ± 1.0
Microsomal	3.01 ± 0.31	5.80 ± 0.47	6.97 ± 0.63	5.27 ± 0.35	2.61 ± 0.5
Supernatant $_I$	0.61 ± 0.21	1.69 ± 0.46	2.53 ± 0.24	2.79 ± 0.28	3.43 ± 0.1

$$\text{Specific Activity} = \frac{\text{CPM (0.5 ml. eluate)}}{A_{260} \text{ (0.1 ml. eluate) x 100}}$$

is already appreciable five minutes after injection of the label. At that time
the ribonuclease of the other cell fractions shows very little labeling. The
specific activity of microsomal ribonuclease reaches a maximum at fifteen
minutes and decreases thereafter. These data have led to the conclusion that
the microsomal portion of the acinar cell is the site of synthesis of pancreatic
ribonuclease in the living mouse.

Siekevitz and Palade (109) investigated the in vivo incorporation of
DL-leucine-1-C^{14} into the trichloroacetic acid precipitable proteins of cell
fractions obtained from guinea pig pancreas. These workers observed that the
microsomal proteins are labeled more rapidly than the proteins of other cell
fractions. Recently, Siekevitz and Palade (110) presented a preliminary
report which indicated that the ribonucleoprotein particles of the microsomal

fraction of guinea pig pancreas are the site of synthesis of the protein chymotrypsinogen.

It can be seen in Table VIII that the ribonuclease of the supernatant $_I$ fraction shows the second most rapid rate of labeled amino acid incorporation. Fifteen minutes after injection the specific activity of the enzyme in that fraction is significantly greater than either nuclei or zymogen granule fraction ribonuclease. However, by thirty minutes the specific activity of ribonuclease in the zymogen granule fraction is elevated sufficiently to exceed that of the supernatant enzyme by a significant margin. These same data, presented pictorially in Graph VII, show that the specific activity values of the supernatant enzyme have begun to plateau at fifteen minutes while the specific activity of zymogen granule enzyme continues to gain radioactivity for a much longer period of time.

Subfractionation of supernatant $_I$.

Two possible interpretations which would account for the distribution of radioactivity in the ribonuclease of the supernatant and zymogen granule fractions are: 1) A precursor-product relationship exists between the enzymes of the two fractions. Thus, the ribonuclease of the supernatant fraction may be an intermediate in the transport of the enzyme from the site of synthesis in the microsomes to the site of storage in the zymogen granules. 2) On the other hand, if a clean separation of the soluble and microsomal portions of the homogenate was not attained by the centrifugal forces which have been applied, there might be a sufficient number of microsomal particles remaining in the soluble fraction to account for the observed data.
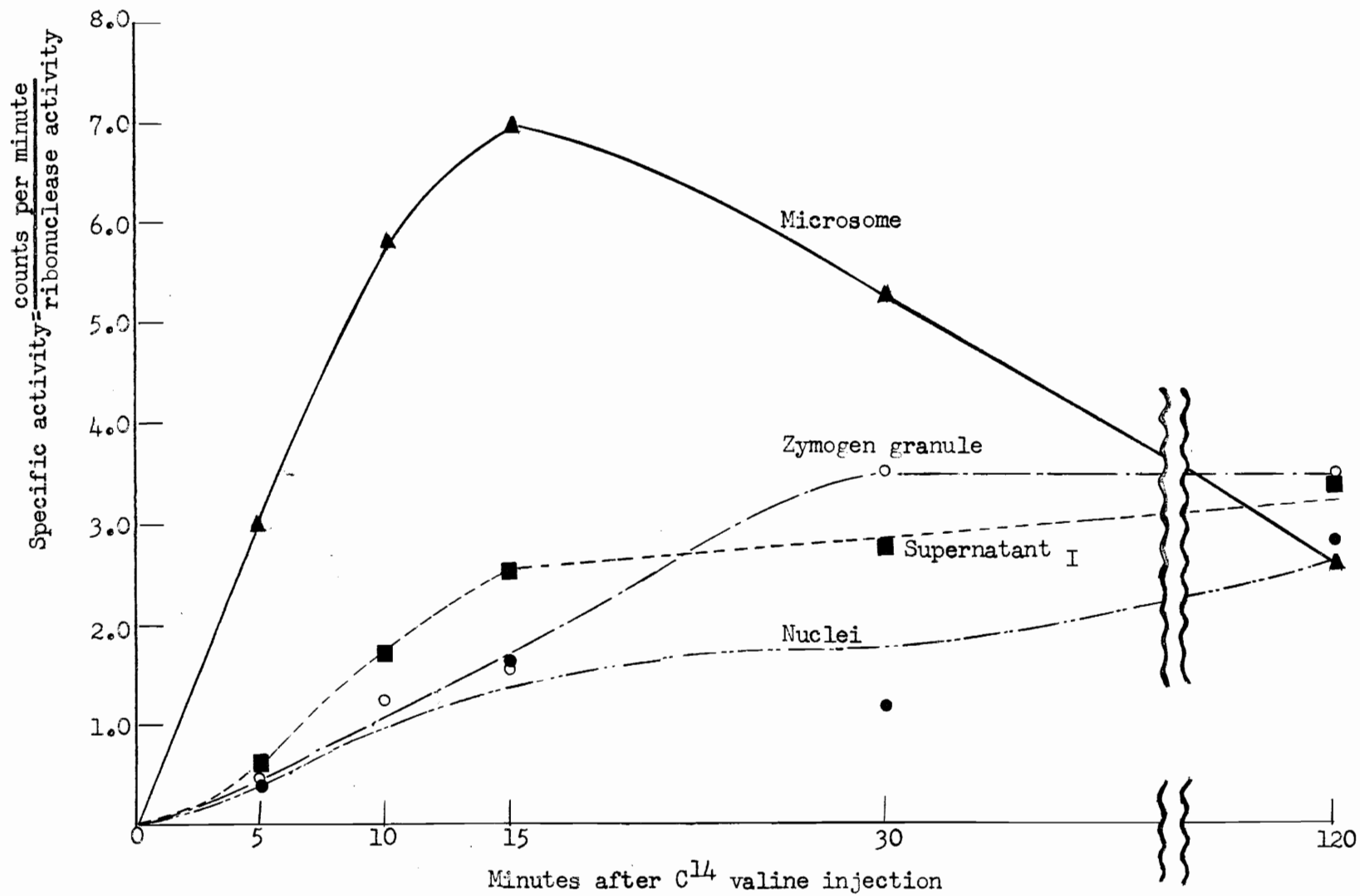
Graph VII. Time Course of Intracellular Ribonuclease Labeling

In order to test the second possibility, a further fractionation of the soluble portion of the cell was carried out. Prolonged centrifugation of a supernatant $_I$ fraction (fifteen minutes after isotope injection) yielded two postmicrosomal fractions and a high speed supernatant fraction (supernatant $_{II}$) (see "Materials and Methods"). If supernatant $_I$ were contaminated with microsomal particles, one would expect the specific activity of the ribonuclease in the first postmicrosomal fraction to be greater than that of either the second postmicrosomal fraction or the final supernatant $_{II}$.

The data shown in Table IX indicate that the supernatant $_I$ fraction is not grossly contaminated with highly labeled microsomal ribonuclease.

Table IX. Subfractionation of Supernatant $_I$

Cell fraction	Specific activity	Ribonuclease content[*]
Supernatant $_I$	2.53 ± 0.24[**]	202 units
Postmicrosome $_I$	2.32 ± 0.28	99
Postmicrosome $_{II}$	1.25 ± 0.23	24
Supernatant $_{II}$	2.37 ± 0.24	37

[*] not treated with H_2SO_4

[**] value taken from Table VIII

Interpretation of the data.

On the basis of the above data it is proposed that the ribonuclease which is synthesized in the microsomal portion of the acinar cells of mouse pancreas is transferred to the soluble portion of the cell before reaching the zymogen granules.

It is interesting to note that Laird and Barton (90) have arrived at a similar conclusion from a kinetic study of the pilocarpine-induced depletion of amylase from the various portions of the mouse pancreas cell. These workers have proposed that amylase is synthesized in the microsomal portion of the cell, released from the site of synthesis in soluble form, and condensed into the zymogen granules where it is stored awaiting secretion.

Sjöstrand and Hanzon (111), from an electron microscopic investigation of the ultrastructure of the exocrine cells of mouse pancreas, have commented on the intimate relationship between the Golgi apparatus and the zymogen granules of the acinar cells. Further, they describe granules in the Golgi zone which appear to represent a whole series of stages of formation from the most minute granules to the well defined zymogen granules. It is conceivable that the soluble proteins of the cell are condensed at this locus to form the zymogen granules.

Siekevitz and Palade (109) have suggested the possibility of the transfer of labeled proteins from the microsomes to other cell fractions. Recently, on the basis of electron micrograph data, they have proposed that the zymogen granules are formed within the endoplasmic reticulum (microsomal fraction) (112). In such a case no soluble intermediate fraction would be present. Therefore, the data presented in this thesis does not support that interpretation.

An estimation of the time required for a complete protein molecule to be assembled from precursors can be made from the data presented (See graphs V, VI, and VII). Since a considerable amount of labeled ribonuclease is present in the microsomal fraction five minutes after intraperitoneal injection of the labeled amino acid, the time required for the formation of labeled enzyme is necessarily less than five minutes. The time required for the homogenate, the microsomal fraction, and the microsomal ribonuclease to become maximally labeled has been plotted on a comparable scale in Graph VIII. Although a greater number of values would allow calculation of a more precise value, it can be seen that the radioactivity is present in the homogenate and the microsomal fraction for 2.9 - 3.4 minutes before it appears in the ribonuclease molecule. This treatment of the data assumes that the specific activity of the protein formed is directly proportional to the radioactivity present in the tissue or cell part at the time of synthesis. It is reasonable to assume that the time difference between these curves is the interval required for the labeled amino acid to be assembled into the intricate structure of the finished, enzymatically active protein.

Craddock and Dalgliesh (63) report that no lag period is present in the incorporation of labeled amino acids into the ribonuclease of in vitro rat pancreas slices. These workers, however, did not conduct studies of the first few minutes after introduction of the labeled amino acids.

Junqueira et al. (64) cannulated the common biliary and pancreatic duct of a carbamylcholine-stimulated rat and found a fifty minute lag between the intravenous injection of C^{14}-glycine and the appearance of labeled proteins in the pancreatic secretions. Radioactivity of the secretory proteins reached
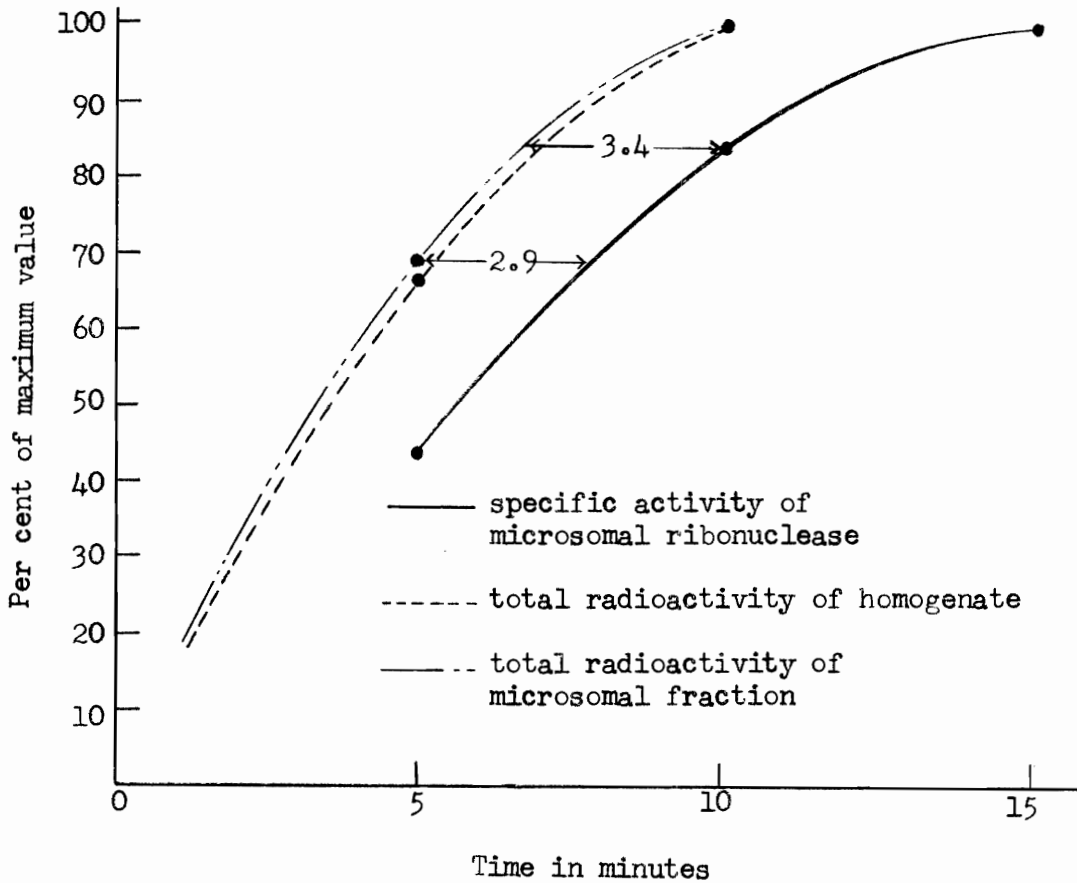
Graph VIII. A Comparison of the Rates of Radio-
activity Uptake.

a maximum approximately two hours later. Such a lag period could be the

time required for the transfer of proteins through the soluble portion of

the cell and for their subsequent concentration into discreet zymogen

granules.

Keller, Cohn and Neurath (113) have recently reported similar studies

of three enzymes in the secretions of bovine pancreas. Maximum specific

activity was obtained in these proteins between three and four hours after

intravenous injection of labeled amino acid, although labeled enzymes were

present during the first one hour sample. Since cholinergic stimulation of

the animal was not used in the latter study, the time that the enzymes were stored prior to the secretion may have been prolonged.

One may conclude that the time from which the secretory enzyme leaves the site of synthesis in the microsomes until it is secreted by the extrusion of zymogen granules is approximately forty-five or fifty minutes in a rapidly secreting pancreas. During the interim the enzyme is undergoing transfer and storage within the secretory cell. A more detailed study of the specific activity of zymogen granule ribonuclease by the methods described in this thesis, especially at time periods later than thirty minutes, could be used to determine the time relationships of the secretory process in a more detailed manner.

SUMMARY

Methods have been developed for the study of amino acid incorporation into a specific protein under established conditions of net synthesis. The results of these studies have been reported and discussed.

Synthesis and storage of the secretory proteins of mouse pancreas have been shown to occur between fifteen to twenty-one hours after pilocarpine injections. The concentration of amylase, ribonuclease, and total pancreatic proteins has been shown to increase rapidly during that period. Deoxyribonucleic acid as well as ribonucleic acid content of the pancreatic tissue does not increase during the period of protein synthesis.

Injection of the labeled amino acid, valine, into pilocarpine treated mice eighteen hours after stimulation, followed by chromatographic resolution of the components of an extract of pancreas, has allowed a demonstration of radioactive labeling of the enzyme ribonuclease under conditions of net synthesis. The ratio of radioactivity to enzymatic activity has been used to define a specific activity for that enzyme.

Analyses of the total radioactivity present in the pancreatic tissue as well as the total radioactivity of each of four cell fractions have been carried out. It was found that ten minutes after intraperitoneal injection of isotope approximately ten per cent of the radioactivity is present in the pancreas. Radioactivity appears most rapidly in the microsomal fraction of the cell during the first ten minutes but at later periods the soluble portion of the cell becomes more intensely labeled.

The distribution of labeled ribonuclease in the various parts of the pancreatic cells has been studied as a function of time. The data indicate that the microsomal portion of the acinar cells is the site of formation of the secretory enzyme, ribonuclease, in the living mouse. The distribution of labeled ribonuclease in the other cell fractions suggests that the newly formed enzyme is transferred to the zymogen granules by way of the soluble portion of the cell.

From a consideration of the rates of isotope uptake into the total homogenate and the microsomal fraction, as well as the rate of appearance of labeled ribonuclease in the microsomal fraction, it has been estimated that the labeled amino acid is present in the microsomes for about three minutes before it appears in the pancreatic enzyme. It has been proposed that this time lag is the period required for the elaboration of the finished, enzymatically active protein, ribonuclease, in the microsomal portion of the cells.

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Research Proposals

Allan J. Morris

1. An investigation of the role of 3-hydroxyanthranilic acid in the oxidative metabolism of tryptophan.

2. A study of the distribution of elastase with special reference to biological function.

3. An investigation of a factor or factors present in the nuclei of Acetabularia which control cytoplasmic synthesis of proteins.

4. To conduct a study designed to elucidate the individuality or interconversion of chromatographically distinct ribonucleases.

5. To investigate the possible role of malonyl CoA in the synthesis of unsaturated fatty acids.

6. To study the breakdown of serum albumin under physiological conditions.

Curriculum Vitae

Born: June 26, 1926 at Linn Grove, Iowa

High School: Graduated from Linn Grove Consolidated School May 1944

Military Service: U. S. Navy August 1944 to December 1946 (Honorable Discharge)

Marital Status: Married September 1, 1956

     Wife: Frances S. Morris

     Children: Scott A. Morris

Colleges Attended: Iowa State College, Ames, Iowa

     Graduated July 1954 with B. S. degree in chemistry

     Honors: Pi Mu Epsilon

       Phi Kappa Phi

       The honor student in chemistry of class of 1955

     University of Utah, Salt Lake City, Utah

     Graduated August 1959 with M. A. degree in biochemistry

       Thesis: "Some Studies of 5-Hydroxytryptophan Metabolism"

     Graduated August 1959 with PhD degree in biochemistry

       Thesis: "Biosynthesis of Pancreatic Ribonuclease"

     Honors: Sigma Xi

Publications: Rat Growth Experiments with 5-Hydroxy-DL-Tryptophan, Fed. Proc. 16,
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    Preparation of 5-Hydroxy-L-and D-Tryptophan, J. of Org. Chem, 22,
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