PART I - GLUTAMINE METABOLISM IN BRAIN

PART II - PROTEIN SYNTHESIS AND SECRETION BY GLANDULAR TISSUES IN VITRO

A thesis

by

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Preface

Two separate and independent lines of investigation are described in this thesis, which is divided into two parts.

Part I deals with glutamine metabolism in brain. This work and related studies are described in the first four chapters of this thesis.

Part II is concerned with *in vitro* studies on protein synthesis and secretion by glandular tissue. Both enzyme synthesis and the incorporation of radioactive amino acids into protein were investigated with pancreas tissue. The work has been presented in two main sections. Section A deals with studies on the synthesis of lipase and ribonuclease by pigeon pancreas slices. Section B describes studies on enzyme synthesis and labelled amino acid incorporation by pigeon and mouse pancreas.

The numbering of pages, Chapters, Figures and references to the literature is continuous throughout the thesis.

PART I

GLUTAMINE METABOLISM IN BRAIN

CHAPTER 1

GENERAL INTRODUCTION

A wide body of information exists on the metabolism of glutamic acid, and its amide, glutamine, in nervous tissue, and the subject has been critically examined in several recent reviews (1, 2, 3). It is proposed in this chapter to outline briefly some of the observations relating, in the main, to the work described in the next two chapters.

Krebs in 1935 (4) showed that brain cortex slices of vertebrates, among other tissues, synthesize glutamine from L-glutamic acid and ammonia, in the presence of glucose. The reaction is endothermic, dependent on energy-giving reactions and inhibited by cyanide. Glutamine synthesis is inhibited markedly by D-glutamic acid, and slightly by DL-hydroxyglutamic acid. Under optimum conditions brain cortex synthesizes 1-2% of its dry weight of glutamine per hour, as compared with 10-20% for kidney.

Leuthardt and Bujard (5) in 1947, found that guinea pig and rat liver homogenates synthesized glutamine from glutamate and ammonium ions. The reaction was shown to be dependent on adenosinetriphosphate (ATP). Frei and Leuthardt (6) used the highly specific bacterial glutamic acid decarboxylase (7), for assaying glutamic acid and glutamine (8), in an investigation

of glutamine synthesis in the particulate and supernatant fractions of rat liver homogenate. They obtained little synthesis with mitochondrial preparations, and with the centrifugate (obtained at 1500 g.) or supernatant fractions of the homogenate alone, in the presence of ATP. Glutamine synthesis occurred when the supernatant and centrifugate were recombined.

Speck (9, 10) in 1947 and 1949 reported investigations on glutamine formation in dilute dispersions of fresh pigeon liver and in acetone-dried powders of pigeon liver. He found that purified extracts of pigeon liver powders catalyze a stoichiometric reaction between ATP, glutamate and ammonia (11). The reaction requires magnesium or manganese. The reaction products are glutamine, adenosinediphosphate and inorganic phosphate. Glutamine synthesis is inhibited by low concentrations of fluoride, and by methionine sulfoxide. Hydroxylamine, hydrazine and methylamine also combine with glutamate in this system.

Elliott (12) in 1948 discovered, independently of Speck, an enzyme system in sheep brain which synthesizes glutamine from glutamic acid, ATP and ammonia. The properties of a partially purified preparation of this enzyme system are essentially similar to those of the liver system studied by Speck, and Elliott drew the same basic conclusions as the former author on the synthesis of glutamine (12, 13).

Glutaminase, the enzyme which splits glutamine into glutamic acid and ammonia was investigated by Krebs (4) in various mammalian tissues, and he distinguished between a "brain type"

and "liver type" of glutaminase. Brain glutaminase shows optimal activity at a pH value of about 8.5 and requires phosphate or other anions for activation. Both D- and L-glutamic acid inhibit brain glutaminase strongly and competitively. The properties of glutaminase suggest that it is inactive under physiological conditions; Krebs felt the available evidence favoured the assumption that the splitting enzyme obtained in tissue extracts is a fragment of the synthesizing systems, and is concerned only with synthesis physiologically (14).

During the past three or four years, various authors have revealed a new enzymic reaction leading to the formation of glutamylhydroxamic acid. (15, 16, 17, 18). The enzyme, termed glutamotransferase, catalyzes the exchange of the amide group of glutamine for either isotopic ammonia (19) or hydroxylamine. Thus hydroxylamine and glutamine, in the presence of the enzyme system, produce glutamylhydroxamic acid plus ammonia. Stumpf and associates have found this transferase activity in pumpkin seedlings (16, 17, 18), and Schou and co-workers have demonstrated it in extracts of acetone-dried powders of brain, liver, kidney and tumour tissue (15). Plant and animal enzyme preparations require phosphate or arsenate and manganese. Only catalytic amounts of either the tri- or di-phosphate of adenosine are required, in contrast to the large amounts required by the glutamine-synthesizing system.

Elliott (20) very recently obtained a highly purified

protein preparation from green peas and compared its glutamine-synthesizing and transferase activity at different stages of purification. The results, although suggesting the possibility of two distinct enzymes, were not conclusive. Glutaminase activity was not demonstrable in the extracts of acetone-dried powders of plant or mammalian tissue.

The observations on the widespread occurrence of enzyme systems effecting the transfer of the glutamyl radical of glutathione and γ-glutamyl peptides to other amino acids, and of the glutamyl and aspartyl radical from ammonia to other amines, in plants, animals and microorganisms, has led to the postulation of a role of glutamine and glutathione in peptide and protein synthesis (21, 22).

Braganca, Schucher and Quastel (23), in testing the effect of DL-α-methylglutamic acid on various brain preparations, found that this compound is a potent inhibitor of rat brain glutaminase, and obtained evidence suggesting that this analogue of glutamic acid may act as a substrate for the glutamine-synthesizing system of acetone-dried powders of beef brain, giving rise, presumably, to α-methylglutamine. Lichtenstein, Ross and Cohen (24, 25) independently observed that DL-α-methylglutamic acid inhibits kidney glutaminase, and obtained results with the glutamic acid analogue and sheep brain extracts which led them to the same conclusion as the above authors. They also found that α-methylglutamic acid inhibits the glutamotransferase system

of sheep brain (24).

Borek and Waelsch (26) have demonstrated that DL-hydroxyglutamic acid acts as a powerful antimetabolite against glutamic acid in bacterial metabolism, and suggested a probable interference with glutamine synthesis. Ayengar and Roberts (27) have reported that DL-α-methylglutamate inhibits the utilization of L-glutamate by L.arabinosus; the inhibition was reversed by increasing concentrations of L-glutamate. The authors suggested that DL-α-methylglutamate exerts its inhibitory action on the growth of the bacteria by preventing the amidation of glutamic acid.

CHAPTER II

MATERIALS AND EXPERIMENTAL METHODS

A - INCUBATION AND PREPARATORY TECHNIQUES

1. Fresh Tissue Preparations

Rat brain was employed in most of the experiments; guinea pig and pigeon brain were also tested. Homogenates, minces and slices of the tissues were prepared. An all-glass homogenizer was used for obtaining homogenates; minces were made with a sharp, fine scissors and passed through cheesecloth; slices were prepared with the Stadie-Riggs slicing equipment. Brain cortex was used for slices and whole brain for the other preparations. Tissue dispersions were usually prepared in 0.9% NaCl. All operations on the tissue were carried out at about 0°C.

2. Extracts of Acetone-Dried Powders of Beef Brain

Fresh beef brain was obtained at the abattoir and kept at 0°C. Brain cortex was removed with a pair of scissors and homogenized in the Waring blendor with 1-2 volumes of water or 0.3% neutralized cysteine hydrochloride solution. About 10 volumes of acetone were then added, the mixture stirred and allowed to stand for about an hour. The brain powder was then collected by suction filtration, dried *in vacuo*, and stored at 0°C *in vacuo*. All operations were carried out at about 0°C. The activity and stability of the powders varied from one batch to the next. Most of the activity in a given preparation was usually lost after a

week of storage.

Extracts of the powders were prepared by a modification of the procedure of Elliott (13). Acetone-dried grey matter of sheep brain was extracted for 10 minutes with 10 volumes of 0.3% cysteine-hydrochloride solution neutralized to pH 5-5.5. After centrifuging, the supernatant was filtered through cotton wool, cooled to 4°C, and mixed with 0.2 volume of 0.1M sodium acetate buffer at pH 4.2. The precipitate obtained was centrifuged down, washed twice by suspending in cysteine-hydrochloride solution, and redissolved in half the volume cysteine-hydrochloride solution of the original extract by adjusting the pH to 6.8 with NaOH. The substitution of cysteine-hydrochloride solution for the water in the original procedure resulted in greater enzymic activity.

3. Incubation Conditions

Incubations were performed in conventional Warburg manometric vessels. In experiments with brain powders, the standard complete system consisted of the following. Quantities of extract, equivalent to 80-100 mg. acetone-dried grey matter of beef brain in 0.5 ml. 0.3% neutralized cysteine-hydrochloride solution; 0.025M $MgSO_4$; 100 µg. ammonium ions (as ammonium sulphate); 0.02M sodium L-glutamate; 0.06M adenosinetriphosphate (as sodium salt) and 0.028M $NaHCO_3$ were present in the main compartment of the vessel. Any other compounds of which the effects were being studied were added in neutral solution. The total incubation

volume was 3.0 ml. Incubations were usually performed for an experimental period of 40 minutes at 37°C in an atmosphere of 7% CO_2 in 93% N_2.

Experiments with fresh tissue were performed with various incubation media which are given in the next chapter dealing with results.

4. Miscellaneous Reagents and Sources

L-glutamic acid	- Nutritional Biochemicals
Sodium L-Glutamate	- Nutritional Biochemicals
Sodium D-Glutamate	- Nutritional Biochemicals
Cysteine hydrochloride	- Merck and Co., Inc.
Sodium pyruvate	- Nutritional Biochemicals
Sodium Ketoglutarate	- Nutritional Biochemicals
L-Alanine	- Nutritional Biochemicals
Adenosinetriphosphate (Na salt)	- Schwartz Laboratories
*DL-λ-methylglutamate (Na salt)	- Merck and Co., Inc.
*DL-Methionine sulfoximine	- Wallace and Tiernan Products Inc.

* Gift samples of these compounds were generously provided by the firms producing them.

B - DETERMINATION OF AMMONIA

In the course of this work, a method has been developed for the microestimation of ammonia in the presence of tissues, which has proved to be of considerable service in investigations

involving studies of metabolism in the Warburg manometric apparatus where ammonium ions are released or absorbed. The method has now been in use for some time in this laboratory and, having proved itself to be of considerable service, has been submitted for publication.

The principle of the method is essentially the same as that of the micro diffusion procedure of Conway and Byrne (28) in which the ammonia liberated from an alkaline solution is allowed to diffuse into the closed compartment containing acid. In the technique to be described the diffusion of ammonia is allowed to take place in Warburg manometric flasks.

1. Reagents

K_2CO_3 - a saturated solution of potassium carbonate

H_2SO_4 - approximately $\underline{N}$ H_2SO_4 prepared by diluting 1 ml. of concentrated sulfuric acid to 36 ml. with water.

NaOH - approximately $2\underline{N}$ NaOH

Nessler Solution - prepared according to the procedure Umbreit (29).

2. Procedure

A small roll of filter paper soaked with 0.2 ml. $\underline{N}$ H_2SO_4 is placed in the centre well of the Warburg manometric vessel, and 0.3 ml. saturated K_2CO_3 solution is placed in the side arm. The test solution containing ammonia is present in the main compartment of the vessel. The Warburg flask is mounted on the manometer, the manometer tap closed, and the K_2CO_3 solution is tipped into the main

compartment. This raises the pH of the contents to about 10.5. Under these conditions, the ammonia present in the test solution diffuses into the centre well where it is absorbed by the acid on the filter paper. The vessels are shaken at 37°C for 3 hours, a period which has been found sufficient to permit complete diffusion and absorption of quantities of ammonia ranging from 10 µg. to 500 µg.

In experiments involving gas exchanges or the use of bicarbonate buffer, the Warburg apparatus is dismantled at the end of the incubation period and the above procedure followed. Otherwise, the H_2SO_4 and K_2CO_3 may be present in the centre well and side arm, respectively, during the experimental incubation; the need for dismantling the apparatus is thus eliminated.

At the end of the three-hour diffusion period the vessels are removed from the bath and the small rolls of filter paper are taken out from the centre well with forceps and placed in graduated tubes. The acid contents of the centre well are quantitatively removed and added to the graduated tubes by washing five times with distilled water. This manipulation is easily accomplished with the aid of a micropipette. The contents of the tubes are made up to definite volume and aliquots corresponding to 20-30 µg. ammonium ions are taken for the assay by nesslerization. The aliquots are made up to a final volume of 10 ml. with distilled water, 1 ml. Nessler solution and 2 ml. 2N NaOH. The intensity of colour produced is estimated with a 425 mµ. filter in the Fisher colorimeter and the ammonia content of the sample ascertained from

a standard calibration curve.

3. Results and Discussion

The standard curve which was employed for ammonia determinations in the work described below, and in the succeeding sections, is reproduced in Figure 1. Each point on the curve represents the average value of triplicate determinations. Standard samples of ammonia were tested routinely, along with the experimental unknowns, and gave reproducible results throughout the period during which this work was conducted.

Data showing recoveries of ammonia after diffusion from water, and after addition of a tissue (brain) homogenate, are shown in Table I. It will be seen that the recoveries of added ammonia are 100%, within an experimental error not exceeding 5%. The experimental variation is usually of the order ± 2%.

The release of ammonia by non-enzymatic hydrolysis of glutamine under the given experimental conditions is shown in the results of Table II. This non-enzymatic hydrolysis amounts to about 9%. Since the value of the non-enzymatic breakdown is quite consistent, under the given conditions, a correction can be applied for this "blank". It has been shown that the amount of desamidation is proportional to the glutamine concentration, up to about 30% hydrolysis (30).

The release of ammonia from other amides is also shown in the results of Table II. The amides employed in this experiment are apparently not hydrolyzed to any significant extent in the

FIGURE 1

AMMONIA CALIBRATION CURVE

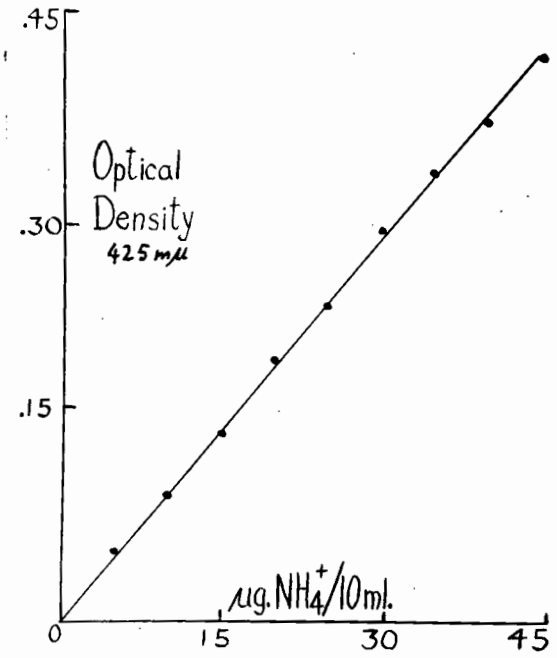

TABLE I

RECOVERIES OF NH_4^+ (ADDED AS $(NH_4)_2SO_4$) IN PRESENCE AND ABSENCE OF TISSUE (RAT BRAIN)

Values corrected for preformed ammonia present in fresh and boiled tissue.
(NH_4^+ in 75 ug. fresh homogenate = 8 ug.;
NH_4^+ in 75 ug. boiled homogenate = 10 ug.)

Recoveries of NH_4^+ in absence of tissue			Recoveries of NH_4^+ in presence of tissue			
ug. NH_4^+ added in 3ml. H_2O	ug.NH_4^+ found	Percent Recovery	3 ml. H_2O containing 75 mg. tissue	ug.NH_4^+ added	ug. NH_4^+ found	Percent Recovery
10.0	10.5	105.0	Fresh homogenate	25.0	26.0	104.0
20.0	19.8	99.0	Fresh homogenate	50.0	49.7	99.4
30.0	30.0	100.0	Fresh homogenate	100.0	98.5	98.5
40.0	41.0	103.0	Boiled homogenate	25.0	23.9	95.6
50.0	51.0	102.0	Boiled homogenate	50.0	52.2	104.4
80.0	83.0	103.7	Boiled homogenate	100.0	102.2	102.2
100.0	99.0	99.0				
100.0	102.0	102.0				
100.0	101.3	101.3				
500.0	508.0	101.6				

TABLE II

RELEASE OF NH_4^+ FROM GLUTAMINE AND OTHER AMIDES

3 ml. water containing:				3 ml. water containing 20 mg. fresh brain homogenate plus substrate				
μM glutamine	μg.amide N (as NH_4^+) present	μg.amide N (as NH_4^+) found	Percent non-enzymic hydrolysis of glutamine	Substrate	μM	μg.amide N (as NH_4^+) present	μg.NH_4^+ found	Percent hydrolysis
6	108	10.0	9.3	L-asparagine	9	162	1	0.6
6	108	9.7	9.0	Benzamide	15	270	6	2.2
15	270	24.3	9.0	Acetamide	300	5400	14	0.3
15	270	26.2	9.6	Nicotinamide	30	540	0	0
30	540	50.8	9.4					
30	540	46.5	8.6					
30	540	52.4	7.8					
30	540	41.4	7.6					
22.5	405	36.5	9.0					
22.5	405	37.0	9.1					
22.5	405	41.4	10.2					
22.5	405	36.6	9.0					

presence of potassium carbonate and brain homogenates.

All ammonia estimations, in the work described in the next chapter, were performed by means of the procedure described above.

The modified microdiffusion method has certain advantages over the usual Conway method when used in conjunction with the Warburg manometric apparatus. Incubations and ammonia determinations may be carried out in the manometric flask, thereby saving time by eliminating the need for transferring material to the special Conway plates.

C - DETERMINATION OF HYDROXAMIC ACIDS

Hydroxamic acid was estimated by the method of Lipmann and Tuttle (31). The intensity of color produced was estimated with a 525 mμ. filter in the Fisher colorimeter, and the hydroxamic acid formation in the test samples ascertained by comparison with a standard calibration curve prepared with succinohydroxamic acid.

D - PAPER CHROMATOGRAPHY

The paper chromatography technique was employed to separate products formed by the brain extract during incubation. The developing solvent consisted of the following:

60 ml. ethanol
20 ml. butanol
7 ml. concentrated NH_4OH
13 ml. H_2O

Both spot and band chromatograms were prepared, for the purposes of preliminary identification, and isolation, respectively, of the reaction products.

The solvent was allowed to ascend overnight for about 16 hours. The paper was dried, sprayed with 0.1% ninhydrin in water-saturated butanol, re-dried at room temperature and heated at 105-110°C for a few minutes so as to permit the reaction between ninhydrin and the amino acids to occur.

The methods employed for tentative identification of the various spots are given in the next chapter.

CHAPTER III

EXPERIMENTAL RESULTS

A - GLUTAMINE SYNTHESIS IN FRESH BRAIN

1. Tissue Dispersions

Homogenates and minces of rat brain, and in a few experiments of pigeon and guinea pig brain, were incubated, at 37°C for periods up to 90 minutes. The following incubation media were used with homogenates: buffer, 0.02M phosphate (pH 7.35) or 0.028M $NaHCO_3$; 0.006M magnesium (as magnesium sulphate); 0.02M glucose; 0.02M sodium glutamate; 100 µg. ammonium ions (as ammonium sulphate). Diphosphopyridine-nucleotide, nicotinamide, adenosinetriphosphate, and calcium and potassium (as the chlorides) were added in some experiments. Krebs-Ringer-phosphate solution (32) was used with minces. The brains were prepared in water, isotonic KCl, 0.9% NaCl and Krebs-Ringer-phosphate solution in various experiments. Volumes of brain dispersion equivalent to 50-200 mg. tissue were employed. The gas phase was 7% CO_2 in 93% O_2 with bicarbonate buffer, and 100% O_2 or air with phosphate buffer. Glutamine synthesis was not obtained under any of these conditions.

2. Brain Slices

In the experiments with brain slices the incubation medium was Krebs-Ringer-phosphate solution (32) to which glucose, ammonia and sodium glutamate, in the concentrations listed above had been added. The gas phase was 100% O_2. Guinea-pig and rat brain cortex

synthesize glutamine (Q_{amide} 1-4.5) (4). The synthesis obtained in the present work was only about one-third of that obtained by Krebs (4), and difficult to estimate accurately by ammonia disappearance in the presence of a high blank.

SUMMARY

Little or no synthesis of glutamine was obtained with fresh brain preparations of various species, under several experimental conditions.

B - EXPERIMENTS WITH EXTRACTS OF BEEF BRAIN POWDERS

1. Glutamine synthesis by Brain Extracts

Table III indicates the conditions under which ammonia is absorbed, in the presence of extracts of acetone-dried powder of beef brain, to form glutamine. Ammonia is not absorbed in the absence of ATP, Mg ions or glutamate. About 70 µg. ammonium ions, equivalent to almost 4µM of glutamine synthesis, were absorbed by the complete system. The brain extracts and solutions of reagents do not contain significant amounts of free ammonia.

2. Inhibition and "Activation" of Glutamine Synthesis

The effects of methionine sulfoximine and DL α-methyl-glutamate on glutamine synthesis by brain extract are shown in the data of Table IV.

TABLE III

AMMONIA ABSORPTION IN PRESENCE OF EXTRACT OF ACETONE-DRIED POWDER OF BEEF BRAIN UNDER VARIOUS INCUBATION CONDITIONS

The complete incubation mixture was the standard system (p. 7) of $MgSO_4$; sodium glutamate; ATP; 100 µg. NH_4^+ and brain extract equivalent to 100 mg. of acetone-dried beef brain powder. 40-minute incubation.

Incubation Conditions	µg. NH_4^+ found	µg. NH_4^+ Absorbed*	µM glutamine Synthesized
Complete system	30	72	4.0
Complete system	33	69	3.8
No Mg^{++}	106	-4	
No glutamate	99	3	
No ATP	102		
No Ammonia	25		
Brain extract alone	3		

* Values are based on µg. NH_4^+ found in the absence of ATP.

TABLE IV

EFFECT OF METHIONINE SULPHOXIMINE AND DL-α-METHYLGLUTAMATE ON GLUTAMINE SYNTHESIS BY BRAIN EXTRACTS

Standard incubation system containing brain extract equivalent to 80 mg brain powder; $MgSO_4$; ATP; sodium glutamate; bicarbonate buffer and 100 µg. ammonium ions. 40-minute incubation.

Additions to Standard systems	µM glutamine synthesized	Percent Inhibition	Percent Stimulation
None	2.3		
None	2.2		
0.005M methionine sulphoximine	1.5	33%	
0.02M methionine sulphoximine	1.2	47%	
0.05M methionine sulphoximine	1.2	47%	
0.01M α-methylglutamate	2.7		20%
0.05M α-methylglutamate	3.2		42%
0.01M α-methylglutamate	3.4		51%

Methionine sulfoximine, at all concentrations tested, inhibited glutamine synthesis. The inhibition was not reversed by methionine. Methionine was tested since it has been shown to delay or suppress convulsions produced by methionine sulfoximine in rabbits and dogs (34, 35). Pace and McDermott (33) have

reported that the synthesis of glutamine from glutamate in the presence of brain extracts prepared according to Elliott (13) is slightly depressed by methionine sulfoximine. No figures were given, and the note dealt in greater detail with the more marked inhibition by methionine sulfoximine of the glutamotransferase activity of the preparations.

Glutamine synthesis, on the other hand, was apparently stimulated in the presence of high concentrations of α-methylglutamate. It was anticipated that α-methylglutamate might compete with glutamate for the enzyme system synthesizing glutamine. The results of Table IV suggested that α-methylglutamate may actually act as a substrate for the glutamine-synthesizing system of brain extracts, and give rise, presumably, to α-methylglutamine. Various experiments were designed to test this possibility, and the results are presented below.

3. Ammonia Absorption by Glutamate and α-Methylglutamate

The uptake of ammonia in the presence of brain extract and varying concentrations of L-glutamate or DL-α-methylglutamate was tested; the results are shown in Table V. Considerable quantities of ammonia are absorbed in the presence of α-methylglutamate, as well as glutamate. The amount of ammonia absorbed by DL-α-methylglutamate is less than that absorbed by an equivalent concentration of L-glutamate.

TABLE V

AMMONIA ABSORPTION IN PRESENCE OF L-GLUTAMATE AND DL-α-METHYLGLUTAMATE AND BRAIN EXTRACT

Varying concentrations of L-glutamate or DL-α-methylglutamate were incubated with brain extracts equivalent to 80 mg. of acetone-dried powder of beef brain for 40 minutes under standard incubation conditions.

L-glutamate Concentration	μM NH_4^+ Absorbed	DL-α-methylglutamate Concentration	μM NH_4^+ Absorbed
0.040M	4.8	0.160M	2.90
0.020M	3.66	0.080M	2.94
0.010M	3.12	0.040M	2.56
0.005M	2.78	0.010M	1.88
0.0025M	2.40	0.005M	1.30

Affinity of substrates for enzyme

The results shown in Table V, and similar results of experiments in which ammonia uptake by L-glutamate and DL-α-methylglutamate in the presence of a single brain extract were compared, suggest that glutamic acid is more active than its α-methyl derivative as a substrate for the glutamine-synthesizing enzyme. However, it is not possible to determine accurately the relative affinities of the two substrates for the enzyme, since the influence of the D-isomer of DL-α-methylglutamic acid is unknown. A strict comparison of the two substrates would require L-α-methylglutamate, and this isomer has not been isolated from the racemic mixture..

4. Ammonia Absorption by Mixtures of Glutamate and DL-α-methyl-glutamate.

Experiments were performed to observe whether the enzyme responsible for glutamine synthesis is also responsible for ammonia absorption in presence of DL-α-methylglutamate. Concentrations of L-glutamate and DL-α-methylglutamate which "saturated" the enzyme system or systems involved (as determined by concentration-activity relations of the type shown in Table VI) were mixed, and the effects of admixture on ammonia absorption determined. The data from one such experiment are shown in Table VI.

TABLE VI

AMMONIA ABSORPTION BY MIXTURES OF L-GLUTAMATE AND DL-α-METHYLGLUTAMATE IN PRESENCE OF BRAIN EXTRACT

The substrates were incubated with brain extract equivalent to 80 mg. of beef brain powder for 40 minutes in the standard incubation medium.

Substrates	Concentration	μM NH_4^+ absorbed
L-glutamate	0.04M	4.90
DL-α-methylglutamate	0.04M	3.17
DL-α-methylglutamate plus L-glutamate	0.04M 0.04M	4.70
L-Glutamate	0.002M	2.70
DL-α-methylglutamate	0.005M	1.39
DL-α-methylglutamate plus L-glutamate	0.005M 0.002M	2.79

The amount of ammonia absorbed by an incubation mixture of L-glutamate and DL-α-methylglutamate is not greater than the higher of the values obtained when either of the amino acids are incubated separately. No additive effects are noted. This result indicates that the same enzyme is involved in the absorption of ammonia by both amino acids.

5. Effect of Methionine Sulfoximine and NaF on Ammonia Absorption by Glutamate and α-methylglutamate

Sodium fluoride is a potent inhibitor of the brain (13) and liver (11) glutamine-synthesizing systems. Methionine sulfoximine, as shown earlier, inhibits glutamine synthesis considerably with beef brain extracts. It was of interest to observe the effects of these agents on ammonia absorption by glutamate and α-methylglutamate. The results of such an experiment are shown in Table VII. Ammonia absorption by both amino acids was inhibited to about the same extent by the convulsant, and by NaF. This result provides further evidence that the same enzyme catalyzes the absorption of ammonia by both amino acids and favors the view that α-methylglutamine, as well as glutamine, may be synthesized by beef brain extracts.

TABLE VII

EFFECTS OF METHIONINE SULFOXIMINE AND SODIUM FLUORIDE ON AMMONIA ABSORPTION BY GLUTAMATE AND α-METHYL GLUTAMATE IN PRESENCE OF BRAIN EXTRACT

The substrates were incubated with brain extract equivalent to 80 mg. of beef brain powder for 40 minutes under standard incubation conditions. Glutamate and α-methylglutamate concentrations were 0.02M.

Substrate	Inhibitor	μM NH_4^+ Absorbed	Percentage Inhibition
L-glutamate	None	3.22	
L-glutamate	Sodium Fluoride 0.02M	0.0	100%
L-glutamate	Methionine Sulfoximine 0.01M	1.83	43%
DL-α-methylglutamate	None	1.66	-
DL-α-methylglutamate	Sodium fluoride 0.02M	0.0	100%
DL-α-methylglutamate	Methionine Sulfoximine 0.01M	0.55	66%

6. Hydroxamic Acid Formation in Presence of Glutamate and α-methylglutamate

Upon replacement of ammonia with hydroxylamine, the formation of glutamylhydroxamic acid from glutamate, in the presence of fortified extracts of liver (11) and brain (13) can be readily demonstrated by a colorimetric method (31).

Experiments were performed in which ammonia was replaced in some vessels by hydrolylamine, using both glutamic and α-methyl-glutamic acids as substrates, and hydroxamic acid formation (31) and ammonia absorption were determined after incubation with brain extract. The results of two such experiments are shown in Table VIII.

TABLE VIII

GLUTAMYLHYDROXAMIC ACID FORMATION AND AMMONIA ABSORPTION BY GLUTAMATE AND DL-α-METHYLGLUTAMATE IN PRESENCE OF BRAIN EXTRACT

The amino acids (0.02M) were incubated with either 100 µg. NH_4^+ or 100 µg. hydroxylamine* and brain extract equivalent to 100 mg. of beef brain powder, for 40 minutes in the standard incubation medium.

Expt. No.	Amino Acid	µM NH_4^+ absorbed	µM hydroxamic acid formed (as succino-hydroxamic acid equivalent)
1	L-glutamate	2.1	2.1
	DL-α-methyl-glutamate	1.5	1.8
2	L-glutamate	1.7	1.8
	DL-α-methyl glutamate	1.0	1.3

* The hydroxylamine hydrochloride was twice recrystallized from water, and neutralized before use. Sufficient material was added to give 100 µg. hydroxylamine.

Ammonia absorption and hydroxamic acid formation, in the presence of both amino acids, are closely paralleled in both experiments. No hydroxamic acid was formed in the absence of either ATP or amino acids. Presumably α-methylglutamylhydroxamic acid is formed by the brain extract from α-methylglutamate and hydroxylamine. Similar results have been obtained by Lichtenstein (24) et al with sheep brain extracts.

These results are in accord with the observations on ammonia absorption, and indicate that α-methylglutamate acts as a substrate for the glutamine-synthesizing enzyme of beef brain extracts. Results to be discussed, obtained by paper chromatography, provide evidence that the reaction product is α-methylglutamine.

7. Chromatographic Experiments with Incubation Mixtures

The products formed by the brain extract in the presence of ammonia and either L-glutamate or DL-α-methylglutamate were investigated by the paper chromatogram technique. After incubation, samples of the incubation mixture were spotted on paper. Part of the incubation mixture was first deproteinized, by heating for about two minutes in a boiling water bath, and samples of the supernatant obtained after centrifugation were spotted on paper. After chromatographing by the procedure described earlier (p.12) a spot was obtained at a position distinct from that occupied by glutamine, L-glutamate, or DL-α-methylglutamate. The R_f values were as follows:

L-glutamate, R_f = 0.21

L-glutamine, R_f = 0.33

DL-α-methylglutamate, R_f = 0.25

"α-methylglutamine", R_f = 0.42

Band chromatograms were also made of the incubation mixture for the purpose of obtaining sufficient material for other tests. Narrow strips of the various bands were cut out and treated with ninhydrin to locate the different compounds. The strips were then re-attached precisely to their original position on the paper, and the bands of paper corresponding to the positions of the various compounds were cut away. The bands were then treated with 2N NaOH to hydrolyze labile amides, and the ammonia produced was estimated by the technique used in all this work.

The results (Table IX) show that bands corresponding to glutamine, and to the product formed after interaction of DL-α-methylglutamate and ammonia, release much larger quantities of ammonia than is released by the bands corresponding to L-glutamate and DL-α-methylglutamate. The differences are particularly pronounced in Experiment 2 of Table IX, in which the paper "blank" is relatively small.

Samples of the band chromatograms were also heated for 1.5 hours at 100°C in 0.01M phosphate buffer at pH 6.5, and the solutions then re-chromatographed. Following this treatment, the spots in the positions characteristic for glutamine and the new compound failed to appear. Heat treatment did not eliminate the spots characteristic for glutamate and α-methylglutamate. Heating under the above conditions hydrolyzes glutamine to pyrollidone-carboxylic acid (36, 37) and apparently alters the ninhydrin-reacting group of the new compound, perhaps by a similar reaction.

TABLE IX

RELEASE OF AMMONIA FROM CHROMATOGRAMS UPON TREATMENT WITH STRONG ALKALI

Bands of paper of equal area, corresponding to the positions of the various compounds in the incubation mixture, were incubated in Warburg vessels with 3 ml. 2N NaOH for 3 hours at 37°C, and the ammonia released was estimated.

Band corresponding to	μg. NH_4^+ released Expt. 1	Expt. 2 *
Paper "blank"	25	10
L-glutamate	30	13
Glutamine	65	50
DL-α-methylglutamate	33	15
α-methylglutamine	55	45

* The paper used for chromatograms was washed with 0.1N NaOH before use.

These results are consistent with the conclusion that an amide, probably α-methylglutamine, is formed during the reaction of DL-α-methylglutamate and ammonia, in the presence of brain extract and ATP.

Summary

1. Extracts of acetone-dried powders of beef brain, in the presence of ATP and magnesium ions, synthesize glutamine from L-glutamic acid and ammonia.

2. Ammonia is actively absorbed in the above system when L-glutamic acid is replaced by DL-α-methylglutamic acid.

3. The absorption of ammonia by this system in the presence of mixtures of L-glutamate and DL-α-methylglutamate is not additive.

4. Methionine sulfoximine and sodium fluoride inhibit by about the same extent the absorption of ammonia by glutamate and by α-methylglutamate.

5. Hydroxamic acid formation is obtained, both with glutamate and with α-methylglutamate, when ammonia is replaced during incubation by hydroxylamine.

6. Paper chromatography results indicate the synthesis of a new compound from α-methylglutamate and ammonia, with an R_f distinct from that of glutamate, glutamine or α-methylglutamate.

7. The new compound releases ammonia on treatment with alkali; and does not appear on chromatograms after being subjected to conditions which completely hydrolyze glutamine.

8. The results provide evidence that the glutamine-synthesizing enzyme of beef brain extracts catalyzes the absorption of ammonia by α-methylglutamate, and that the product formed is probably α-methylglutamine.

C- EXPERIMENTS WITH BRAIN GLUTAMINASE

1. Glutaminase Activity of Homogenates

The rate of breakdown of glutamine, with respect to time, in the presence of a fixed amount of rat brain homogenate, is shown in the data of Table X. The rate is linear for the first

20 minutes of the reaction.

TABLE X

GLUTAMINASE ACTIVITY OF RAT BRAIN HOMOGENATE

Vessels contained 3 ml. 0.02M phosphate buffer (pH 7.35); 15 μM glutamine and 20 mg. rat brain homogenate prepared in 0.9% NaCl. Incubation at 37°C in air for varying intervals of time. Values corrected for brain blank (3 μg. NH_4^+) and non-enzymatic glutamine hydrolysis (22 μg. NH_4^+).

Time of Incubation (Minutes)	μM NH_4^+ liberated
5	1.15
10	2.28
15	3.33
20	4.46
30	5.77
40	6.90

The effect of variation in the quantity of rat brain homogenate on the rate of breakdown of glutamine, is shown in the results of Table XI. The amount of ammonia liberated is approximately directly proportional to the amount of brain tissue used.

TABLE XI

GLUTAMINASE ACTIVITY OF RAT BRAIN HOMOGENATE

Vessels contained 3 ml. 0.02M phosphate buffer (pH 7.35), 22.5 μM glutamine and rat brain homogenate in 0.9% NaCl. Incubation at 37°C in air for 30 minutes. Values corrected for brain blank and non-enzymatic hydrolysis.

Brain Homogenate	μM NH_4^+ liberated
0	0
10	1.89
20	3.16
30	4.78
40	6.28

2. Inhibition of Glutaminase by DL-α-methylglutamic and D- and L-glutamic acids

It was of interest to observe the effect of DL-α-methylglutamate on brain glutaminase. Glutaminase activity of brain homogenate was tested in the presence of different concentrations of α-methylglutamate. The data of such an experiment are shown in Table XII.

TABLE XII

INHIBITION OF GLUTAMINASE ACTIVITY OF RAT BRAIN HOMOGENATE BY DL-α-METHYLGLUTAMATE

Vessels contained 0.02M phosphate buffer (pH 7.35); 22.5 μM glutamine; 20 mg. homogenate prepared in 0.9% NaCl and varying concentrations of inhibitor. Incubation for 30 minutes at 37°C in air. Values corrected for brain blank and non-enzymatic hydrolysis.

DL-α-Methylglutamate Concentration (M)	μM NH_4^+ Liberated	Percentage Inhibition
0.0	3.80	
0.001	3.71	
0.002	3.38	11%
0.005	2.62	29%
0,01	2.25	39%
0.05	1.33	65%
0.10	0.82	78%
0.20	0.72	81%

The results indicate that DL-α-methylglutamate inhibits brain glutaminase, and the inhibition increases with increasing concentrations of the amino acid. These results are similar to those obtained with dog kidney glutaminase (25).

The inhibition of brain glutaminase by α-methylglutamate is comparable with that produced by D- and L-glutamate (4), as shown by the data of Table XIII. The results indicate a competition

of these amino acids with glutamine for glutaminase.

TABLE XIII

INHIBITION OF GLUTAMINASE ACTIVITY OF RAT BRAIN HOMOGENATE BY DL-α-METHYLGLUTAMIC ACID AND D- AND L-GLUTAMIC ACIDS

Vessels contained 0.02M phosphate buffer (pH 7.35); 22.5 μM glutamine; 20 mg. rat brain homogenate prepared in 0.9% NaCl and inhibitors as indicated. Incubation for 30 minutes at 37°C in air. Values corrected for brain blank and non-enzymatic hydrolysis.

Inhibitor	Concentration	μg. NH_4^+ Liberated	Percentage Inhibition
None	-	3.45	-
D-glutamate	0.01M	0.55	84%
L-glutamate	0.01M	0	100%
DL-α-methyl-glutamate	0.01M	0.93	73%

Summary

Glutaminase activity of brain homogenates is inhibited by DL-α-methylglutamate approximately to the same extent as by D- or L-glutamic acids.

D - MISCELLANEOUS EXPERIMENTS WITH α-METHYLGLUTAMATE

1. Transamination

Braunstein and Kritzmann (38) first provided evidence that animal tissues contain enzymes catalyzing the transfer of the α-amino

nitrogen of one amino acid directly to the carbon skeleton of another amino acid. Transaminating activity has been found in homogenates of various tissues of the rat, including liver and brain (39).

It was of interest to test the effect of α-methylglutamate on the transaminating activity of fresh tissue. Rat brain and liver homogenates were employed. The production of two amino acids was followed; (1) alanine formation from pyruvate and glutamate, (2) glutamate formation from alanine and α-ketoglutarate. Incubations were conducted at 37°C in open conical centrifuge tubes containing 1.0 ml. 0.01M phosphate buffer (pH 7.4), 50-100 mg. tissue and 10-20 μM each of the α-keto and α-amino acids. The amounts of α-methylglutamate added ranged from 5-100 μM in various trials. The incubation time was varied from 15-60 minutes in different experiments. The reaction was stopped by heating the reaction mixture for 5 minutes at 100°C in a water bath. After centrifuging, precise aliquots of the supernatants were spotted on paper and chromatograms prepared by the techniques described previously. The intensity of the spots provided an estimate of the transaminase activity of the samples. DL-α-methylglutamate had no detectable effect, under the various experimental conditions, on the transaminase activities of either brain or liver homogenates.

2. Glutamic Decarboxylase

Awapara and collaborators (40) have isolated, identified and estimated the free γ-aminobutyric acid in the brains of

several species of mammals, including the rat. Their data indicated that glutamic acid is converted into γ-aminobutyric acid by decarboxylation.

Attempts were made to isolate γ-aminobutyric acid by paper chromatography, after incubation of rat brain homogenates with glutamic acid, alone and with α-methylglutamate. Mere traces of γ-aminobutyric acid (spots from brain homogenates were compared with synthetic material) were obtained, and the possible effects of α-methylglutamate could not be assessed under these experimental conditions.

Summary

α-methylglutamate did not affect the transaminase activity of liver or brain homogenates, under various experimental conditions.

CHAPTER IV

DISCUSSION

A ready explanation for not obtaining glutamine synthesis with brain homogenates cannot be offered, but tentative suggestions may be made. Krebs (4) found that the amount of glutamine synthesized by brain slices is only ten percent of that of kidney slices and about twenty-five percent of that of retina. The synthetic activity of tissue homogenates, in which most of the cellular structure is destroyed, is usually considerably lower than that of tissue slices, whose cells are relatively intact. The glutamine-synthesizing activity is concentrated in brain cortex. Since homogenates of whole brain were used, only a small fraction of the tissue employed contained the synthesizing enzyme. Disruption and dilution of cells, in a tissue not too active under optimal conditions, may account in part for lack of synthetic activity.

Brain homogenates are particularly rich in enzymes which destroy the pyridine nucleotide and adenylic acid coenzyme systems (41). The integrity of these systems is of crucial importance to synthetic reactions. Rapid destruction of added ATP by adenosine-triphosphatase, and irreversible breakdown of the adenylic system by adenylic deaminase, may account for lack of glutamine synthesis on energetic grounds.

The high glutaminase activity of brain homogenates is another important consideration in accounting for the absence of glutamine synthesis in this system. Although glutaminase is strongly inhibited by glutamic acid, Krebs (4) points out that

"the equilibrium of the reaction" catalyzed by the hydrolytic enzyme "lies at practically complete hydrolysis of the amide (in physiological solution)", and "no trace of glutamine is formed from glutamic acid and ammonia in the presence of glutaminase". It is quite possible that a net synthesis of glutamine could not be obtained with brain homogenates because the glutamine, which may be synthesized at a slow rate, can be slowly but completely hydrolyzed due to the high glutaminase activity of the preparations.

It is interesting that α-methylglutamic acid can act as a substrate for the glutamine-synthesizing enzyme, particularly in view of the high substrate specificity reported for the latter (13). Elliott's work (13) indicates that the amino group and both carboxyl groups must be available for activation of glutamic acid by the enzyme. The work reported here shows that the α-hydrogen of glutamic acid is not essential for enzyme activation.

The evidence presented here, coupled with analogy to the synthesis of glutamine from glutamic acid and ammonia, favours the concept that the product produced with α-methylglutamate as substrate is α-methylglutamine. It is not possible to distinguish between the synthesis of glutamine and α-methylglutamine, with the available methods for glutamine estimation. Chemical synthesis of α-methylglutamine has not yet been achieved. Should this compound become available, one could determine whether or not synthetic α-methylglutamine, and the product produced by beef brain extracts, are identical. Isolation and characterization of

the reaction-product of a large-scale incubation, as performed by Elliott with glutamine (13), will provide final proof of the enzymatic synthesis of α-methylglutamine.

The absorption of ammonia by α-methylglutamate in the presence of beef brain extract is an example of a fruitful combination between enzyme and "unnatural" substrate. Results have been obtained with more complex systems, in which an analogue of the natural substrate is tested for competition with the latter, and the specificity barrier is completely bypassed, in that the analogue acts as a substrate. Levine and Tarver (42) provided evidence that ethionine, an analogue of methionine, is incorporated into tissue protein, and Mitchell and associates (43) found that 8-azaguanine is incorporated into pentose nucleic acid of both tumor and normal tissue. These findings bring up questions as to the effect of such syntheses on the cell, and whether these events are common or rare.

From the response of α-methylglutamate with the glutamine-synthesizing system, one might have anticipated its inhibitory effect on glutaminase activity. It is of interest that DL-α-methylglutamate administered to mice produces audiogenic seizures, and that a reduction in the number of seizures occurs when glutamic acid is given (27). The fact that DL-α-methyl-glutamate and L-glutamate compete in the brain for ammonia absorption may afford an explanation for this phenomenon.

The inhibition of ammonia absorption by both L-glutamate and DL-α-methylglutamate in the presence of DL-methionine sulfoximine is interesting because this compound produces convulsions in several species of mammals, when given at the proper dose levels (34, 35, 44). Methionine sulfoximine structurally resembles methionine and glutamine, and may thus be a metabolic antagonist to either or both of these amino acids. Methionine was not found to reverse the inhibition of glutamine synthesis by beef brain extracts. The inhibitory effect of methionine sulfoximine on the glutamine-synthesizing system of brain extracts suggests the possibility that the glutamic acid-glutamine system may be involved somewhere in the biochemical changes leading to convulsive seizures.

Transamination and decarboxylation of glutamic acid involve functional groups attached to the α-carbon of glutamic acid, and it was of interest to observe the effect of α-methylglutamic acid on these systems. Transamination was unaffected by α-methylglutamate, and this result emphasizes the importance of the α-hydrogen for the transaminating enzymes, in effecting combinations with substrates. Lichtenstein et al (25), prompted possibly by similar considerations to the above, found on testing DL-α-methylglutamic acid with crystalline glutamic dehydrogenase, that it acted neither as a substrate nor as an inhibitor of the enzyme.

The experiments testing the effect of α-methylglutamate on glutamic decarboxylase were inconclusive, since the decarboxylase

system employed in the work was too feeble. However, Roberts (45) has demonstrated that the decarboxylation of glutamic acid by mouse brain or bacterial extracts is inhibited by DL-α-methyl-glutamic acid. A more detailed study indicated the inhibition to be competitive (46). It was suggested that α-methylglutamic acid associated with the enzyme at three loci, but decarboxylation could not occur because of the absence of the α-hydrogen.

The results reported, here, and those of other workers, on the effect of α-methylglutamic acid on enzyme systems involving glutamic acid and glutamine, illustrate the usefulness of this compound as a tool in further investigation of the mechanisms involved in the metabolism of glutamic acid and its amide.

GENERAL SUMMARY - Part I

A modified microdiffusion method, employing the Warburg manometric apparatus, has been developed for the estimation of ammonia in the presence of tissue.

Data has been presented, using this method, which shows that ammonia may be recovered, under various experimental conditions, with an error of 5%.

Little or no glutamine synthesis was obtained with several preparations of fresh brain, under various experimental conditions.

DL- α-methylglutamate had no effect on the transaminase activity of liver or brain homogenates, under various experimental conditions.

Extracts of acetone-dried powders of beef brain, in the presence of ATP and magnesium ions, synthesize glutamine from L-glutamic acid and ammonia.

Ammonia is actively absorbed in the above system when L-glutamic acid is replaced by DL- α-methylglutamic acid. The absorption of ammonia by mixtures of L-glutamate and DL- α-methylglutamate is not additive.

Methionine sulfoximine and sodium fluoride inhibit by about the same extent the absorption of ammonia by glutamate and by α-methylglutamate.

Hydroxamic acid formation is obtained, both with glutamate and with α-methylglutamate, when ammonia is replaced during incubation

by hydroxylamine.

Paper chromatography results indicate the synthesis of a new compound from α-methylglutamate and ammonia by beef brain extracts, with an R_f distinct from that of glutamate, glutamine or α-methylglutamate.

The new compound releases ammonia on treatment with alkali, and does not appear on chromatograms after being subjected to conditions which completely hydrolyze glutamine.

The results provide evidence that the glutamine-synthesizing enzyme of beef brain extracts catalyzes the absorption of ammonia by α-methylglutamate, and that the product formed is probably α-methylglutamine.

The results were discussed with regard to their biological significance in glutamic acid-glutamine metabolism..

PART II

PROTEIN SYNTHESIS AND SECRETION BY GLANDULAR TISSUES IN VITRO

CHAPTER V

GENERAL INTRODUCTION

The problem of protein synthesis has been approached by several different lines of attack, and a considerable body of information has been accumulated. However, the precise mechanisms involved in protein synthesis are still unknown. Early attempts to obtain protein synthesis *in vitro* with crude proteolytic enzyme preparations have been continued more recently with crystalline enzymes. The availability of radioactive isotopes has stimulated many *in vivo* and *in vitro* studies on amino acid incorporation into mixed tissue proteins. The modern concept of biological energetics has been applied to peptide bond synthesis in many interesting studies. Most recently, interest has centred around demonstrating the synthesis of specific proteins or enzymes, *in vitro*, and the mechanisms involved. It is proposed to discuss these main lines of research with particular emphasis on the observations relating to the work described in the succeeding chapters.

Investigations with Proteolytic Enzymes

Plastein, an insoluble material formed from concentrated solutions of peptic digests by the action of pepsin at pH 4, was discovered in 1886 by Danilewski (47). Wasteneys and Borsook (48, 49), among numerous other workers, investigated plastein formation. The need for concentrated peptic digests was confirmed, and amino and carboxyl groups were shown to disappear during

plastein formation. The earlier results obtained with impure enzyme preparations have been confirmed more recently with crystalline pepsin (50) and with trypsin or papain acting on peptic digests of insulin (51). Many investigators considered plastein to be a product of synthesis. It was suggested that protein synthesis in general may be catalyzed by proteolytic enzymes (49).

Folley (52) in 1932 questioned whether peptide bonds were reconstituted during plastein formation; ultracentrifugal studies, recently confirmed by Ecker (53), indicated average molecular weights of not over 1,000 for plasteins. Virtanen and associates (54, 55) consider, on the basis of X-ray diagrams and other evidence, that the molecular weights of plasteins produced by pepsin are of the order of 5,000. Northrop (56) studied the properties of plastein produced from peptic digests of enzymes (pepsin or trypsin). The plastein showed no enzymatic activity and did not possess the general properties of the enzyme protein from which it was derived. It is most unlikely that plasteins resemble natural proteins in many other ways than being polypeptides.

Tauber (57, 58) recently demonstrated enzymatic synthesis of protein-like material with molecular weights, based on ultracentrifuge measurements, of 250,000-500,000. The polymers were produced upon incubating concentrated solutions of Witte peptone (57), or peptic digests of a wide variety of proteins (58), with small quantities of crystalline chymotrypsin at pH 7. The energy

for the synthetic reaction was considered to be provided by precipitation of the reaction products.

Resynthesis of proteins from proteolytic digests upon submitting the digests and the proteolytic enzyme to high pressures (5-10,000 atmospheres) has been reported by Bresler and co-workers (59, 60, 61, 62, 63, 64, 65). The enzymatic, immunological (62), and hormonal (63, 64) properties of the original proteins, in whole or in part, were found to be present in the synthetic products. Linderstrøm-Lang found that application of high pressures facilitates hydrolysis of proteins due to the volume contraction occurring during protein hydrolysis (66); this observation is not in accord with the above studies.

The early work of Bergmann and Fraenkel-Conrat (67) on the enzymatic synthesis of peptides has been pursued more recently by many workers (68, 69, 70, 71, 72). These investigations have shown that acylamino acid anilides are formed upon incubation of acylamino acids and substituted anilides with chymotrypsin or papain. The use of the above substrates favour peptide synthesis because of the insolubility of the reaction products and the low free energy of formation of a peptide bond from non-ionized amino acids. Di- and tri-peptides of methionine, in 10-20% yield, have been obtained by Brenner and associates upon incubation of a wide variety of methionine esters with crystalline chymotrypsin at pH 9 (73).

The above efforts to demonstrate enzymatic synthesis of

peptides and proteins *in vitro*, were mainly based on establishing conditions to reverse proteolysis. The equilibrium in hydrolytic reactions catalyzed by proteolytic enzymes is 99% in the direction of hydrolysis(74). Conditions designed to favour peptide bond synthesis, which include high buffer concentrations (up to 1M),(71), high concentrations of substrates, and formation of insoluble products, are far removed from the environment of physiological systems. Bergmann, (67) nevertheless, suggested that the mechanism of peptide and protein synthesis *in vivo* may be similar to the synthesis of peptides by model systems. Whether or not this is true, work with these systems indicates that proteolytic enzymes are potentially capable of catalyzing peptide bond synthesis.

Energy relations in peptide bond synthesis

Since proteins are about 99% hydrolyzed at equilibrium (74), protein synthesis is dependent on an available source of energy. The free energy of formation of peptide bonds has been calculated by various authors (74, 75, 76), and is of the order of 3,000 calories. One of the significant advances of recent years has been the elucidation of the participation of high-energy phosphate bonds in biosynthetic processes (78, 79, 80). The demonstration by Lipmann (81, 82) that acetylation of sulfanilamide by cell-free tissue extracts depends on the stoichiometric utilization of ATP suggested that compounds containing high-energy phosphate participate in the synthesis of amide linkages.

Peptide bond synthesis has been shown by various workers

to be coupled with oxidative reactions in respiring tissue or to be dependent on ATP. Synthesis of the peptide p-aminohippuric acid, from p-amino benzoic acid and glycine, by respiring liver and kidney homogenates has been demonstrated by Cohen and McGilvery (83, 84, 85). The reaction occurs anaerobically only in the presence of ATP. Other demonstrations of the requirement of ATP as a source of energy in the formation of peptide and amide bonds have been made in the synthesis of hippuric acid (86), glutamine (10, 11, 13) (see p.2), citrulline (87), glutathione (88, 89, 90), and ornithuric acid (91). Chantrenne (92) has provided evidence for the participation of coenzyme A in hippuric acid synthesis.

Hanes and co-workers (93, 94) have shown with mammalian tissue enzymic transpeptidation reactions involving glutathione or γ-glutamyl peptides as "donors" and various amino acids as "acceptors"; an enzyme from plant tissue effected transpeptidation reactions involving normal α-aminoacyl peptides. Evidence for the catalysis of transamidation (transpeptidation) reactions by proteolytic enzymes (papain, chymotrypsin), resulting in elongation of peptide chains, has been provided by Fruton and his associates (95, 96, 97, 98). The enzyme (glutamotransferase) catalyzing transamidation reactions between glutamine and hydroxylamine or ammonia has been demonstrated with various preparations (15, 16, 17, 18) (see p.3). These findings led Hanes <u>et al</u> (93) and Fruton <u>et al</u> (95, 98), independently, to a similar hypothesis attempting to link the enzymatic catalysis of transpeptidation

reactions to the role of ATP in the biosynthesis of glutamine and glutathione. According to this view, the latter two compounds, which are formed with participation of high-energy phosphate, as discussed above, represent the substrates through which the energy of the phosphate bond is channeled into the formation of peptide bonds, and hence proteins (22).

Incorporation of isotope-labelled amino acids into proteins

The investigations of Schoenheimer and his colleagues (99) on protein turnover in vivo, using deuterium and N^{15} labelled amino acids and ammonia, demonstrated that the rate of protein breakdown and resynthesis in an animal in nitrogen equilibrium is much greater than was proposed in Folin's theory of endogenous and exogenous metabolism. The half-life of liver proteins, as determined by the isotope technique, is about 7 days (100, 101); estimates of the half-life of total protein in the rat and man, gave figures of 17 and 80 days, respectively (102).

The increasing availability of radioactive isotopes has stimulated studies on the incorporation of labelled amino acids into protein in vitro and has provided interesting information. This subject has been reviewed by Borsook in 1950 (103). Various tissue preparations such as slices, homogenates, and isolated cytoplasmic components incorporate labelled amino acids into their protein moiety. Embryonic and malignant tissues, incorporate labelled amino acids more rapidly than normal adult tissues (104, 105, 106). Mechanical damage to cells reduces the rate at which

their proteins take up labelled amino acids by 4- to 40-fold (107, 108).

For most amino acids studied, incorporation was shown to be dependent on an intact supply of energy provided by oxidative reactions in respiring systems. Frantz et al found anaerobic conditions (109) and 2:4-dinitrophenol (110) inhibit alanine uptake by rat liver slices, and Winnick et al (111) showed that glycine is not incorporated by rat liver homogenates anaerobically. Arsenate, azide, cyanide and fluoride were shown to inhibit incorporation of methionine into E.coli by Melchior et al (112); inhibition by these respiratory poisons of glycine, leucine and lysine uptake by bone marrow cells and rat diaphragm was observed by Borsook et al (113).

Lysine uptake, however, into guinea pig liver homogenate, or any of its fractions, is not inhibited by anaerobic conditions, and only partly inhibited by respiratory poisons (114). Two different enzyme systems are apparently involved. Histidine incorporation by bone marrow cells is also only partly inhibited anaerobically (103).

Winnick observed that homogenates of embryonic rat liver lost most of their incorporative ability upon dialysis (115). Most of the activity could be restored by adding ATP, Mg ions and a mixture of unlabelled L-amino acids to the dialyzed homogenates. Siekevitz (116) fractionated liver homogenates and found that the greatest rate of labelled alanine incorporation occurred in the microsome fraction. Significant incorporation did not occur when

either isolated microsomes or mitochondria were incubated with labelled alanine; incubation of both fractions together resulted in incorporation. Inactivation of the mitochondria by heat or the presence of 2:4-dinitrophenol inhibited incorporation. The results indicate that the oxidative activity of the mitochondria and the concomitant phosphorylation are responsible for amino acid incorporation into protein, and that the actual incorporation occurs in the microsomes.

Labelled amino acids that are not normal constituents of animal proteins have been tested, in vivo and in vitro, to determine whether unnatural amino acids would be incorporated. α-Aminoadipic acid when injected into mice, was not incorporated into the proteins (117). However, the unnatural amino acid was catabolized at approximately the same rate as lysine. Rat tissue proteins, however, incorporate ethionine in vivo (42). The incorporation is only about one-tenth of that of natural amino acids, and the half-life is less; this finding is nevertheless of great interest. D-lysine, the one amino acid of the D configuration studied, was not incorporated (114).

Simpson (118) has recently studied the release of amino acids from proteins by labelling the proteins of the intact rat with either S^{35}-methionine or C^{14}-leucine and then following the release of these amino acids from the proteins of liver slices. The liberation of the labelled amino acids from protein was inhibited by conditions which limit the production or use of energy (anaerobiosis,

cyanide, dinitrophenol). Since energy is required both for the incorporation and the release of amino acids from protein, it was suggested that the two processes might be interrelated. However, other possible interpretations were not precluded by the author.

Studies on protein synthesis and amino acid incorporation in tissue cultures have been made by Wimmick and associates (119, 120). It was found that labelled amino acid incorporation into the tissue proteins is more rapid under conditions of tissue autolysis than under conditions favourable to growth. Rapid turnover of labelled amino acids occur in tissue cultures in the absence of growth (119). Most of the radioactivity of labelled proteins from the nutrient medium could be transferred to heart tissue culture protein without the release of free amino acids. Amino acid analogues (ethionine, o-fluorophenylalanine and β-3-thienylalanine) and dinitrophenol inhibited, in varying degree, protein synthesis, growth and amino acid incorporation. However, even the most potent inhibitors did not prevent transfer of radioactivity from labelled protein in the medium to tissue protein. The results indicate that embryonic heart cultures can use protein of the nutrient medium extensively, and without completely hydrolyzing it. The utilization presumably represented in part a turnover type of process (120).

The exact nature of the processes involved in amino acid incorporation into tissue protein is unknown, but there is considerable evidence to indicate that peptide bond synthesis may occur (108, 110, 121, 132, 133, 135). In this regard, Brunish and Luck (122)

have shown that amino acids can become firmly bound to proteins without the participation of enzymes. Preparations of inert liver histones "incorporated" labelled amino acids, when incubated together, in vitro, at temperatures up to 100°C. The labelled histones withstood various tests for genuine incorporation; no carbon dioxide was released on treatment with ninhydrin. However, incorporation of radioactivity was very small. The results indicate the need for caution in isotopic work, especially in systems with very low incorporative activity, but do not necessarily discredit previous work. Borsook (103) is inclined to the view that "turnover and synthesis of protein are the same process and that in both processes proteins are built up from peptides as intermediates rather than in the case of turnover by a template exchange mechanism". Investigations on the synthesis of specific proteins are more desirable, and some work along these lines is described below.

Synthesis of Specific Proteins and Enzymes in vitro

A net synthesis of amylase has been obtained by Hokin (123, 124) upon incubation of pigeon pancreas slices in physiological saline. The rate of amylase synthesis was increased about two to threefold by the addition of a mixture of 20 amino acids or acid-hydrolyzed casein supplemented with tryptophan. Synthesis of the enzyme was shown to be an energy-requiring process and was inhibited by various respiratory poisons. It was found that every nutritionally essential amino acid present in crystalline amylase was required for its maximal synthesis (125). Methionine, which is the only essential

amino acid absent from crystalline amylase, was the only essential amino acid not required for amylase synthesis.

Peters and Anfinsen (126) have demonstrated a net production of serum albumin upon incubation of chicken liver slices in bicarbonate medium; an immunological technique was developed for determining small amounts of serum albumin in slices and their incubation media. The formation of serum albumin was also found to be dependent upon a supply of energy within the cell. The rate of albumin production was not significantly affected by various hormones or oxidizable substrates. Addition of amino acids did not affect the rate of albumin synthesis, in contrast to the observations with amylase (125). The authors concluded that the liver slice system is complete in itself with regard to its supply of amino acids and energy-yielding compounds. Demonstration of the incorporation of large amounts of radioactive carbon dioxide into the dicarboxylic amino acids of the albumin provided further evidence that the production of albumin represents formation of new protein (126, 127).

Ranney and London (128) showed that <u>in vitro</u> formation of antibody may be obtained in the liver and spleen, but not in the kidney tissue, of animals actively synthesizing immune bodies. It was also indicated that the antibody produced incorporates labelled glycine. The evidence presented in this work and the above (126) indicates that labelled amino acids are taken up into newly formed protein. Such results support the supposition that protein synthesis, and not merely some exchange-equilibrium or adsorption, is measured

in studies with isotopic amino acids. However the possibility does exist, and is actually indicated by the studies on lysine incorporation in homogenates (113), that certain amino acids in particular locations in protein are unusually labile and undergo rapid exchange.

The investigations discussed above on amino acid incorporation and specific protein synthesis have dealt mainly with aspects of intracellular location of incorporating enzymes, energy and substrate requirements (particularly amino acids) and the action of inhibitors. These studies provide little information on possible intermediate compounds or later reactions, beyond the primary combination of amino acids, or the "peptidization" step suggested by Lipmann (80). Various authors have proposed that the intermediates in protein synthesis may be peptides or non-specific proteinogens (protein precursors) (103, 129, 130). The composition of several large peptides found in tissues, which may represent stages in the formation of protein, have been studied by Borsook and associates (131).

Anfinsen and Steinberg (132, 133) have approached experimentally the problem of whether all amino acids are incorporated into a given protein at the same time (template theory) (134), in studies in vitro on ovalbumin synthesis in minced oviduct tissue in the presence of labelled carbon dioxide. Crystalline radioactive ovalbumin was isolated and split by a specific enzymatic method into a hexapeptide and "plakalbumin". The aspartic acid isolated from the peptide fraction had a much higher specific

activity than the average specific activity of aspartic acid residues in the remaining protein fraction (132). The investigation was extended to labelled ovalbumin prepared in vivo (133), and the radioactivity of amino acid residues besides alanine was examined in peptides produced by peptic digestion of ovalbumin, as well as by plakalbumin formation. Non-uniform protein labelling was demonstrated in these studies and the authors thus ruled out a complete template mechanism for protein synthesis. A stepwise synthetic process was suggested, involving the formation of amino acid derivatives, probably peptides, as intermediates. A recent report on similar findings with peptic digests of radioactive crystalline ribonuclease has prompted Anfinsen to suggest that a stepwise synthetic process may be a general mechanism for protein synthesis (135).

Tracer studies of serum albumin formation by Peters (136) indicate that amino acids are in intermediate compounds for a measurable period of time before forming the final protein. It was found that radioactive carbon appears in serum albumin 15 to 20 minutes later than in the total protein of chicken liver slices; unlabelled albumin continues to appear during this period. The intermediates do not resemble serum albumin in either serological, electrophoretic, or solubility behavior, and the conversion to albumin apparently occurs by a process which causes the simultaneous appearance of all these properties. These observations are in accord with the findings discussed above.

Protein Secretion

Most of the information on the mechanism of protein secretion has been obtained from cytological studies on the secretory cycle of digestive glands. The existing knowledge on this subject has been exhaustively collected and lucidly presented by Babkin (137). The term secretion is often employed to describe both the formation and discharge of proteins by secretory cells. Secretion will be used here to signify only the discharge of proteins.

Heidenhaim (140) and Langley (141) were the first investigators to recognize that glandular cells undergo a series of internal changes, and to establish the relation between the histological changes and the enzyme content of glandular tissue. Glandular cells contain large granules which diminish both in size and number during secretion and are restored in the resting state. The enzyme content of glandular tissue (e.g. pancreas) is proportional to the granule content of the secretory cell. It is therefore considered that the secretory enzymes are present in the granules.

The large granules of pancreas cells, which were isolated by centrifugation by Bracket and Jeener (142), were found to have various enzymes (amylase, trypsin, arginase, ribonuclease, phosphatases and dipeptidases) partly bound to them. Khesin (143) isolated the larger cytoplasmic granules from perfused rat liver by centrifugation and obtained serum albumin in the centrifugate. Albumin was released into the surrounding medium by rat liver slices on aerobic incubation but not anaerobically. The process under aerobic conditions was likened to secretion of proteins by various glandular tissues.

The *in vitro* secretion of pepsin by isolated dog stomach was first reported by Edwards and Edwards (144). Acetylcholine, eserine or pilocarpine increased secretion. Hokin (124) has demonstrated that cholinergic drugs stimulate amylase secretion by pigeon pancreas slices; the process is dependent upon a supply of energy. Villarreal (145) has found that pepsin secretion by mouse stomach *in vitro*, is stimulated threefold over the basal level by carbamylcholine. Davies and associates (146) have demonstrated histologically the discharge of secretory glanules from isolated cat pancreas upon incubation in media containing pancreozymin or acetylcholine with eserine.

SECTION A (CHAPTERS VI AND VII)

ENZYME SYNTHESIS AND SECRETION BY PIGEON PANCREAS SLICES

CHAPTER VI

MATERIALS AND EXPERIMENTAL METHODS

A - INCUBATION MEDIA

1. Media

Two media were used: (a) the bicarbonate saline of Krebs and Henseleit (147) gassed with 7% CO_2 in 93% O_2; (b) Krebs Medium III (148) gassed with either 100% O_2 or 100% N_2. Krebs Medium III was modified by replacing the sodium salts of the organic acids by an equivalent quantity of NaCl (124); the modified medium will be called "Medium III". All media contained glucose in a final concentration of 0.2%.

2. Amino Acid Mixtures

In experiments on the effects of amino acids, the following mixtures were usually used. Mixture I - ("Aminosol") - a 5% solution of a partially acid-hydrolyzed preparation of fibrin consisting of approximately two-thirds free amino acids and one-third peptides. Mixture II - composed of the amino acids found "essential" for maximal amylase synthesis (125). Mixture III - composed of amino acids not required for amylase synthesis. Commercial amino acids (Merck and Nutritional Biochemicals) were used for preparing Mixtures II and III.

The components (and final concentrations in the incubation medium) of Mixtures II and III are shown in Table XIV. Where Mixture I was used, 0.2 ml. of the 5% solution was added to a final

volume of 3.0 ml. so that the final Aminosol concentration was 0.34%. Stock solutions of Mixtures II and III were prepared by dissolving the amino acids in Krebs Medium III and neutralizing with NaOH; all amino acid mixtures were stored in the deep freeze at -10°C.

TABLE XIV

STANDARD AMINO ACIDS MIXTURES

(Concentrations are those in incubation vessels in routine expts.)

Mixture II		Mixture III	
Amino Acid	Conc'n (M)	Amino Acid	Conc'n (M)
L-Tryptophan	0.002	L-Proline	0.002
L-Arginine	0.002	DL-Aspartate	0.004
L-Lysine	0.002	L-Cystine	0.002
L-Leucine	0.002	L-Glutamate	0.002
L-Histidine	0.002	DL-Norleucine	0.004
L-Tyrosine	0.002	L-Hydroxyproline	0.002
DL-Valine	0.004	L-Citrulline	0.002
DL-Isoleucine	0.004	L-Ornithine	0.002
DL-Threonine	0.004	Glycine	0.002
DL-Phenylalanine	0.004	DL-Serine	0.004
		L-Glutamine	0.002
		DL-Methionine	0.004

3. Miscellaneous Reagents

Various reagents used in this work, and the manufacturers are listed below. Stock solutions of the first four reagents

were prepared in "Medium III" and stored at -10°C.

Carbamylcholine Chloride	- British Drug Houses
Acetylcholine Chloride	- Merck Co.
Eserine Sulphate	- T. & H. Smith Co.
Atropine Sulphate	- British Drug Houses
Sodium Ribonucleate	- Nutritional Biochemicals
2:4-dinitrophenol	- Merck Co.
Tributyrin	- Fisher Chemicals
Aminosol	- Abbott Laboratories

B - PREPARATION AND INCUBATION TECHNIQUES

1. Preparation and Incubation of Tissues

The general procedure employed was that described by Hokin (124, 125); some of the details are given below. The enzyme content of the pancreas was depleted by providing a continuous supply of food (ground oats), and injecting 150 ug. carbamylcholine intramuscularly about 60-75 minutes prior to decapitation. Low initial enzyme levels were usually obtained by this procedure so that distinct increases in enzyme activity could be demonstrated.

The pancreas was stored in "Medium III" at 0°C for a few minutes prior to slicing. Tissue slices of both the large and small lobes of the pancreas were employed, and divided evenly in each incubation vessel. At least 70 mg. wet weight of tissue

were used per vessel, and in most experiments, 100-150 mg. wet weight of tissue were employed. The amount of tissue incubated was kept approximately the same for each cup in any one experiment. Slices were stored dry in a chilled humidified crystallizing dish before being placed in the vessels. Surface slices were not used. The tissue was kept thoroughly chilled from the time of its removal until the vessels were placed in the bath, a period of about 45 minutes. Incubations were usually performed for 2.5 hours at 37.5°C. The total volume of incubating medium was always 3.0 ml.

In most of the experiments gas exchanges were recorded, incubations being carried out in conventional Warburg manometer flasks containing a roll of filter paper soaked in 20% NaOH, in the center well, to absorb CO_2 produced by the tissue slices during incubation. In the few experiments in which Q_{O_2} values were not recorded, incubations were performed in 25 ml. stoppered Erlenmeyer flasks at 37.5°C. The flasks were agitated during incubation in the same manner as the Warburg vessels by being attached to a movable rack.

In experiments on enzyme secretion the pancreas slices were incubated for 30 minutes at 37.5°C in 25 ml. stoppered conical flasks containing 3 ml. of oxygenated saline with 0.2% glucose. After this preliminary incubation the slices were

transferred to the experimental vessels for the main incubation.

2. Preparation of the Tissue and Medium for Enzyme Assays

The technique followed in this work has been described (124). An aqueous tissue extract was obtained by grinding the slices with sand and water. In many of the experiments in which enzyme secretion was not studied, enzyme assays were performed on pooled samples of the incubation medium and the tissue extract after suitable dilution for each enzyme. For a given enzyme the same dilution was used for each sample in any one experiment. In experiments on enzyme synthesis and secretion diluted samples of the medium and tissue were assayed separately.

Most of the assays could not be done on the day of incubation and the diluted samples were stored in the refrigerator (5°C). It was found that the lipase and ribonuclease activities of the samples decreased during cold storage but could be maintained by freezing. The enzyme solutions were therefore kept frozen until immediately before assay. Freezing did not affect amylase activity. This enzyme has been found to be stable for up to 5 days when stored in dilute solution in the refrigerator (124).

C - ASSAYS OF ENZYME ACTIVITY

1. Amylase Assay

Amylase activity was assayed by the modified (124) method of Smith and Roe (149). The starch-iodine colors were read in a Beckman Spectrophotometer at 620 mu. The amylase activities of the medium and tissue were expressed in units of Smith and Roe (149) per mg. initial dry weight.

2. Lipase Assay

Lipase activity was determined manometrically by the method of Rona and Lasnitzki (150). The procedure was as follows.

A 6% (v/v) aqueous emulsion of tributyrin was prepared with the aid of a glass homogenizer[1]. 0.5 ml. of this emulsion was pipetted into the Warburg flask. 2.0 ml. of 0.1 M $NaHCO_3$ which had been gassed with 7% CO_2 in 93% N_2 were then added. 0.5 ml. of the dilute enzyme extract (representing about 0.1-0.5 mg. wet weight of tissue) was pipetted into the sidearm of the vessel. The vessels were gassed for 15 minutes with 7% CO_2 in N_2 in a Warburg bath at 37.5°C. The enzyme extract was then tipped from the sidearm into the main compartment of the vessel. Manometer

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[1]In later stages of this work the tributyrin was added directly to the flasks to give a final concentration of 1%; the sensitivity of the assay was not affected by this procedure.

readings were taken every 10 minutes, the first reading being discarded.

Working with dilute tissue extracts the rate of CO_2 evolution was linear for periods up to 90 minutes, and the total gas output, for a given time, was proportional to the enzyme concentration. No CO_2 was evolved by any of the reaction components, alone, or in combination, in the absence of tissue extract. In several standardization experiments the assay was found to be accurate within about 20%.

Lipase Unit

For convenience of notation in this work, enzyme activity has been expressed in terms of arbitrary units, as defined below. A lipase unit is that amount of lipase which upon incubation at 37.5° in 3.0 ml. of 0.067 M bicarbonate buffer containing 1% tributyrin, in an atmosphere of 7% CO_2 in N_2 will produce 100 µl. of CO_2 per hour.

3. Ribonuclease Assay

A spectrophotometric assay was developed for the determination of ribonuclease which was found applicable to crude aqueous extracts of small quantities of tissue. In this method the optical density of the products of ribonucleic acid digestion which are soluble in acidified ethanol are determined by measuring the optical density of the solution in the ultraviolet at 260 mu. The procedure and reagents employed are described below.

Purification of Ribonucleic Acid

Commercial yeast ribonucleic acid contains considerable quantities of degraded fragments soluble in acetic acid in alcohol. These fragments absorb strongly in the ultraviolet thereby giving high blank readings. Purification of the ribonucleic acid by dialysis gave a product relatively free of ultraviolet-absorbing components soluble in acidic ethanol..

100 ml. of a 15% solution of sodium ribonucleate were dialyzed in cellophane sacs against running tap water for about 24 hours and then against several lots of distilled water for about six hours. The sac contents were then precipitated with 7.5 volumes of acetic acid in ethanol (1 part of glacial acetic acid and 13.8 parts of ethanol) at 0°C. After standing for 1 hour at 0°C the precipitated ribonucleic acid was collected by centrifugation, washed twice with ether and dried in vacuo. 6 g. of a fine brownish powder were obtained, which showed negligible alcohol-soluble ultraviolet-absorbing material under the conditions of the assay. A 0.5% solution of this ribonucleic acid in 0.02M phosphate buffer at pH 7.5 was employed for the ribonuclease assays.

Ribonucleic acid precipitating reagent

This reagent was prepared by mixing 935 ml. of 95% ethanol, 60 ml. glacial acetic acid and 5 ml. 2N NaOH. The pH of the mixture is about 4.2. This mixture is a modification of one used by Volkin and Carter (151) for extraction of nucleic acids from tissue. The precipitating reagent functioned in two ways: (a) ribonuclease activity was immediately stopped by the reagent

due to precipitation of the undigested ribonucleic acid and protein present in the incubation mixture; (b) the alcohol-soluble products liberated by the action of ribonuclease were estimated directly by measuring the optical density of the clear supernatant obtained after centrifugation of the precipitate.

Assay Procedure

0.5 ml. of a 1% ribonucleic acid solution in 0.04M phosphate buffer was pipetted into centrifuge tubes. 0.5 ml. of a dilute solution of ribonuclease was then added and the tube incubated at 37.5° for a given interval of time. At the end of the incubation period 7.0 ml. of precipitating reagent at 0°C were pipetted into the incubation mixture. In the control tube 0.5 ml. of the dilute enzyme solution was added to 7.0 ml. of the precipitating agent. It was established that upon incubation of either ribonucleic acid solution or enzyme solution alone, prior to addition of the precipitating reagent, the optical density of the supernatant obtained after centrifugation did not change.

After an hour of standing at 0°C, the tubes were centrifuged for 15 minutes and the supernatant solutions decanted. High-speed centrifugation for one minute at about 15,000 g produced clear, easily-decantable supernatants. However, if the low speed angle centrifuge was used, at least 15 minutes of centrifugation was necessary to obtain firm packing of the precipitate and a supernatant free of fine, flocculent particles.

Optical density readings were taken of the supernatants,

at 260 mu in a Beckman Model DU spectrophotometer against the precipitating reagent as a blank. The difference in optical density between the incubated and the unincubated samples was a measure of the ribonuclease activity of the unknown. When the enzyme activity was so great as to give optical density values greater than 0.5, the supernatants were diluted with precipitating reagent and the readings then obtained were multiplied by the dilution factor. The optical density was found to be directly proportional to the dilution.

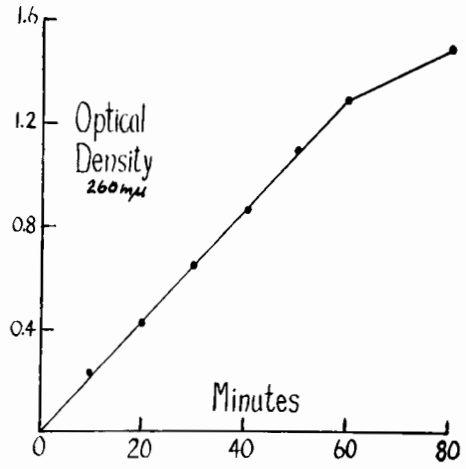
Standardization of Assay

Experiments were run with crystalline ribonuclease (Worthington Biochemicals Corp.) and crude aqueous pancreas extracts to standardize the assay. At low crystalline enzyme concentrations (0.05 ug./ml. and less) the optical density is directly proportional to the time of incubation for periods up to 60 minutes. The results of one such experiment are shown in Figure 2. For a fixed time, the optical density of the supernatant is directly proportional to the amount of crystalline enzyme used.

Similar results were usually obtained with aqueous extracts of pigeon pancreas, although in a few preparations direct proportionality was not obtained. However, working with amounts of tissue not exceeding 10 mg. wet weight/ml., the optical density of the supernatant is linear with respect to time, and, for a fixed time, linear with respect to tissue concentration. The relationship between tissue (enzyme) concentration and optical

FIGURE 2

RIBONUCLEASE ASSAY



Incubation of 0.05 ug./ml. crystalline ribonuclease with 0.5 ml. purified yeast ribonucleic acid at 37.5°C for various time intervals. Optical density readings obtained on a 5-fold dilution of samples of supernatants and readings multiplied by 5.

density is shown in Figure 3.

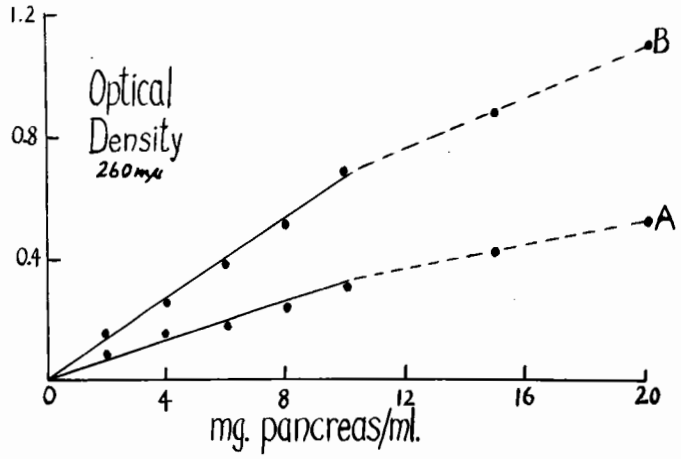
In several standardization experiments, designed to test the sensitivity of the method, the assay was found to be accurate within about 5%, using crystalline ribonuclease, and about 15% using crude tissue extracts.

Ribonuclease Unit

A ribonuclease unit is defined here as that amount of ribonuclease which upon 30 minutes of incubation at 37.5°C in 1.0 ml. of 0.5% purified yeast ribonucleic acid solution in 0.02M phosphate buffer at pH 7.5 will produce an increase of 0.01 optical density units at 260 mμ. in the supernatant obtained by the described assay procedure.

FIGURE 3

RIBONUCLEASE ASSAY



Incubation of varying amounts of aqueous extracts of pigeon pancreas with 0.5% purified yeast ribonucleic acid at 37.5°C.

Curve A = 30 minute incubation
Curve B = 60 minute incubation

CHAPTER VII

EXPERIMENTAL RESULTS

A - ENZYME SYNTHESIS

1. Reliability of Lipase and Ribonuclease Assays in Slices

It has been shown that amylase is evenly distributed in pigeon pancreas and completely extracted by the procedure employed (124). Similar results were obtained for lipase and ribonuclease (Table XV). Freshly prepared slices, of varying weight, from different parts of the pancreas, were extracted in the same volume of water and the extracts tested for their lipase and ribonuclease activities. The results shown in Table XV and subsequent tables have been calculated for initial dry weight using the ratio 0.23. This ratio was obtained by weighing unincubated slices before and after drying at about 105°C.

The agreement in values for lipase and ribonuclease activities of different slices from each lobe of the pancreas is within 20%, and 15%, respectively, although the difference between the large and small lobe average value for both enzymes is greater than 20%. The values for both enzymes fall well within the range of variation of the assay procedures, which means that within these limits, lipase and ribonuclease are completely extracted and evenly distributed in the pancreas.

TABLE XV

DISTRIBUTION OF LIPASE AND RIBONUCLEASE

IN PIGEON PANCREAS SLICES

(No carbamylcholine given)

	Enzyme activity expressed in units defined in text	
Dry wt. of slice (mg.)	Lipase Units/ mg. initial dry wt.	Ribonuclease Units/ mg. initial dry wt.
Large lobe		
10.8	68	147
12.3	64	135
20.0	62	138
22.8	76	130
34.2	74	148
Small lobe		
15.4	52	103
15.9	52	110
37.2	58	112

2. Effect of Incubation on Lipase and Ribonuclease Activity of Pancreas Slices

Increase in enzyme activity with time of incubation

Aerobic incubation of pancreas slices in saline for three hours approximately doubles the total (sum of medium and tissue) amylase, lipase and ribonuclease activities of the slices (Table XVI).

TABLE XVI

RATES OF AMYLASE, LIPASE AND RIBONUCLEASE SYNTHESIS IN PRESENCE AND ABSENCE OF ADDED AMINO ACIDS

Incubations in bicarbonate saline and amino acid Mixtures II and III where indicated. Gassed with 7% CO_2 in 93% O_2.

Incubation Time (hours)	Additions	Total Enzyme Activity of Medium and Tissue per mg. initial dry wt.					
		Amylase Units		Lipase Units		Ribonuclease Units	
			Δ		Δ		Δ
0	None	15		20		10	
1	None	22	7	24	4	13	3
2	None	27	12	32	12	15	5
3	None	32	17	37	17	20	10
1	Amino Acids	32	17	32	12	17	7
2	Amino Acids	43	28	41	21	22	12
3	Amino Acids	56	41	57	37	31	21

This indicates that all three enzymes are synthesized in pancreas slices. When a complete mixture of amino acids is added to the incubation medium the total activity of each of the three enzymes increases more than two-fold during 3 hours of incubation. Thus amino acids stimulate markedly the above increases in lipase and ribonuclease activities as well as amylase activity. The rates of increase in the activities of all three enzymes with respect to time of incubation are quite constant.

Inhibition of enzyme synthesis by 2:4 dinitrophenol (DNP)

If the increase in enzyme activity is true enzyme synthesis one would expect the reaction to be inhibited by substances which inhibit the production of usable energy by the cell. This possibility was tested, and the results of an experiment are shown in Table XVII.

TABLE XVII

EFFECT OF 2:4-DINITROPHENOL ON THE SYNTHESIS OF AMYLASE, LIPASE AND RIBONUCLEASE IN PRESENCE AND ABSENCE OF ADDED AMINO ACIDS

Incubations in "Medium III" containing 0.2% glucose, amino acid Mixtures II and III where indicated, 2×10^{-4}M 2:4-dinitrophenol where indicated. Gas phase 100% O_2.

Incubation Time (hours)	Additions	Total Enzyme Activity of Medium and Tissue per mg. initial dry wt.		
		Amylase Units	Lipase Units	Ribonuclease Units
0	None	15	13	22
0	None	13	10	21
2.5	DNP and amino acids	13	11	14
2.5	DNP	16	13	16
2.5	None	25	30	21
2.5	None	26	25	25
2.5	None	28	35	23
2.5	Amino acids	50	57	32
2.5	Amino acids	49	51	34
2.5	Amino acids	58	66	37

In the presence of 2 x 10^{-4}M 2:4 dinitrophenol (DNP) the amylase and lipase activities do not increase during incubation, either in the presence or in the absence of added amino acids. The ribonuclease activity, however, decreases by about 30% after incubation with DNP, both with and without added amino acids.

Control experiments have shown that DNP does not inactivate ribonuclease. Mg and Ca ions, which are present in the medium and are inhibitors of ribonuclease activity (152), do not have any measurable effect on ribonuclease activity at the concentrations in which they are present. It appears, therefore, that the decrease in ribonuclease activity after incubation is due to a partial destruction of this enzyme during incubation. In calculating the results for ribonuclease activity, the values obtained after incubation with DNP were therefore taken as a measure of initial ribonuclease activity.

Incubation of slices in saline media produced an average increase of about 80%, 150% and 50%, in amylase, lipase and ribonuclease activity, respectively, over the average activity of the unincubated and DNP controls (Table XVII). When a mixture of 22 amino acids was present during incubation the above increases were at least doubled.

The stimulating effect of amino acids on the increases in lipase and ribonuclease activity during incubation (Tables XVI and XVII) is further evidence that these two enzymes, as well as amylase, are synthesized by pigeon pancreas, <u>in vitro</u>.

The observations with DNP indicate that the increases in lipase and ribonuclease activities represent enzyme synthesis and

not an activation of some inactive form of the enzymes during incubation, since conversion of a proenzyme (inert precursor) to the active form is not an energy requiring process (130).

3. Effect of Different Mixtures of Amino Acids on Enzyme Synthesis

Comparison of lipase and ribonuclease synthesis in presence of a complete and incomplete mixture of amino acids

Table XVIII shows the results of an experiment designed to test broadly whether the amino acid requirements for lipase and ribonuclease synthesis differ from those of amylase.

TABLE XVIII

COMPARISON OF LIPASE AND RIBONUCLEASE SYNTHESIS IN PRESENCE OF DIFFERENT AMINO ACID MIXTURES

Incubations in bicarbonate-saline containing 0.2% glucose, amino acid Mixtures II, or II and III, where indicated. Gas phase 7% CO_2 in O_2

Incubation Time (hours)	Additions	Total Enzyme Activity of Medium and Tissue per mg. initial dry wt.		
		Amylase Units	Lipase Units	Ribonuclease Units
0	None	53	40	48
2.5	Mixtures II and III ⁂	49	45	34
2.5	Mixture II	89	87	58
2.5	Mixture II	85	92	54
2.5	Mixture II	87	83	52
2.5	Mixtures II and III	86	88	57
2.5	Mixtures II and III	90	83	53
2.5	Mixtures II and III	84	95	53

⁂ Gas phase 7% CO_2 in N_2. Yellow phosphorus in side arm of Warburg vessel.

The increase in activities of all three enzymes is the same when the incubation is performed in the presence of amino acid Mixture II ("essential" for amylase synthesis), or in the presence of all 22 amino acids, ie., amino acid Mixtures II and III.

The nutritionally essential amino acid, methionine, found unnecessary for maximal amylase synthesis (125), is absent from amino acid Mixture II, and apparently has no measurable effect on the synthesis of lipase and ribonuclease. Although methionine is not present in crystalline amylase (125), it is present in crystalline ribonuclease to the extent of about 4.5% (153). The amino acid constituents of pancreatic lipase are unknown, since crystallization of lipases has not been accomplished. It is probable that the methionine content of the slices was sufficiently high to prevent this amino acid from becoming the limiting factor in the synthesis of lipase or ribonuclease, by whatever mechanism the synthesis of these enzymes may proceed. Preincubated slices, in which the endogenous amino acid content has been reduced, are best suited for studying the effects of individual amino acids on enzyme synthesis; some experiments with such "depleted" slices will be reported in a later section.

The ribonuclease activity of slices incubated anaerobically is about 30% lower than that of unincubated slices; the ribonuclease activity of the anaerobically-incubated sample was therefore used

as the control value. In this experiment the increase in enzyme activity after incubation was greatest for lipase (about 95%), intermediate for amylase (about 75%), and again least for ribonuclease (about 60%).

The initial levels of all three enzymes in the experiment of Table XVI were about two or three times those shown in Table XVII, and hence the percentage increase in enzyme activity after incubation is not as great. However, the absolute increases in the activities of all three enzymes are about the same as those shown in the results of Table XVII. The data in Tables XVII and XVIII and results shown later indicate that ribonuclease is synthesized only about 1/3 to 1/2 as rapidly as amylase and lipase.

Effect of amino acids and partially hydrolyzed fibrin on enzyme synthesis

A comparison was made of the effects of a complete amino acid mixture and Aminosol (a partial hydrolysate of fibrin containing 1/3 small peptides) on enzyme synthesis. The results are presented in Table XIX. The synthesis of each of the three enzymes is the same in the presence of either of the mixtures. The results indicate that the peptides present in the partially-hydrolyzed fibrin are not superior to free amino acids as substrates for enzyme synthesis. These results become of particular interest when compared later with the effect of the partial hydrolysate of fibrin on labelled amino acid incorporation by pancreas slices.

TABLE XIX

COMPARISON OF ENZYME SYNTHESIS IN PRESENCE OF AMINO ACIDS AND PARTIALLY HYDROLYZED FIBRIN (AMINOSOL)

Incubation in "Medium III" containing 0.2% glucose, 2×10^{-4}M dinitrophenol where indicated. Mixtures I and II = 22 amino acids; Mixture I = Aminosol. Gas phase 100% O_2.

Incubation Time (hours)	Additions	Total Enzyme Activity of Medium and Tissue/mg. initial dry wt.		
		Amylase Units	Lipase Units	Ribonuclease Units
0	None	32	20	39
2.5	Mixture I and DNP	32	24	27
2.5	DNP	27	20	24
2.5	Mixture I	62	56	38
2.5	Mixture I	63	56	41
2.5	Mixture I	63	48	41
2.5	Mixture II and III	56	62	36
2.5	Mixture II and III	62	56	38
2.5	Mixture II and III	63	48	42

4. Differential Stimulation of Enzyme Synthesis by Amino Acids

Tables XX and XXI provide data indicating that amino acids, in some experiments, do not stimulate the synthesis of all three pancreatic enzymes under investigation in the same manner.

TABLE XX

SYNTHESIS OF AMYLASE, LIPASE AND RIBONUCLEASE IN PRESENCE AND ABSENCE OF ADDED AMINO ACIDS

Incubation in "Medium III" containing 0.2% glucose, Amino acid Mixtures I and II where indicated. Gas phase 100% O_2.

Incubation Time (hours)	Additions	Total Enzyme Activity of Medium and Tissue per mg. initial wet wt.		
		Amylase Unit	Lipase Unit	Ribonuclease Unit
0	None	22	6	9
2.5	None	39	10	12
2.5	Amino acids	65	18	13
2.5	Amino acids	67	20	13
2.5	Amino acids	65	18	12

The results of Table XX show that although added amino acids stimulated amylase and lipase synthesis in the usual manner, by about 150% and 200% respectively, the amino acids had no significant effect upon ribonuclease synthesis. The ribonuclease control was an unincubated slice in this experiment, and the increase in ribonuclease activity is undoubtedly higher than indicated due to the previously-discussed decrease observed in ribonuclease activity after incubation in the absence of an energy source. The absolute increase in both lipase and ribonuclease activity was particularly small in this experiment, being only about 1/4 of the values usually obtained. The results for amylase are typical of those usually obtained.

TABLE XXI

SYNTHESIS OF AMYLASE, LIPASE AND RIBONUCLEASE IN PRESENCE AND ABSENCE OF ADDED AMINO ACIDS

Incubation in bicarbonate saline containing 2×10^{-4} M dinitrophenol where indicated, amino acid Mixture I (Aminosol) where indicated, 0.2% glucose. Gas phase 7% CO_2 in O_2.

	Incubation Time (hours)	Additions	Total Enzyme Activity of Medium and Tissue per mg. initial dry wt.		
			Amylase Units	Lipase Units	Ribonuclease Units
Expt.1	0	DNP	36	15	11
	2.5	None	56	31	32
	2.5	Amino acids	80	34	33
Expt.2	0	None	29	8	8
	2.5	None	53	19	22
	2.5	Amino acids	67	19	23

The results of Table XXI indicate that lipase synthesis also is not always stimulated by amino acids. Amino acids stimulated amylase synthesis by about 120% and 60% in two separate experiments, but had no effect on either lipase or ribonucelase synthesis. Aminosol was used to supply amino acids in the experiments illustrated in Table XXI, but the difference in the pattern

of enzyme synthesis cannot be attributed to this source, since it was shown above (Table XIX) that Aminosol does not differ from the other amino acids in stimulating enzyme synthesis. The use of bicarbonate saline in these experiments cannot account for the differential stimulation since synthesis of all three enzymes was stimulated by amino acids upon incubation in bicarbonate saline in other experiments (Table XVI).

The observations illustrated in Tables XX and XXI indicate that each of the digestive enzymes can, to some extent, be synthesized independently of the others, and that their total amino acid requirements differ. The differential stimulation to synthesis by amino acids is probably due to sufficient stores of amino acids in the tissue for maximal synthesis of some, but not all of the enzymes. Experiments with slices "depleted" of their amino acid stores by preincubation, will provide evidence for this interpretation.

Summary

1. The synthesis of lipase and ribonuclease by pigeon pancreas slices has been studied.

2. The lipase and ribonuclease content of pancreas tissue may be depleted considerably by abundant feeding of the pigeons and injection of carbamylcholine before killing.

3. Increases in the total lipase and ribonuclease activities (sum of medium and tissue) occur when depleted pancreas slices are incubated aerobically in physiolyical saline.

4. The increases in total lipase and ribonuclease activities are not obtained anaerobically or in the presence of 2:4-dinitrophenol.

5. The increases in total lipase and ribonuclease activities are the same when a mixture of 10 amino acids ("essential" for amylase synthesis) or a complete mixture of 22 amino acids is present during incubation.

6. Small peptides, obtained from a partial hydrolysate of fibrin, are no more effective than amino acids in stimulating amylase, lipase and ribonuclease synthesis.

7. The increases in the total lipase and ribonuclease activities are usually, but not always, greater in saline containing amino acids, whereas amylase activity is always greater in the presence of amino acids.

8. The above observations indicate that pigeon pancreas slices synthesize lipase and ribonuclease <u>in vitro</u>, if respiration or the energy derived therefrom is intact.

B - ENZYME SYNTHESIS AND SECRETION

Since amylase, lipase, and ribonuclease are synthesized by pigeon pancreas slices, it was decided to observe whether drugs stimulating amylase secretion (124) would also stimulate lipase and ribonuclease secretion. The results of several experiments designed to answer this question are shown in Tables XXII and XXIII. Several interesting features about the system arise out of these experiments, and they will be discussed under separate headings.

1. Stimulation of Lipase and Ribonuclease Secretion by Cholinergic Drugs

In the experiments on enzyme secretion, the cholinergic drug was present in all the vessels; the addition of atropine to the control vessels prevented the secretory action of the drugs (154). Aerobically, in the presence of carbamylcholine alone, the amylase, lipase and ribonuclease activities of the medium are at least twice that observed in the presence of carbamylcholine and atropine (Table XXII) and this indicates that carbamylcholine stimulated the secretion of all three enzymes in a parallel manner. Anaerobically, the stimulation of enzyme secretion by carbamylcholine does not occur. This indicates that enzyme secretion is an energy-requiring process.

The results of Table XXIII are in general similar to those of Table XXII. Acetylcholine with eserine produces an increase of about 50-100% in the activities of all three enzymes in the medium,

TABLE XXII

SYNTHESIS AND SECRETION OF AMYLASE, LIPASE AND RIBONUCLEASE

Medium contained Krebs Medium III; 0.2% glucose; 10 ug./ml carbamylcholine; amino acid Mixture I where indicated; 100 ug./ml. atropine where indicated. Yellow phosphorus placed in sidearm of Warburg vessel when N_2 used as gas phase. 30 minutes preliminary incubation in oxygenated "Medium III" containing 0.2% glucose.

Expt. 1 - Pigeon received 150 ug. carbamylcholine *in vivo*. Expt. 2 - No carbamylcholine administered *in vivo*

			Enzyme Activity per mg. initial dry weight											
			Amylase Units				Lipase Units				Ribonuclease Units			
Expt. No.	Gase Phase	Additions	Medium	Tissue	Total * (added)	Total ** (assayed)	Medium	Tissue	Total * (added)	Total ** (assayed)	Medium	Tissue	Total * (added)	Total ** (assayed)
1	100% N_2	Mixture I	11			34	8			34	2	10	12	12
	100% O_2	Atropine	14			44	8			38	2	9	11	14
	100% O_2	Mixture I atropine	13	57	70	74	10	48	58	58	2	13	17	20
	100 % O_2	Mixture I	35	35	70	73	24	40	64	61	5	10	17	20
2	100% N_2	Mixture I	9	33	42	42	6			28	2	13	15	14
	100% O_2	Atropine	11	42	53	51	6			28	2	14	16	16
	100% O_2	Mixture I atropine	11	69	80	83	6	46	52	62	2	15	17	22
	100% O_2	Mixture I	33	44	77	87	16	36	52	64	5	14	19	23

* The values for total enzyme activity in these columns are the sum of the activities obtained for medium and tissue.
** The values for total enzyme activity shown in these columns were obtained by assaying pooled samples of medium and tissue.

TABLE XXIII

SYNTHESIS AND SECRETION OF AMYLASE, LIPASE AND RIBONUCLEASE

Medium contained "Medium III"; 0.2% glucose; 10 ug./ml. acetylcholine; 100 ug./ml. esserine; amino acids where indicated; 2 x 10^{-4}M 2:4-dinitrophenol where indicated; 100 ug./ml. atropine where indicated. Gassed with 100% O_2. 30 minutes preliminary incubation at 37.5° in oxygenated Medium III containing 0.2% glucose. 2 hours main incubation.

Expt. 1 - Pigeon received 150 ug. carbamylcholine *in vivo*. Expt. 2 - No carbamylcholine administered *in vivo*.

Expt. No.	Sample	Additions	Enzyme activity per mg. initial dry weight								
			Amylase Units			Lipase Units			Ribonuclease Units		
			Medium	Tissue	Total *	Medium	Tissue	Total *	Medium	Tissue	Total *
1	1	Mixture I dinitrophenol	12	28	40	10	12	22	5	14	19
	2	Atropine	16	33	49	14	14	28	6	16	22
	3	Atropine Mixture I	15	48	63	12	18	30	8	19	27
	4	Mixture I	26	35	61	22	14	36	12	17	29
2	1	Mixture I dinitrophenol	17	44	61	10	16	26	4	15	19
	2	Atropine	20	44	64	12	18	30	5	14	19
	3	Atropine Mixture I	26	55	81	16	30	46	6	26	32
	4	Mixture I	42	42	84	24	24	48	10	23	33
	5	Atropine hydrolyzed Mixture I	26	51	77	18	24	42	5	24	29
	6	Hydrolyzed Mixture I	57	24	81	32	22	54	11	19	30

* The values for total enzyme activity are the sum of the medium and tissue activities.

over the corresponding control values obtained with atropine and acetylcholine. The activities of the enzymes in the media containing 2:4-dinitrophenol and acetylcholine are about the same or somewhat lower, than the atropine control levels; this indicates that dinitrophenol inhibits secretion.

It will be noted that in vitro enzyme secretion occurs to the same extent irrespective of whether or not carbamylcholine is administered in vivo. The initial enzyme levels of the undepleted pancreases used in these experiments were about the same as the enzyme levels of the depleted.

2. Effect of Preincubation on Enzyme Synthesis and Secretion

In the experiments on enzyme secretion a preliminary half-hour incubation was performed in order to remove from the slices considerable quantities of all three enzymes which rapidly enter the medium, and probably arise in the main, from damaged cells (154). The results obtained in enzyme secretion studies are much more consistent when this procedure is followed.

The preincubation procedure brings out an important effect with regard to enzyme synthesis (Tables XXII and XXIII). The synthesis of all three enzymes by preincubated slices in the absence of added amino acids is in most cases either very small, or completely abolished. With added amino acids, however, the synthesis of all three enzymes is considerable in most experiments. Thus added amino acids may stimulate enzyme synthesis five or tenfold over saline controls, when preliminary incubation of the slices is performed.

The maximum stimulation of enzyme synthesis by amino acids in non-preincubated slices is only two- or three-fold. These results provide evidence that enzyme synthesis obtained in the absence of added amino acids is dependent on the endogenous free amino acids of the slices, and that preliminary incubation of the slices reduces the quantity of these free amino acids to such an extent that the enzyme synthesizing mechanism is markedly disturbed.

Comparison of amylase synthesis of preincubated and non-preincubated slices of the same pancreas

The possibility was considered that factors necessary for enzyme synthesis, other than amino acids, may be lost by the slices during preincubation. Table XXIV shows the results of an experiment testing this possibility. In the presence of amino acids amylase synthesis in preincubated slices is only about 15% less than in the non-preincubated slices. In the absence of added amino acids, preincubation reduces amylase synthesis by 60%. Amino acids stimulate amylase synthesis about two-fold in non-preincubated slices, whereas a five-fold stimulation is obtained with preincubated slices. These results correspond with those of Tables XXII and XXIII and indicate that the main effect of preincubation is to deplete the tissue of amino acids.

3. Effect of Stimulation of Enzyme Secretion on Enzyme Synthesis

Under the conditions of these experiments, the rates of synthesis of amylase, lipase and ribonuclease are not significantly

TABLE XXIV

AMYLASE SYNTHESIS IN PREINCUBATED AND NON-PREINCUBATED PANCREAS SLICES

Incubation in "Medium III" containing 0.2% glucose, amino acid mixtures as indicated. Gassed with 100% O_2. Preliminary incubation for 30 minuts at 37.5°C in oxygenated "Medium III" containing 0.2% glucose for Group I.

Treatment of Slices	Main Incubation Time (hours)	Additions	Total Amylase Activity (Smith & Roe Units) per mg. initial dry wt.	Amylase Synthesized (Smith & Roe Units) per mg. initial dry wt.
	0	None	18	
I Preincubated	2.0	None	21	4
	2.0	Mixture I	40	22
	2.0	Mixtures II plus III	38	20
	0	None	20	
II Non-Preincubated	2.0	None	30	10
	2.0	Mixture I	43	23
	2.0	Mixtures II plus III	45	25

affected by carbamylcholine or acetylcholine with eserine. These results are in accord with results obtained with amylase on depleted glands (124). Glands depleted of their enzyme content by *in vivo* carbamylcholine administration might already be expected to be synthesizing at a maximal rate. However the synthesis of all three enzymes was not stimulated by cholinergic drugs in the experiments in which carbamylcholine was not given *in vivo*. It thus appears that stimulation of secretion does not stimulate enzyme synthesis, *in vitro*. To avoid undue repetition, comments on the results on enzyme synthesis and secretion are reserved for the general discussion (Chapter X).

Summary

1. The discharge of lipase and ribonuclease by pancreas slices into the medium is greater in the presence of carbamylcholine or acetylcholine (with eserine) than in the presence of either of these drugs plus atropine.

2. This discharge of enzyme is dependent on oxygen and is inhibited by 2:4-dinitrophenol.

3. The above observations indicate that pigeon pancreas slices actively secrete amylase, lipase and ribonuclease, in a parallel fashion, *in vitro*.

4. Preincubation of pancreas slices in glucose-saline reduces or abolishes the synthesis of amylase, lipase and ribonuclease obtained on incubation in saline without amino acids, and markedly enhances the synthesis of all three enzymes in saline containing

amino acids.

5. Amylase synthesis is only slightly reduced in slices which have undergone preliminary incubation.

6. The synthesis of amylase, lipase and ribonuclease is not appreciably affected by stimulation of secretion in pancreas slices of pigeons which have, and have not, received carbamylcholine *in vivo*.

SECTION B (CHAPTERS VIII AND IX)

GLYCINE-1-C^{14} INCORPORATION AND ENZYME SYNTHESIS BY PANCREAS TISSUE IN VITRO

CHAPTER VIII

MATERIALS AND EXPERIMENTAL METHODS

A - INCUBATION MEDIA

1. Inorganic Media and Amino Acid Mixtures

The inorganic media used in the work reported in this section were the same as those described in Chapter VI-A. In experiments on the effects of amino acids and peptides the following mixtures were used: (1) Aminosol, a 5% solution of a partial hydrolysate of fibrin containing peptides and amino acids, in a final concentration during incubation of 0.34%; (2) hydrolyzed Aminosol (see below), in a final concentration during incubation of about 0.3%; (3) Amino acid Mixture IV - composed of 20 amino acids listed in Table XXV. Glycine was omitted from this mixture so as not to dilute the radioactive glycine used in this work. Amino acid Mixture IV was prepared by dissolving commercial amino acids in "Medium III" and neutralizing with NaOH. Stock solutions of these mixtures were stored in the frozen state at about -10°C.

2. Preparation of Hydrolyzed Aminosol

Aminosol was hydrolyzed and purified in the following manner. 25 ml. of concentrated HCl (about 12N) were added to 25 ml. of Aminosol and the mixture autoclaved at 15 lbs. pressure for one hour. The hydrolysate was decolourized by shaking with activated charcoal and separated from the charcoal by filtration. The clear hydrolysate was then concentrated down to a volume of about 2 ml.

TABLE XXV

STANDARD AMINO ACID MIXTURES

(Concentrations are those in incubation vessels in routine expts.)

MIXTURE V

Amino Acid	Conc'n (M)	Amino Acid	Conc'n (M)
DL-Aspartate	0.002	L-Proline	0.001
L-Arginine	0.001	L-Hydroxyproline	0.001
L-Cysteine	0.001	DL-Norleucine	0.002
L-Glutamate	0.001	DL-Methionine	0.002
L-Histidine	0.001	L-Tyrosine	0.001
DL-Isoleucine	0.002	L-Tryptophan	0.001
L-Citrulline	0.001	DL-Threonine	0.002
L-Ornithine	0.001	DL-Phenylalanine	0.002
L-Leucine	0.001	DL-Serine	0.002
L-Lysine	0.001	DL-Valine	0.002

by vacuum distillation. The syrupy concentrate was diluted with about 3 ml. water and vacuum distilled to a volume of about 2 ml. This procedure was repeated three times to remove most of the HCl. The hydrolysate was then neutralized with NaOH and made up to a concentration of about 5% with water. In making up the final solution an allowance of 5% was made for losses of amino acids by adsorption on the charcoal. The solution contained about 0.4% NaCl arising from neutralization of the residual HCl.

3. Radioactive Materials

The specific activity of the glycine-1-C^{14} [1.] used was about 80,000 counts per minute per mg. (c/m/mg.). A portion of the radioactive glycine was diluted 1:4 with non-radioactive glycine and a stock solution of 10 mg/ml. glycine was prepared with distilled water. A 10 mg/ml. solution of undiluted radioactive glycine was similarly prepared. These solutions were stored at -10°C. 1 mg. glycine (exactly 0.1 ml. solution) was used in a total volume of 3.0 ml. incubation medium, so that the glycine concentration was 0.0044M and the radioactivity either 20,000 or 80,000 counts per minute of C^{14} per vessel.

C^{14}-carboxyl-labelled alanine (specific activity about 15,000 c/m/mg.) was used in some experiments. 0.10 ml. of a 20 mg/ml. solution was employed in a total incubation volume of 3.0 ml.

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1. I am indebted to Dr. D. Douglas of the Atomic Chemistry Division of the Montreal General Hospital Research Institute for preparing the radioactive glycine and alanine used in this work.

P^{32} was obtained from Atomic Energy of Canada Ltd., Chalk River, Ontario. Approximately 10 μC P^{32} as phosphate was used per vessel.

B - TISSUE TECHNIQUES

The procedures employed in preparing and incubating pigeon pancreas were described in Chapter VI-B. New procedures adopted in this work are described below. The volume of incubation fluid was always 3.0 ml. Incubations were performed at 37.5°C for 2 hours unless otherwise specified.

1. Preparation of Mouse Pancreas

In the experiments with mice, pancreases of 4 to 6 animals were used per incubation vessel. 400-600 mg. of tissue were usually obtained. One half of the tissue was set aside as unincubated controls in some experiments, in order to follow enzyme synthesis.

The mice employed were an albino variety which have been raised at the Montreal General Hospital Research Institute for the past five years and are highly inbred. (In one of the experiments a group of albino male mice (23-25 gm.) obtained from the Royal Victoria Hospital were used.) Male animals weighing 25-35 gm. were employed. In any one experiment mice were selected whose weights did not vary by more than three gm. These animals were then kept in the same cage for the 24-hour fasting period. Water was provided during the fast.

Pilocarpine was used to stimulate pancreatic secretion *in vivo* in some experiments. 0.1 mg. of 0.1% pilocarpine-hydrochloride was injected intraperitoneally 90 minutes before sacrifice. The response to this cholinergic drug occurred a few minutes after injection, in the form of profuse sweating, defecation, lachrymation, etc. The dose of pilocarpine used was never lethal. The data of Daly and Mirsky (155) on the effects of pilocarpine on enzyme levels in mouse pancreas provided the basis for drug dosages.

The mice were killed by decapitation, and the abdominal wall penetrated laterally on the left side with a sharp scissors. In the mouse the spleen and pancreas are associated anatomically; both organs were removed with minimum damage to the pancreas. The spleen was then dissected away, and the pancreas stored in a chilled, closed crystallising dish, humidified by moist filter paper. In experiments requiring more than a dozen mice, assistance was usually obtained. One operator killed and opened the mice, and the other removed and stored the pancreases.[1] Two dozen pancreases were obtained in about 20-25 minutes in this manner. The different pancreases were arranged so that each vessel would have samples of tissue removed both at the beginning and the end of the preparation.

Pancreases were sliced in half by the Stadie-Riggs method and pooled samples were weighed on the torsion balance. Whole mouse

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[1] My thanks are due to Mr. Andrew Taussig of this Institute for his help in this procedure.

pancreas was also incubated in some experiments. Incubation procedure was the same as described in Chapter VI-B.

2. Treatment of Tissue after Incubation

The method described earlier of grinding the tissue with sand after incubation could not be employed in the isotope experiments. A suspension of radioactive protein, free of any other solid particles was required in these experiments and procedures were adopted to obtain such a product.

In preliminary isotope experiments the tissue was ground with water in an all-glass homogenizer. The results obtained were very inconsistent. It was found that a considerable quantity of fine glass particles was produced when an all-glass homogenizer was used. The assay of radioactivity was dependent on weighing a few mg. of pure protein accurately and this was not possible because of the presence of variable amounts of glass in the samples. A commercial homogenizer consisting of a heavy-duty glass tube fitted with a Teflon pestle which produced no particles during grinding was employed in this work.

After incubation, the tissue was homogenized for about 2-3 minutes in 5 ml. of ice-cold water. Homogenization was performed at several thousand r.p.m. and interrupted at frequent intervals to chill the homogenate in ice and thus keep proteolytic activity to a minimum. Aliquots of the homogenate were collected at this stage for enzyme assays and the balance treated as described below.

3. Miscellaneous Determinations

In determinations of the specific activity of acid-soluble organic phosphate, the procedure followed was identical with that described by Hokin and Hokin (154).

Amylase, lipase and ribonuclease were assayed by the methods described in Chapter VI-C.

C - TECHNIQUES WITH RADIOACTIVE ISOTOPES

1. Preparation of Protein for Plating

The radioactive protein was separated from other cellular constituents by a procedure, essentially similar to that described by Winnick (121), which is given below.

One volume of cold 20% trichloroacetic acid was added to the homogenate. The precipitate was centrifuged, and washed once with 6 ml. of cold 5% trichloroacetic acid. This was followed by extraction with 5% trichloroacetic acid at 90° for 15 minutes. The residue was then washed with 5% trichloroacetic acid at room temperature. The protein precipitate was extracted once with 6 ml. of 95% ethanol, twice with 3:1 ethanol-ether in a water bath at 60-65° for 5 minutes, and once with ether. Centrifugation was performed after each extraction, to pack the fine protein precipitate.

2. Plating and Counting Technique

The plating and counting technique was developed for small quantities of protein from pancreas. The purified protein suspen-

sion was centrifuged and the ether decanted. The protein was then resuspended in about 1 ml. of a mixture of 4:1 chloroform-ether. Preliminary tests for a suitable suspending agent were made with various organic solvents including ethanol, acetone, petroleum ether, chloroform and various mixtures of these. The chloroform-ether mixture proved most satisfactory and was routinely employed.

Aluminum discs with edges raised 1-2 mm. and an area of 3.8 sq. cms. were used for plating the radioactive materials. The discs were placed on a large glass plate adjusted to a perfectly horizontal position, to ensure even settling of the protein. The protein suspension was introduced on the disc by means of a Pasteur pipette, and the ether-chloroform mixture allowed to evaporate at room temperature. Homogeneous films of protein were usually obtained by this procedure, although slight cracking occurred in some samples. After drying, the plates were weighed and counted. Counts obtained on cracked and intact protein layers did not vary significantly.

Counting was performed with a Geiger-Muller end-window counter attached to a scale-of-sixty-four scaler and an automatic timer. The counting equipment used was kindly loaned by the Dept. of Chemistry, McGill University. The background count of the instrument was about 15-20 counts per minute over a period of one year, and was very consistent over any one-day period. All samples from any one experiment were always counted on the same day. Since radioactive decay is a random process, its measurement is limited by statistical fluctuations inherent in counting data. In the

observation of events occurring at random, it can be shown that $\sigma = \sqrt{N}$ where σ = standard deviation and N = number of events observed. All samples with appreciable radioactivity were counted sufficiently long so that σ was no greater than 2.5% of N.

The actual count obtained was corrected for background and then for self absorption by dividing by the appropriate value taken from the curve in Figure 4 (see below for preparation of curve). Division of the corrected count by the mg. of protein on the plate gave the specific activity of the protein, ie., counts per minute per mg. protein (c/m/mg.)

3. Preparation of Self-Absorption Correction Curve for C^{14} - Protein on Aluminum

Radioactive protein for the absorption curve was obtained in the following manner. Bicarbonate saline (147) was prepared containing 0.02M sodium pyruvate, 0.2% glucose and 0.75 mg. glycine (15,000 c/m) per ml. solution. Two 125-ml conical flasks, each containing 1 g. rat liver slices and 10 ml. of the above were gassed with 7% CO_2 in 93% O_2 and incubated for 4 hours at 37.5°C. The protein was extracted, plated, and counted by the procedure described above. Table XXVI shows the counts obtained from plates containing various quantities of protein.

The data of Table XXVI were plotted (mg. protein vs. counts/minute) and the linear portion of the resulting curve, which represents the true activity of the samples, extrapolated (according to 156). A self absorption curve for protein was then constructed expressing the ratio of apparent activity to true activity (in percent)

as a function of mg. protein. This curve, shown in Figure 4, was used for correcting the counts for C^{14} self absorption.

TABLE XXVI

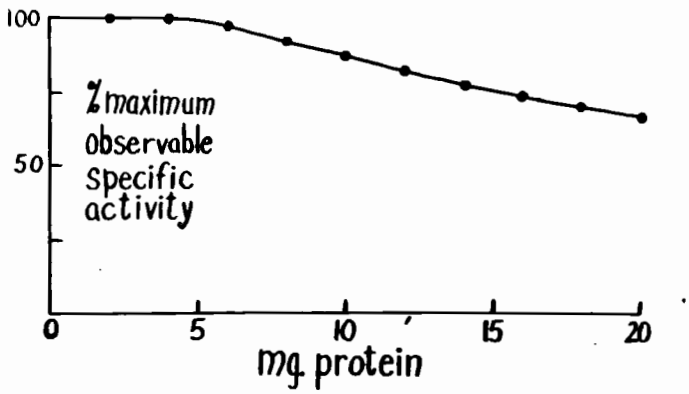
MEASURED RADIOACTIVITY FROM C^{14}-PROTEIN WITH VARYING QUANTITIES OF PROTEIN (VARYING THICKNESS OF PROTEIN SAMPLES)

Protein Plated (mg.)	Counts/Minute
2.10	26.0
2.15	31.3
2.55	36.2
2.75	39.5
3.00	42.3
3.70	48.4
4.20	55.0
4.40	59.6
4.60	60.1
4.75	64.4
5.10	67.3
5.45	74.1
5.70	76.6
7.10	91.2
8.15	101.0
10.60	123.2
11.55	133.1
14.40	152.0
17.75	173.3
19.60	182.4

FIGURE 4

SELF ABSORPTION CORRECTION CURVE

FOR PROTEIN ON ALUMINUM



Points represent calculated values (See text p.97)

CHAPTER IX

EXPERIMENTAL RESULTS

A - EXPERIMENTS WITH PIGEON PANCREAS SLICES

1. Effect of Amino Acids on Glycine-C^{14} Incorporation and Enzyme Synthesis

Table XXVII shows the results of an experiment on the rate of radioglycine incorporation into pigeon pancreas slices, in the presence and absence of added amino acids. The rate of glycine incorporation was linear over a two-hour period, both in the presence and absence of added amino acids. A complete mixture of amino acids increased the rate of glycine incorporation by about 15% over that observed in the absence of the amino acid mixture. The radioactivity incorporated per mg. protein after two hours of incubation was about 0.04% of the total radioactivity present during incubation. 13.2 μM of labelled glycine was present in the vessel; the rate of incorporation was thus about 3 μM glycine/g. protein/hr.

The rate of glycine incorporation by pancreas may be compared with related rate figures reported in the literature. Rat diaphragm (157) and rabbit bone marrow cells (158) incorporate glycine (initial concentration 0.004M) at rates of approximately 0.7 and 5 uM glycine/g. protein/hr., respectively. Embryonic and malignant tissues take up labelled amino acids much faster than normal adult tissues (104, 105), and bone marrow cells belong with embryonic

TABLE XXVII

EFFECT OF AMINO ACIDS ON GLYCINE-C^{14} INCORPORATED AND AMYLASE SYNTHESIS BY PIGEON PANCREAS SLICES

Incubations in bicarbonate saline containing: 0.2% glucose, Amino acid Mixture IV where indicated; 5.5×10^{-5}M carbamylcholine, 1 mg. glycine-C^{14} (20,000 c/m/mg.). Gassed with 7% CO_2 in 93% O_2. Pigeon received 150 ug. carbamylcholine intramuscularly 60 minutes before sacrifice.

Incubation Time (Minutes)	Additions	Specific Activity of Protein c/m/mg. Protein	Stimulation by Amino Acids	Total Amylase Activity (Medium plus Tissue) Units per mg. initial dry. wt.	Amylase Units Synthesized per mg. initial dry wt.	Stimulation by Amino Acids
0	-			23		
40	-	2.8				
40	Amino Acids	3.1	11%			
80	-	4.9				
80	Amino Acids	5.9	20%			
120	-	8.2		34	11	
120	Amino Acids	9.0	10%	47	24	120%

tissue. It thus appears that pigeon pancreas incorporates labelled glycine about as rapidly as growing tissues. As will be seen later, incorporation rates varied considerably with the pancreases of different pigeons, so that the value calculated above, though representative, is only to be considered as an order of magnitude.

One might expect that the specific activity of the radioglycine added to the incubation mixture would be somewhat reduced as a result of dilution with unlabelled endogenous glycine contributed by the pancreas slices. The following calculation of the amount of glycine contributed in this manner shows it be to insignificant. The average free amino nitrogen of several pigeon pancreases was found to be 36 mg N/100 gm. tissue, and about 50% of this was considered to be due to glutamic acid plus glutamine (125). Taking the average nitrogen content of amino acids as 16%, as in protein, and assigning the value 120 as the molecular weight of the "average" amino acid, the molar concentration of amino acids (exclusive of glutamic acid plus glutamine) in pigeon pancreas is $\frac{6.25 \times 0.018 \times 10}{120}$, or of the order of 0.01M. Assuming glycine to make up as much as 10% of this free amino acid pool, the glycine concentration of pigeon pancreas is of the order of 0.001M. The volumes of tissue used in the experiments was about 3-5% of the total incubation medium; this medium was 0.0044M in glycine. Hence the dilution of radioglycine by tissue glycine would appear to be of the order of only 1%.

The data of Table XXVII indicate that amylase synthesis was rather small in this experiment; the rate of synthesis could not be measured accurately after short incubation periods. However, the synthesis obtained after two hours of incubation was considerable, both in the presence and absence of added amino acids, and the stimulation to amylase synthesis by added amino acids was about 120%. This result is typical of those discussed in detail in Chapter VII. The main point of interest here is that amino acids stimulated amylase synthesis about eight times more than they stimulated radioglycine incorporation.

The results of another experiment on the time course of radioglycine incorporation and enzyme synthesis are shown in Table XXVIII. Samples were incubated for longer intervals of time in this experiment and the synthesis of lipase, ribonuclease and amylase were followed at each interval. In this experiment, amino acids stimulated radioglycine incorporation by an average value of 30%. Amino acids more than doubled the synthesis of amylase, lipase and ribonuclease. It is thus clear from the results shown in Tables XXVII and XXVIII that amino acids stimulate enzyme synthesis to a far greater extent than they stimulate peptide bond synthesis, as measured by amino acid incorporation into total protein.

2. Effect of Anaerobic Conditions and Dinitrophenol on Glycine-C^{14} Incorporation

Experiments were performed to ascertain to what extent the uptake of labelled glycine into pancreas protein is an active process requiring a supply of energy. The data of Table XXIX show

TABLE XXVIII

EFFECT OF AMINO ACIDS ON GLYCINE-C^{14} INCORPORATION AND ENZYME SYNTHESIS BY PIGEON PANCREAS SLICES

Incubations in "Medium III" containing: 0.2% glucose, amino acid Mixture IV where indicated, 1 mg. glycine (20,000 c/m/mg.) Gassed with 100% O_2. Pigeon received 150 ug. carbamylcholine intramuscularly one hour before killing.

Incubation Time (hours)	Additions	Specific Activity of Protein, c/m/mg. Protein	Stimulation by amino acids	Enzyme Synthesized per mg. initial dry wt.					
				Amylase Units	Stimulation by amino acids	Lipase Units	Stimulation by amino acids	Ribonuclease Units	Stimulation by amino acids
1	-	3.4		7		3		2	
1	Amino acids	4.6	35%	17	143%	8	160%	5	150%
2	-	7.8		13		9		5	
2	Amino Acids	10.0	28%	28	116%	22	144%	11	120%
3	-	10.4		17		14		9	
3	Amino Acids	13.2	26%	41	141%	34	143%	21	133%

TABLE XXIX

EFFECT OF ANAEROBIC CONDITIONS AND 2:4-DINITROPHENOL ON INCORPORATION OF GLYCINE-C^{14} INTO PIGEON PANCREAS SLICES

Incubations in "Medium III" containing: 0.2% glucose, 0.0044M glycine-C^{14}, 2×10^{-4}M 2:4-dinitrophenol where indicated. Pancrease of 48-hour fasted pigeon was used in Expt.1 and of fed pigeon in Expt. 2. 2 hour incubation.

	Gase Phase	Additions	Glycine-C^{14} Added	Specific Activity of Protein (c/m/mg.). Protein	Reduction in Specific Activity
Expt. 1	100% O_2	None	20,000 counts/ min.	6.5	
	100% N_2 *	None	"	0.13	98%
Expt.2	100% O_2	None	80,000 counts/ min.	8.0	
	100% O_2	2:4-dinitrophenol	"	0.52	99%

* Yellow phosphorus placed in sidearm of Warburg vessel.

the effects of anaerobic incubation and aerobic incubation with 2:4-dinitrophenol on the incorporation of labelled glycine. Glycine incorporation was virtually completely inhibited under these conditions. The traces of radioactivity found in the protein may have been due to adsorption of free labelled glycine by the protein, or possibly, to an actual incorporation of the glycine into the protein mediated by the small amount of energy produced during incubation by glycolysis. The results show that incorporation of radioglycine by pigeon pancreas slices is dependent on respiration, and the energy derived from it; these observations are in conformity with similar results obtained with various tissues in vitro (Chapter V).

3. Effect of Cholinergic Drugs and Aminosol on Glycine-C^{14} Incorporation

Studies reported previously (124) and those described in Chapter VII-B demonstrate that stimulation of enzyme secretion by cholinergic drugs does not stimulate enzyme synthesis by pigeon pancreas in vitro. It was considered of interest to study the effects of cholinergic drugs on labelled glycine incorporation into total protein under various experimental conditions.

The pancreases of both fed and fasted pigeons were used. Feeding stimulates the gland to secrete. Glands of fasted pigeons are non-secreting and rich in enzyme stores. The functional state of the pancreas was reflected by its appearance; glands from fasted animals were usually small, pale and friable, while those of fed pigeons were reddish-pink and larger.

The results shown in Table XXX were obtained with the pancreases of pigeons fasted, for approximately 72 hours prior to killing. Glycine incorporation into total protein was inhibited by carbamylcholine in Expt.2, and unaffected in Expt.1, of Table XXX. The inhibition was completely reversed in the presence of a partial hydrolysate of fibrin containing peptides (Aminosol). The peptide-containing mixture stimulated glycine incorporation only moderately in non-secreting slices (slices incubated without carbamylcholine). However, the effect of Aminosol, in reversing the inhibition produced by carbamylcholine in Expt.2, might represent a direct stimulation of incorporation in the secretory slices. A series of experiments were undertaken to observe the effects of the peptide-containing fibrin hydrolysate (Aminosol) on the incorporation of labelled glycine into total protein of pancreas slices stimulated to secrete by cholinergic drugs.

Table XXXI summarizes the results of a number of experiments performed with the pancreases of pigeons fasted for 48 hours prior to killing. Acetylcholine, the natural cholinergic agent, was employed in conjunction with eserine (cholinesterase inhibitor). The effects of varying the concentrations of these agents were studied in the different experiments although the concentrations used were all such as to cause a maximal stimulation of secretion (Table XXXII). The use of atropine, which prevents the action of cholinergic agents, in the control vessels made it possible to determine whether the results obtained with the cholinergic agents were related to their physiological effects.

TABLE XXX

EFFECT OF CARBAMYLCHOLINE ON INCORPORATION OF GLYCINE-C^{14} INTO

PANCREAS SLICES OF FASTED PIGEONS IN THE PRESENCE AND ABSENCE OF AMINOSOL

Incubations in bicarbonate saline containing: 0.2% glucose, 1 mg. glycine-C^{14} (20,000 c/m/mg.), 5.5×10^{-5} M carbamylcholine where indicated, 0.34% Aminosol where indicated. Gassed with 7% O_2 in 93% N_2. 2 hour incubation. Pigeons fasted for 72 hours prior to sacrifice.

Expt. No.	Additions	Specific Activity of Protein, c/m/mg. Protein	Decrease in Specific Activity of Protein with carbamylcholine	Specific Activity of Protein, c/m/mg. Protein	Increase in Specific Activity with Aminosol
		WITHOUT AMINOSOL		WITH AMINOSOL	
1	None	10.2		10.9	7%
	Carbamylcholine	9.8	4%	11.9	21%
2	None	8.0		10.0	20%
	Carbamylcholine	5.8	28%	10.8	69%

TABLE XXXI

EFFECT OF ACETYLCHOLINE WITH ESERINE ON INCORPORATION OF GLYCINE-C^{14} INTO PANCREAS SLICES OF FASTED PIGEONS IN PRESENCE OF AMINOSOL AND VARIOUS AMINO ACID MIXTURES

Incubations in "Medium III" containing: 0.2% glucose, amino acid mixtures as indicated, cholinergic agents as indicated, 1 mg. glycine (20,000 c/m/mg.). Gassed with 100% O_2. 2 hour incubation. Pigeons fasted for approximately 48 hours prior to killing. Es = Eserine; AcCh = Acetycholine; At = atropine; S.A. = Specific Activity; C.A. = Cholinergic agents; A.A. = Amino acids

Expt. No.	Additions	S.A. of Protein, c/m/mg. protein	Decrease in S.A. with C.A.	S.A. of Protein, c/m/mg. protein	Increase in S.A. with C.A.	Increase in S.A. produced by Aminosol with C.A. ¤
		With hydrolyzed Aminosol		With Aminosol		
1	1 ug./ml. Es, 5.5×10^{-6} M AcCh, 10 ug./ml. At	6.2		6.5		
	1 ug./ml. Es, 5.5×10^{-6} M AcCh	2.4	61%	6.5	0%	129%
2	100 ug./ml. Es, 5.5×10^{-6} M AcCh, 100 ug./ml. At	2.8		3.3		
	100 ug./ml. Es, 5.5×10^{-6} M AcCh	2.1	25%	4.5	36%	121%
3	None	10.0		9.4		
	10 ug./ml. Es, 5.5×10^{-5} M AcCh	5.6	44%	9.4	0%	68%
		With Amino Acid Mixture IV				
4	100 ug./ml. Es, 5.5×10^{-4} M AcCh, 100 ug./ml. At	4.0		4.3		
	100 ug./ml. Es, 5.5×10^{-4} M AcCh	3.8	5%	7.0	63%	84%
5	100 ug./ml. Es, 5.5×10^{-4} M AcCh 100 ug./ml. At	9.9		11.6		
	100 ug./ml. Es, 5.5×10^{-4} M AcCh	7.1	28%	11.2	4%	58%

¤ Values calculated by comparing corresponding figures in columns 3 and 5

An Aminosol solution in which the peptides had been broken down to free amino acids by acid-hydrolysis (Chapter VIII-A) was used in most experiments to determine the role of the peptides in the Aminosol mixture.

The following three paragraphs discuss the main effects demonstrated by the results of Table XXXI.

Acetylcholine with eserine, in secreting pancreas slices (no atropine), inhibited glycine incorporation by 30-60% in the presence of either amino acids or hydrolyzed Aminosol in four out of five experiments. The degree of inhibition does not appear related to the concentrations of cholinergic agents in the range studied. It will be noted that incorporation rates in the absence of cholinergic agents varied as much as threefold with different pancreases.

Aminosol, in pancreas slices stimulated to secrete by acetylcholine and eserine, stimulated glycine incorporation into total protein by 36 and 63% in two out of five experiments. Although in the other experiments a direct stimulation of glycine incorporation was not obtained with Aminosol, plus cholinergic agents, the inhibition produced by the cholinergic agents in the presence of hydrolyzed Aminosol or amino acids were in every experiment completely reversed in the presence of the peptide containing mixture.

In non-secreting pancreas slices (cholinergic agents plus atropine) glycine incorporation did not vary significantly with the addition of Aminosol or amino acid mixtures. However, in secreting pancreas slices (acetylcholine and eserine) in the presence of

Aminosol, the incorporation of glycine into total protein was 60-130% greater than that obtained with secreting slices in the presence of amino acid mixtures.

Table XXXII summarizes the results of another group of experiments on radioglycine incorporation into total protein of pancreas slices of fed pigeons stimulated to secrete in the presence and absence of Aminosol. Both carbamylcholine and acetylcholine with eserine were employed to stimulate secretion in these experiments. Atropine was present in the control vessels. Some degree of inhibition of radioglycine incorporation by cholinergic agents in the presence of hydrolyzed Aminosol or amino acids was obtained in these slices. Aminosol, in pancreas slices of fed pigeons which were stimulated to secrete, stimulated glycine incorporation by 28-35% in three out of four experiments. Incorporation of glycine into total protein of secreting pancreas slices was increased by 38-100% in three out of four experiments when Aminosol replaced amino acid mixture. The results of Tables XXX, XXXI and XXXII are in general similar with regard to the effect of the peptide-containing mixture (Aminosol) on glycine incorporation in secreting and non-secreting pancreas slices. The results will be interpreted in Chapter X.

4. Effect of Acetylcholine on the Turnover of Acid-Soluble Phosphate Esters and Amylase Secretion by Pigeon Pancreas Slices

It was thought that high concentrations of cholinergic drugs might inhibit glycine incorporation by interfering with energy

TABLE XXXII

EFFECT OF CHOLINERGIC AGENTS ON INCORPORATION OF GLYCINE-C^{14} INTO PANCREAS SLICES OF FED PIGEONS IN PRESENCE OF AMINOSOL AND VARIOUS AMINO ACID MIXTURES

Incubations in "Medium III" containing: 0.2% glucose, amino acid mixtures as indicated, cholinergic agents as indicated. Expts. 1 to 3 - 1 mg. glycine (80,000 c/m/mg.); Expt. 4 - 1 mg. glycine (20,000 c/m/mg.). Gassed with 100% O_2. 2 hour incubations. 150 ug. carbamylcholine injected intramuscularly into pigeon in expt. No.1 Es = Eserine; AcCh = Acetylcholine; cc = Carbamylcholine; At = Atropine; S.A. = Specific Activity; C.A. = Cholinergic Agents; A.A. = Amino acids

Expt. No.	Additions	S.A. of Protein, c/m/mg. Protein	Decrease in S.A. with C.A.	S.A. of Protein, c/m/mg. Protein	Increase in S.A. with C.A.	Increase in S.A. Produced by Aminosol with C.A. *
		With Hydrolyzed Aminosol		With Aminosol		
1	$5.5x10^{-4}$M cc, 100 ug./ml. At	57.8		53.9		
	$5.5x10^{-4}$M cc	50.4	13%	68.9	28%	38%
2	$5.5x10^{-4}$M cc, 100 ug./ml. At	58.0		65.0		
	$5.5x10^{-4}$M cc	54.0	7%	90.0	39%	67%
3.	$5.5x10^{-6}$M AcCh, 1 ug./ml. Es, 10 ug. ml. At	23.2		25.4		
	$5.5x10^{-6}$M AcCh, 1 ug./ml. Es	17.3	25%	34.2	35%	100%
		With Amino Acid Mixture IV				
4.	$1.7x10^{-6}$M AcCh, 100 ug./ml. Es 10 ug./ml. At	15.6		13.6		
	$1.7x10^{-6}$M Es, 100 ug./ml. Es	13.4	14%	13.9	2%	4%

* Values calculated by comparing corresponding figures in columns 3 and 5

production in pigeon pancreas. Even though respiration of pigeon pancreas slices was not affected by cholinergic drugs in any of the experiments, it was possible that phosphorylation was, and in order to test this pancreas slices were incubated with P^{32}, in the presence of various concentrations of acetylcholine with eserine, and the specific activities of the acid-soluble phosphate esters determined. Enzyme secretion was also measured in these experiments. The results of such an experiment are shown in Table XXXIII.

Enzyme secretion was stimulated half-maximally by eserine alone and maximally by eserine in combination with 10^{-6} M acetylcholine. Acetylcholine did not affect the specific activity of the acid-soluble phosphate esters at concentrations as high as 10^{-3} M. Eserine was also without effect. The results suggest that cholinergic drugs do not interfere with energy production in pigeon pancreas slices.

Summary

1. Glycine-1-C^{14} is incorporated rapidly into the total protein of pigeon pancreas slices during aerobic incubation. Although there is a wide variation between the rates of glycine incorporation by pancreas slices of different pigeons, the rate of incorporation in slices of a single pancreas is linear for at least 3 hours. The average rate of incorporation (4 μM glycine/g. protein/hour) is about equal to the rate reported for rabbit bone marrow cells and six times faster than the rate reported for rat diaphragm.

2. Glycine-C^{14} incorporation into pigeon pancreas slices is inhibited 98-99% after incubation in the absence of oxygen or with

TABLE XXXIII

EFFECT OF VARIOUS CONCENTRATIONS OF ACETYLCHOLINE ON AMYLASE SECRETION AND INCORPORATION OF P^{32} INTO ACID-SOLUBLE ORGANIC PHOSPHATE BY PIGEON PANCREAS SLICES

Incubation in "Medium III" containing: 0.2% glucose, about 10 μC P^{32} as inorganic phosphate, 100 μg./ml. eserine where indicated. Acetylcholine as indicated. Gassed with 100% O_2. Pigeon fasted 72 hours before killing. Slices preincubated for 40 minutes before main incubation in 3 ml. oxygenated "Medium III" containing 0.2% glucose. Main incubation 80 minutes.

Additions	Amylase Activity of Medium (Smith & Roe Units) per mg. initial dry wt.	Amylase Secreted (Smith & Roe Units) per mg. initial dry wt.	Specific Activity of Acid-Soluble Organic Phosphate c/m/μg.
None	17		189
Eserine	23	6	206
Eserine, AcCh* ($1x10^{-7}$ M)	23	6	222
Eserine, AcCh ($1x10^{-6}$ M)	29	12	204
Eserine, AcCh ($1x10^{-4}$ M)	28	11	210
Eserine, AcCh ($1x10^{-3}$ M)	27	12	194

* AcCh = Acetylcholine

2:4-dinitrophenol; this indicates that the incorporation is an energy-dependent process.

3. In pancreases of pigeons depleted of enzymes by in vivo carbamylcholine administration a complete mixture of amino acids stimulates glycine incorporation by 15-30%. Synthesis of amylase, lipase and ribonuclease, however, is stimulated 3-6 times more than is labelled glycine incorporation by the complete amino acid mixture.

4. Glycine incorporation into non-secreting pancreas slices occurs at the same rate in the presence of added amino acids, Aminosol or hydrolyzed Aminosol. In pancreas slices incubated with or without amino acids, radioglycine incorporation may be inhibited as much as 60% by the action of cholinergic drugs. These inhibitions are reversed in the presence of Aminosol. Aminosol may stimulate the incorporation of glycine into the protein of secreting pancreas slices. The incorporation of glycine into the total protein of secreting pancreas slices may be 130% greater in the presence of Aminosol than in the presence of amino acid mixtures.

5. Eserine alone, and acetylcholine at concentrations up to 10^{-3}M have no effect on the specific activity of acid-soluble phosphate esters of pancreas slices incubated with P^{32}. This indicates that the inhibitory effects of these cholinergic agents on glycine incorporation is not caused by an inhibition of energy production.

B - EXPERIMENTS WITH MOUSE PANCREAS

1. Effect of Acetylcholine and Eserine on C^{14} Glycine and Alanine Incorporation into Mouse Pancreas Slices

It was considered of interest to observe the effect of cholinergic drugs on labelled amino acid incorporation into mouse pancreas slices. Amylase synthesis may be followed *in vitro* in mouse pancreas; amino acids stimulate synthesis in pancreases of fasted mice (157). The results of two such experiments with glycine are shown in Table XXXIV. Pancreases of mice fasted for 24 hours were used.

Acetylcholine and eserine inhibited glycine incorporation, by over 80% in Expt. 1 of Table XXXIV. In Expt. 2 the inhibition produced by eserine alone was as great as with acetylcholine and eserine; in either case the inhibition was only about 20%. Eserine is apparently effective without added acetylcholine in inhibiting glycine incorporation. The inhibitory effect of eserine may either be due to the direct action of the agent itself, or more probably, to the accumulation of endogenous acetylcholine in amounts sufficient to cause the inhibitory effect. Aminosol was without effect on the incorporation of glycine into the protein of secreting mouse pancreas.

Similar results to those obtained with glycine-C^{14} were obtained with alanine-1-C^{14}. In an experiment employing the pancreases of fed mice C^{14}-alanine incorporation was inhibited about 60% by acetylcholine with eserine (Table XXXV).

TABLE XXXIV

INCORPORATION OF GLYCINE-C^{14} INTO MOUSE PANCREAS IN PRESENCE AND ABSENCE OF CHOLINERGIC DRUGS

Incubation in "Medium III" containing: 0.2% glucose, Aminosol, 0.0044M glycine (80,000 counts per vessel in Expt.1; 20,000 counts per vessel in Expt.2). Gassed with 100% O_2. 2 hour incubation.

Mice used in Expt.2 were an albino strain from the Royal Victoria Hospital. Mice used in both experiments were fasted for 24 hours prior to killing. 4 pancreases per vessel.

Expt. No.	Additions	Specific Activity of Protein, c/m/mg. Protein	Decrease in Specific Activity of Protein with Cholinergic Agents
1	$1x10^{-4}$M Acetylcholine, 100 ug. per ml. eserine, 100 ug. per ml. atropine	25.3	
		24.4	
	$1x10^{-4}$M Acetylcholine, 100 ug. per ml. eserine	4.2	83%
		4.4	
	None	11.6	
2	$1x10^{-4}$M Acetylcholine, 100 ug. per ml. eserine, 100 ug. per ml. atropine	11.5	
	100 ug. per ml. eserine	8.9	23%
	$1x10^{-4}$M Acetylcholine, 100 ug. per ml. eserine	9.3	20%

TABLE XXXV

INCORPORATION OF ALANINE-C^{14} INTO MOUSE PANCREAS SLICES

IN PRESENCE AND ABSENCE OF ACETYLCHOLINE WITH ESERINE

Incubation in "Medium III" containing: 0.2% glucose, Aminosol, 2 mg. alanine (15,000 c/m/mg.). Gassed with 100% O_2. 3 hour incubation. The mice were not fasted. Pancreases of 4 mice per vessel.

Additions	Specific Activity of Protein, c/m/mg.	Decrease in Specific Activity of Protein with Cholinergic Agents
None	33.8	
None	36.5	
$1x10^{-4}$M acetylcholine, 10 ug. per ml. eserine	13.9	60%
$1x10^{-4}$M acetylcholine, 10 ug. per ml. eserine	12.7	64%

These inhibitions are typical of those obtained with pigeon and mouse pancreas slices and indicate that the inhibitory effects of cholinergic drugs are not unique to glycine. Aminosol did not stimulate radioalanine incorporation into protein of secreting mouse pancreas.

Incorporation of labelled alanine into liver slices of fed mice was also measured. The livers of three fed mice were used. Each vessel contained slices of liver (300-400 mg.) from only one mouse. The specific activities of the labelled protein obtained were 5.1, 4.3 and 2.9. The average specific activity was therefore about one-tenth of that obtained with protein of fed mouse pancreas (Table XXXV).

2. Effect of Various Concentrations of Carbamylcholine on Glycine Incorporation and Amylase Synthesis and Secretion

Since eserine alone was as effective as acetylcholine with eserine in inhibiting glycine incorporation (Table XXXIV) it was desirable to stimulate secretion in the absence of eserine and observe the effect on glycine incorporation. The effect of various concentrations of carbamylcholine on glycine incorporation into pancreas protein and amylase synthesis and secretion are shown in the results of Table XXXVI. Carbamylcholine did not inhibit glycine incorporation into mouse pancreas protein at concentrations of less than 10^{-4}M. Above this concentration an inhibition of close to 50% was obtained. Amylase synthesis was unaffected by varying concentrations of carbamylcholine. Secretion of amylase was obtained with carbamylcholine concentrations of 10^{-6}M or greater. It will be noted that glycine incorporation was not inhibited by a concentration of carbamylcholine which stimulated amylase secretion maximally (10^{-6}M). This indicates that the inhibitory effect of carbamylcholine on glycine incorporation into mouse pancreas is not related to its capacity to stimulate secretion. In contrast to the situation in the pigeon pancreas, where Aminosol reverses the inhibition of glycine incorporation by cholinergic drugs, in the mouse pancreas carbamylcholine inhibits glycine incorporation even in the presence of Aminosol.

3. Effect of Acetylcholine and Eserine on Uptake of Free Glycine into Mouse Pancreas

It was thought that the inhibitory effect of cholinergic

TABLE XXXVI

EFFECT OF VARIOUS CONCENTRATIONS OF CARBAMYLCHOLINE ON GLYCINE-C^{14} INCORPORATION AND ENZYME SYNTHESIS AND SECRETION BY MOUSE PANCREAS SLICES

Incubations in "Medium III" containing: 0.2% glucose, Aminosol, 1 mg. glycine (80,000 c/m/mg.). Gassed with 100% O_2. 2 hour incubation. Mice fasted for 24 hours and injected intraperitoneally with 1 mg. pilocarpine in 0.1 ml. water 90 minutes before killing. Pancreases of 6 mice per vessel.

Vessel	Additions	Specific Activity of protein, c/m/mg. protein *	Average Value	Decrease in Specific Activity with carbamylcholine	Smith and Roe Units of Amylase per mg. initial dry wt.	
					Synthesized	Secreted
1	100 ug. per ml. atropine	50.0 48.7 49.8	49.5		19	-
2	10^{-10} M Carbamylcholine	52.5 50.2 53.4	51.7		23	1
3	10^{-8} M Carbamylcholine	51.1 50.7 47.8	49.9		18	2
4	10^{-6} M Carbamylcholine	53.4 49.2 51.7	51.4		21	7
5	10^{-4} M Carbamylcholine	25.0 24.3 26.8	25.4	49%	19	7
6	10^{-2} M Carbamylcholine	27.9 28.0 29.5	27.8	44%	23	7

* Plates prepared in triplicate

drugs on labelled amino acid incorporation into protein might be due to their affecting the rate of entry of amino acids into the cell. One method of testing this possibility was to measure the radioactivity of the trichloroacetic acid used to extract and wash the radioactive proteins from the homogenized slices after incubation. Labelled glycine which had entered the cell and was not incorporated into protein could be determined in this manner. The results of such an experiment are shown in Table XXXVII.

Eserine alone inhibited glycine incorporation into protein by over 50%, and acetylcholine with eserine produced an inhibition of over 70%. The inhibition by acetylcholine with eserine was the same in mice that received, and mice that did not receive, pilocarpine in vivo. The radioactivity of the acid-soluble fraction was essentially the same in all four samples. The results of this preliminary experiment indicate that acetylcholine and eserine do not inhibit glycine incorporation into protein by interfering with the passage of glycine across the cell membrane. A more strict measurement of the effect of these drugs on the time course of free glycine accumulation within the cells would be necessary in order definitely to establish this point.

4. Experiments with Whole Mouse Pancreas

Since mouse pancreas is a very spongy and porous-appearing tissue, it was anticipated that sufficient oxygenation could be obtained by whole pancreases during incubation. The Q_{O_2} values (8-9) obtained upon incubating whole mouse pancreases were the same as

TABLE XXXVII

RADIOACTIVITY OF TRICHLOROACETIC ACID-INSOLUBLE (PROTEIN) AND TRICHLOROACETIC ACID-SOLUBLE FRACTIONS OF MOUSE PANCREAS SLICES AFTER INCUBATION WITH GLYCINE-C^{14} IN THE PRESENCE AND ABSENCE OF CHOLINERGIC DRUGS

Incubations in "Medium III" containing: 0.2% glucose, amino acid Mixture V, 1 mg. glycine (80,000 c/m/mg.). Gassed with 100% O_2 2 hour incubation. Mice fasted 24 hours prior to killing. 4 pancreases per vessel. Pancreases of vessel 4 obtained from mice injected with 1 mg. pilocarpine 90 minutes before killing.

Vessel	Additions	Specific Activity, c/m/mg. Protein (acid-solube fraction)	Decrease in Specific Activity over control (Vessel 1)*	c/m/mg. initial wet wt. tissue of acid-soluble fraction
1	None	69.1 68.6		76
2	100 ug. per ml. eserine	31.2 29.0	56%	68
3	100 ug. per ml. eserine, $1x10^{-4}$M Acetylcholine	19.9	71%	71
4	100 ug. per ml. eserine, $1x10^{-4}$M Acetylcholine	19.7 18.4	72%	69

* The average specific activity of duplicate determinations were used in calculating the results in this column.

those obtained with mouse pancreas slices and similar to those of pigeon pancreas slices. Representative results on the rate of oxygen consumption by whole and sliced mouse pancreases are shown in Figure 5. Whole pancreases respired steadily for periods up to 6 hours at a rate of about 2/3 that obtained during the first 15-30 minutes. A similar decrease in the rate of oxygen consumption was observed with mouse pancreas slices with a further slight decrease after three hours.

5. Ribonuclease Activity of Mouse Pancreas

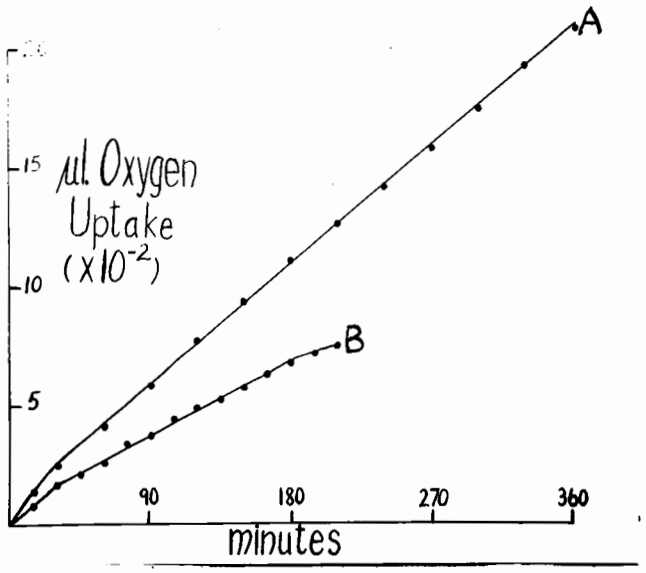
It was considered of interest to observe whether the ribonuclease activity of mouse pancreas increases after incubation. In assaying aqueous mouse pancreas extracts for ribonuclease, it was found that the ribonuclease activity of enzyme-depleted (pilocarpine injected) mouse pancreas is about 10 times higher than that of depleted pigeon pancreas. Table XXXVIII shows the results of an experiment with mouse pancreas slices, in which the ribonuclease activities of the incubated and unincubated samples were measured.

The ribonuclease activity increased by 10-20% over the activity of the unincubated controls. Despite the small percentage increase in activity, the absolute increases in ribonuclease activity are several times greater than the largest increases observed with pigeon pancreas slices (See Chapter VII). Although further work is required to establish the validity of these experimental observations, these results suggest that mouse pancreas slices may synthesize ribonuclease.

FIGURE 5

RESPIRATION OF WHOLE AND

SLICED MOUSE PANCREAS



A - 205 mg. fresh tissue (2 whole pancreases)

B - 127 mg. fresh tissue (sliced pancreases)

Tissue incubated in "Medium III" containing 0.2% glucose and 0.34% Aminosol. Gassed with 100% O_2. Temp. 37.5°C.

TABLE XXXVIII

EFFECT OF AEROBIC INCUBATION ON RIBONUCLEASE ACTIVITY OF MOUSE PANCREAS

Incubation in "Medium III" containing 0.2% glucose and Aminosol. Gassed with 100% O_2. 3 hour incubation at 37.5°C. Pancreases of 3 mice per vessel.

Vessel	Ribonuclease Units: Unincubated Control	Incubated Tissue	Increase after Incubation
1	280	346	66
2	306	331	25
3	286	316	30
4	322	381	59

Summary

1. Glycine-C^{14} is incorporated rapidly into total protein of mouse pancreas slices. The average incorporation obtained after two hours of incubation (8 uM glycine/g. protein) is equal to that obtained with pigeon pancreas slices.

2. Acetylcholine and eserine inhibit the incorporation of labelled glycine into the protein of mouse pancreas slices by 20-80%. The inhibitions produced by eserine alone are almost as great as those produced by acetylcholine with eserine. The inhibitions are obtained both in the presence of amino acids and of Aminosol.

3. Alanine-1-C^{14} is incorporated into mouse pancreas protein *in vitro*. Incorporation is inhibited about 60% by acetylcholine with eserine. The inhibition occurs in the presence of Aminosol.

4. Alanine-1-C^{14} incorporated *in vitro* about ten times more rapidly into mouse pancreas protein than into mouse liver protein.

5. Carbamylcholine, at concentrations of 10^{-4}M or greater, inhibits glycine incorporation into mouse pancreas protein about 50%; no inhibition is obtained with lower concentrations. The inhibitions are obtained in the presence of Aminosol. High concentrations of carbamylcholine do not inhibit amylase synthesis by mouse pancreas slices.

6. The total radioactivity found in the acid-soluble fraction of mouse pancreas slices incubated with and without acetylcholine for 2 hours is the same. This suggests that cholinergic agents do not interfere with the entry of labelled glycine into the pancreas cell.

7. The rate of respiration of whole mouse pancreas is linear, for periods up to 6 hours, after a decrease of about 30% during the first 30 minutes.

8. The ribonuclease activity of the pancreases of mice depleted *in vivo* is about ten times greater than that of the pancreases of pigeons depleted *in vivo*. The ribonuclease activity of mouse pancreas slices increases by 10-20% after aerobic incubation.

CHAPTER X

DISCUSSION

Enzyme Synthesis and Secretion

The experiments on enzyme synthesis have provided evidence that lipase and ribonuclease, as well as amylase, are synthesized *in vitro* by pigeon pancreas. The similar pattern observed in the synthesis of all three enzymes studied makes it most probable that other digestive enzymes, such as the proteases, are also synthesized *in vitro*. Pigeon pancreas slices thus provide a system in which the synthesis of several specific proteins can be studied under controlled conditions. Anfinsen (160), using tracer techniques, obtained evidence that ribonuclease is synthesized in bovine pancreas slices; the net formation of the enzyme was not followed.

Any explanation of the variable effects obtained in some experiments with added amino acids on the synthesis of amylase, lipase and ribonuclease must include several factors. The endogenous amino acid supply of the slices and the quantity of amino acids contributed by proteolysis during the incubation are undoubtedly quite variable and uncontrolled. In the few experiments in which synthesis of lipase or ribonuclease was not stimulated by added amino acids, the rates of synthesis of these enzymes must have been below those rates which would be limited by the tissue supply of amino acids. The fact that amylase synthesis was invariably stimulated by added amino acids indicates a marked dependence of the rate of synthesis of this enzyme on the amino acid concentration.

Preliminary incubation of the slices is of value in studies on the effects of amino acids on enzyme synthesis, since it probably removes a large part of the tissue amino acids. This is indicated by the fact that added amino acids stimulate enzyme synthesis several times more in preincubated than in non-preincubated slices and clearly demonstrates the marked dependence of *in vitro* protein synthesis on amino acids. *In vivo*, the amino acid supply of the tissues is maintained by the circulating blood, and net protein synthesis or protein equilibrium is thus assured. Pancreas tissue *in vitro* can continue its specialized activity of synthesizing large quantities of protein in the presence of an optimal concentration of amino acids.

Evidence has been presented that pigeon pancreas slices actively secrete amylase, lipase and ribonuclease in a parallel fashion. The *in vitro* results with pigeon pancreas are similar to the early *in vivo* observations by Babkin (137) on the parallel secretion of amylase, lipase and trypsin, and those obtained more recently by Baxter (138) and Barrington (139). Presumably the increase in the enzyme activity of the medium in the presence of cholinergic drugs is due to the discharge of the secretory granules by the pancreas cell, as was demonstrated histologically *in vitro* (146).

Many physiologists have assumed that stimulation of enzyme secretion in the pancreas causes an accelerated rate of enzyme synthesis. Several recent *in vivo* studies on the relationship

between ribonucleic acids and enzyme synthesis in the pancreas are based on this view (155, 161, 162, 163, 164, 165). Total enzyme synthesis was not actually measured in any of these *in vivo* investigations, although Daly and Mirsky (155) and de Dekan-Grenson (165) have followed the changes in the tissue content of some of the enzymes after pilocarpine injection. In the studies on enzyme synthesis and secretion reported here increases of approximately threefold in the quantities of amylase, lipase and ribonuclease secreted into the medium did not augment their synthesis. This was observed in the pancreases of fed and fasted pigeons and in the presence and absence of amino acids. The results clearly demonstrate that at least *in vitro* the rate of enzyme synthesis is independent of the secretory activity of the pancreas. The results of the *in vitro* studies on labelled glycine incorporation into pigeon pancreas slices do suggest a relationship between protein synthesis and secretion, and this will be discussed below.

Glycine-C^{14} Incorporation into Protein and Enzyme Synthesis in Secreting and Non-Secreting Pancreas Slices

The experiments on enzyme synthesis and labelled glycine incorporation into protein have indicated that certain differences exist between the incorporating and enzyme-synthesizing systems. The differences obtained with pigeon pancreas slices are particularly interesting and are summarized in Table XXXIX. These results may be interpreted by the simple hypothesis shown diagramatically below,

TABLE XXXIX

ENZYME SYNTHESIS AND GLYCINE-C^{14} INCORPORATION BY PIGEON PANCREAS SLICES UNDER VARIOUS CONDITIONS OF INCUBATION

	Incubation with	Effect on Enzyme Synthesis	Effect on Glycine Incorporation into Total Protein
1	Amino Acids	Increased 100-200%	Increased 10-30%
2	Amino Acids plus simple peptides (Aminosol)	Increased 100-200%	Increased 15-20%
3	Cholinergic drugs plus amino acids	No effect above that obtained with amino acids alone	No stimulation or inhibition of up to 60%
4	Cholinergic drugs plus Aminosol	No effect above that obtained with Aminosol alone	Stimulations of up to 60% or complete reversal of inhibitions produced by cholinergic drugs

for the process of enzyme synthesis.

(a)		(b)		(c)
Amino Acids plus peptides (in secreting gland)		Precursor Protein (plus Amino Acids)		Final Enzyme

It is suggested that two processes are involved in enzyme synthesis in pancreas: (1) formation of precursor protein (not enzymatically active), (2) elaboration of enzyme. The evidence for intermediates or protein precursors has been reviewed in Chapter V.

The major portion of glycine incorporation into pancreas protein probably represents formation of precursor. This view is supported by the following observations. Glycine was found to be rapidly incorporated into pancreas protein *in vitro*; the rate approximates that of growing tissue. Mouse pancreas has been shown to incorporate glycine-N^{15} about twice as fast as liver *in vivo* (164). Results reported here showed that mouse pancreas incorporated alanine-C^{14} almost ten times as rapidly as liver *in vitro*; the difference in incorporation rates between the two tissues is thus even more pronounced *in vitro* than *in vivo*. In the pigeon pancreas amino acid mixtures which stimulated enzyme synthesis as much as threefold had but little effect on glycine incorporation into protein (Table XXXIX). On the other hand, peptides stimulated glycine incorporation in secreting pancreas slices but had no greater effect than amino acids on enzyme synthesis. (Table XXXIX). The response of the glycine incorporating and enzyme synthesizing systems

of pigeon pancreas slices to various incubation conditions is thus quite different. This supports the view that glycine incorporation into total pigeon pancreas protein is not directly related to enzyme synthesis. Glycine incorporation is, however, a very rapid process in pancreas slices, and hence reflects a high rate of peptide bond and protein synthesis occurring in this tissue. Although not directly related the high rate of incorporation is in accord with the active synthesis of enzyme-protein by pancrease tissue. The maximal content of known secretory enzymes of this tissue has been estimated to represent about 20% of its dry weight (155). The high rate of glycine incorporation may thus be regarded as largely due to formation of inert protein precursor, corresponding to non-enzyme protein produced by actively incorporating (growing) tissues.

It appears likely that precursor formation in secreting pancreas slices require peptides. This is supported by the following evidence. The incorporation of glycine into pigeon pancreas slices incubated in the presence of amino acids was inhibited by cholinergic drugs which caused the slices to secrete. In slices stimulated to secrete, peptides (Aminosol) completely reversed these inhibitions and in some cases caused a greater glycine incorporation than that found in non-secreting slices. In the secreting slices peptides caused stimulations of as much as 130%. The inhibition of glycine incorporation in pancreases stimulated to secrete by cholinergic agents complicates the picture, and will be discussed below. It is considered that the inhibitions produced by cholinergic agents mask

the stimulating effect of peptides on glycine incorporation into protein of secreting pigeon pancreas slices. In non-secreting pancreas slices, incorporation rates were the same with amino acids plus peptides as with amino acids alone. These observations favour the view that the precursor synthesizing mechanism is activated in secreting pigeon pancreas slices and requires peptides.

The strong evidence for the role of amino acids in enzyme synthesis has already been discussed. A mixture of peptides and amino acids has no greater effect on enzyme synthesis than have amino acids alone; this supports the view that the enzyme is formed from precursor plus amino acids. If de novo synthesis of enzymes occurred with amino acids as starting materials, one might expect amino acid incorporation into total pancreas protein and enzyme synthesis to occur at a similar rate under various experimental conditions, since both processes represent synthesis of peptide bonds. However, the fact that stimulation of peptide bond synthesis may occur in the absence of stimulation of enzyme synthesis suggests that the formation takes place of intermediate enzyme-precursor protein which ultimately becomes active enzyme. The process of building up precursor material from simple peptides and amino acids to final enzyme-protein is relatively slow when compared with synthesis of enzyme from available precursor. The rates of enzyme synthesis in secreting and non-secreting pigeon pancreases are the same even though under certain conditions rates of precursor synthesis may vary widely. Availability of amino acid limits the rate of enzyme synthesis in

both secreting and non-secreting glands. In the secreting gland, precursor synthesis, requiring peptides, is thrown into action to maintain an optimal supply of precursor for ultimate enzyme synthesis.

Peptides (Aminosol) had no stimulatory effect on glycine incorporation into the protein of secreting mouse pancreas slices. With high concentrations of cholinergic agents, glycine incorporation into mouse pancreas was inhibited in the presence of amino acids or amino acids plus peptides. Both inhibitory and stimulatory phenomena are involved in glands secreting in the presence of peptides, and greater sensitivity to cholinergic agents may explain the results obtained with mice.

The inhibition of glycine incorporation by cholinergic drugs cannot be readily explained. The inhibition produced by eserine alone is almost as great as by acetylcholine with eserine in mouse pancreas. This may mean that the concentration of acetylcholine which accumulates in the presence of eserine is sufficient to produce a considerable inhibition of glycine into the proteins or possibly that eserine may act directly on the glycine incorporating system. The former view is supported by two observations; (1) carbamylcholine, in the absence of eserine, is capable of inhibiting glycine incorporation into protein, although at relatively high concentrations; (2) atropine, which specifically blocks the action of acetylcholine, prevented the inhibitory effects of eserine and acetylcholine.

Cholinergic agents have also been found to inhibit glycerol-1-C^{14}

incorporation into brain glycerophosphatides (166) and the uptake of P^{32} into the ribonucleic acids of mouse and pigeon pancreas (159). It thus appears that cholinergic agents are capable of inhibiting many synthetic reactions. Neither respiration nor acid-soluble phosphate ester turnover was affected by high concentration of cholinergic agents and hence their inhibitory effects do not appear to be due to interference with energy production by the cell. Tentative evidence was provided that the inhibitions are not due to permeability changes in the cell membrane.

The studies on glycine-C^{14} incorporation into protein and enzyme synthesis by pancreas slices suggest that although peptide bond synthesis is probably being measured by both techniques, the product being examined in either case is different. With pigeon pancreas slices, it is proposed that glycine incorporation measures primarily the synthesis of enzymatically-inactive protein, while enzyme synthesis measures the production of specific protein. Information on both processes and on the relation of one process to another should aid in the ultimate understanding of the mechanism of protein synthesis.

GENERAL SUMMARY - PART II

A spectrophotometric method has been described for assaying ribonuclease in crude aqueous extracts of small quantities of tissue.

Lipase and ribonuclease synthesis has been studied in pigeon pancreas slices depleted of their enzyme content by abundant feeding of the pigeons and injection of carbamylcholine before killing.

Increases in the total lipase and ribonuclease activities (sum of medium and tissue) are obtained when depleted pancreas slices are incubated aerobically in physiological saline; this indicates synthesis of these enzymes in vitro.

Lipase and ribonuclease synthesis is not obtained anaerobically or in the presence of 2:4-dinitrophenol.

Lipase and ribonuclease synthesis is usually, but not always, stimulated by amino acids. Consistent stimulations of enzyme synthesis occur with slices which have been preincubated.

A partial fibrin hydrolysate containing small peptides and amino acids is equal, but not superior, to a complete mixture of 22 amino acids in stimulating synthesis of the three enzymes measured.

The synthesis of amylase, lipase and ribonuclease obtained on incubation in saline without amino acids, is reduced or abolished in preincubated slices. In the presence of amino acids synthesis is not significantly reduced by preincubation.

Pancreas slices discharge more lipase and ribonuclease into the medium in the presence of carbamylcholine or acetylcholine (with eserine) than in the presence of either of these drugs plus atropine.

Secretion of lipase and ribonuclease is dependent on oxygen and is inhibited by 2:4-dinitrophenol.

The above observations indicate that pigeon pancreas slices actively secrete amylase, lipase and ribonuclease in a parallel fashion, in vitro.

Synthesis of amylase, lipase and ribonuclease is not appreciably affected by stimulation of secretion in pancreas slices of pigeons which have, or have not, been stimulated to secrete in vivo by carbamylcholine.

Glycine-1-C^{14} is incorporated rapidly into the protein of pigeon and mouse pancreas slices. The average rate of incorporation (4 uM glycine/g. protein/hour) is about equal to the rate reported for bone marrow cells and five times greater than the rate reported for rat diaphragm.

The in vitro incorporation into pigeon pancreas slices is inhibited 98-99% after incubation in the absence of oxygen or in the presence of 2:4-dinitrophenol; this indicates that the incorporation is an energy-dependent process.

Alanine 1-C^{14} is incorporated into mouse pancreas protein in vitro. Alanine is incorporated in vitro about 10 times faster into mouse pancreas protein than into mouse liver protein.

Glycine incorporation into slices of mouse pancreas protein may be inhibited as much as 80% by acetylcholine with eserine. Eserine alone produces almost as great an inhibition as acetylcholine with eserine. The inhibitions are obtained both in the presence of Aminosol and of amino acids. Carbamylcholine inhibits glycine incorporation

into mouse pancreas slices, but only at concentrations of 10^{-4}M or greater.

Amylase synthesis is not inhibited by high concentrations of carbamylcholine.

The total radioactivity found in the acid-soluble fraction of mouse pancreas slices incubated with and without acetylcholine and eserine for 2 hours is the same, indicating tentatively that cholinergic agents do not interfere with the entry of labelled glycine into the pancreas cell.

The rate of respiration of whole mouse pancreas is linear, for periods up to 6 hours, after a 30% decrease during the first 30 minutes.

In pancreases of pigeons depleted of enzymes by in vivo carbamylcholine administration a complete mixture of amino acids, stimulates glycine incorporation by 15-30%. Similar stimulations are obtained with a partial hydrolysate of fibrin (Aminosol) containing small peptides and amino acids. The amino acid mixtures stimulate amylase, lipase and ribonuclease synthesis 3-6 times more than glycine incorporation into total pancreas protein.

Glycine incorporation into non-secreting pancreas slices occurs at the same rate in the presence of added amino acids, Aminosol, or hydrolyzed Aminosol. In pancreas slices incubated in the presence or absence of added amino acids, glycine incorporation into protein may be inhibited as much as 60% by the action of cholinergic

drugs (carbamylcholine or acetylcholine with eserine). These inhibitions are reversed in the presence of a partial hydrolysate of fibrin containing amino acids and peptides (Aminosol). Aminosol may stimulate the incorporation of glycine into the total protein of secreting pancreas slices. The incorporation of glycine into the total protein of secreting pancreas slices may be 130% greater in the presence of Aminosol than in the presence of amino acid mixtures.

Eserine alone, and acetylcholine at concentrations of up to 10^{-3} M have no effect on respiration, and on the specific activity of acid-soluble phosphate esters, of pancreas slices incubated with P^{32}. This indicates that the inhibitory effects of these cholinergic agents on glycine incorporation are not caused by an inhibition of energy production.

The implications of these observations are discussed.

CLAIMS TO ORIGINAL RESEARCH

PART I

1. A modified microdiffusion technique has been developed for the determination of ammonia in the presence of tissue, using the conventional Warburg apparatus.

2. DL-α-methylglutamate has been found to absorb ammonia in the presence of extracts of acetone-dried powders of beef brain and ATP, and to produce a hydroxamic acid when hydroxylamine replaces ammonia during incubation.

3. Methionine sulfoximine and sodium fluoride inhibit the absorption of ammonia by α-methylglutamate.

4. Evidence has been provided indicating that the same enzyme in beef brain extracts is responsible for ammonia absorption by α-methylglutamate and for glutamine synthesis.

5. Chromatographic evidence was obtained which suggests that brain extracts synthesize α-methylglutamine from α-methylglutamate and ammonia.

6. Glutaminase activity of rat brain homogenates is inhibited by DL-α-methylglutamate to the same extent as by D- and L-glutamate.

PART II

1. A spectrophotometric assay has been developed for measuring ribonuclease activity in crude aqueous extracts of small quantities of tissue.

2. It has been found that the total lipase and ribonuclease activities (sum of medium and tissue) of pigeon pancreas slices increase on aerobic incubation in physiological saline, indicating a net synthesis of these enzymes in vitro.

3. Synthesis of lipase and ribonuclease is abolished by 2:4-dinitrophenol or anaerobic incubation.

4. Lipase and ribonuclease is usually, but not always stimulated by amino acids. In slices which have undergone preliminary incubation, consistent stimulations of synthesis are obtained with amino acids.

5. A partial fibrin hydrolysate containing simple peptides and free amino acids is equal, but not superior, to a mixture of 22 amino acids in stimulating the synthesis of amylase, lipase and ribonuclease.

6. Preincubation of pancreas slices reduces or abolishes the synthesis of amylase, lipase and ribonculease obtained on incubation in saline without amino acids; when amino acids are present preincubation does not appreciably depress synthesis.

7. Lipase and ribonuclease are secreted by pigeon pancreases in vitro. The secretion of amylase, lipase and ribonuclease is stimulated to the same extent by cholinergic agents.

8. Secretion is dependent on oxygen and is inhibited by 2:4-dinitrophenol.

9. In vitro stimulation of enzyme secretion is not accompanied by increased enzyme synthesis. This is true both for pancreas slices of pigeons which have or have not received carbamylcholine in vivo, and in the presence as in the absence of amino acids.

10. Glycine-C^{14} is incorporated rapidly (4 μM/g. protein/hr.) into the protein of pigeon and mouse pancreas slices.

11. Glycine incorporation into the protein of pigeon pancreas slices is 98% inhibited upon incubation in the absence of oxygen or in the presence of 2:4-dinitrophenol.

12. Alanine-1-C^{14} is incorporated into mouse pancreas protein in vitro. Mouse pancreas slices incorporate alanine ten times faster than mouse liver slices.

13. Glycine incorporation into slices of mouse pancreas protein may be inhibited up to 80% by acetylcholine (with eserine); eserine alone inhibits almost as much. Inhibitions are obtained both in the presence of Aminosol and of amino acids. Incorporation is inhibited by high concentrations of carbamylcholine. High concentrations of carbamylcholine do not inhibit amylase synthesis.

14. The total radioactivity found in the acid-soluble fraction of mouse pancreas slices incubated for 2 hours with and without acetylcholine and eserine, and with eserine alone, is the same.

15. Whole mouse pancreas respires at a linear rate for periods up to 6 hours, after a 30% decrease during the first 30 minutes.

16. In pancreases of pigeons depleted of enzymes by in vivo carbamylcholine administration a complete amino acid mixture stimulates glycine incorporation into total protein 15-30%; similar stimulations are obtained with a partial hydrolysate of fibrin containing small peptides and amino acids (Aminosol). Amylase, lipase and ribonuclease synthesis is stimulated 3-6 times more than is glycine incorporation by the amino acid mixture.

17. Glycine incorporation into non-secreting pancreas slices occurs at the same rate in the presence of added amino acids, Aminosol, or hydrolyzed Aminosol.

18. Glycine incorporation into the protein of pancreas slices is inhibited by the action of cholinergic drugs. The inhibitions are obtained with slices incubated with or without amino acids, and with hydrolyzed Aminosol. In the presence of Aminosol (amino acids plus simple peptides) the inhibitions are reversed. Aminosol may stimulate the incorporation of glycine into the total protein of secreting pancreas slices. With secreting pancreas slices, glycine incorporation into total protein may be over 100% greater in the presence of Aminosol than in the presence of amino acid mixtures.

19. Eserine alone and high concentrations of acetylcholine (10^{-3} M) affect neither respiration nor the specific activity of acid-soluble phosphate esters of pancreas slices incubated with P^{32}.

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