STUDIES IN PROTEIN SYNTHESIS

with special reference to the Pancreas.

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by

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INTRODUCTION.

P R O T E I N

In both plants and animals a substance is contained, which is produced within the former, and is imparted through their food to the latter. To both, its uses are numberless. It is one of the most complicated substances, it is very changeable in composition under various circumstances, and hence is a source of chemical transformations, especially within the animal body, which cannot even be imagined without it. It is unquestionably the most important of all known substances in the organic kingdom. Without it no life appears possible on our planet. Through its means the chief phenomena of life are produced.

The Chemistry of Vegetable and Animal Physiology.
G.J.Mulder, Translated by Dr. Fromberg, Berwick, 1845.

INTRODUCTION.

Of all the unsolved problems of biology and biochemistry, perhaps the understanding of protein synthesis and its *in vitro* accomplishment is still the greatest. It is now well established, by degradative procedures, that all proteins are composed of amino-acids linked together by peptide bonds. Analytical methods are now available for determining the composition and even the exact amino acid sequence in a protein. Modern work on protein structure indicates that protein molecules differ, not only in their constituent amino acids, but also in the sequence in which they are arranged. In consequence, many different types of synthetic arrangement must be available in every cell in order to account for the variety of protein species which they contain. It is, therefore, necessary for any proposed theory of protein synthesis to be able to explain these specific abilities which apparently are inherent in every cell.

The requirements for protein synthesis are three fold:-

(a) Precursors, i.e. building blocks, which are eventually free amino acids

(b) A mechanism for linking them by peptide bond formation

(c) A means of ensuring a definite order of assembly of these amino acid residues.

Energy of Peptide Bond Formation.

The formation of a peptide from two amino acids is accompanied by an increase in free energy, the average value of 3000 calories per mole being widely quoted. It has been calculated by Linderstrom-Lang (1952) that this figure is not strictly correct. The figure is approximately correct for synthesis of di-peptides from free amino acids, but if di-peptides are united, then the energy of formation of peptide bonds will be smaller than 3000 calories per mole. The possibility exists, therefore, that, if small peptides are produced in significant concentrations, polymerisation of the peptides might occur under favourable circumstances. The coupling of large peptides might occur relatively easily. It would therefore be relevant at this point to consider the precursors of protein synthesis, whether they be simple amino acids, di-peptides or other substances.

Precursors in Protein Synthesis.

The problem of the nature of the precursors of protein synthesis resolves itself into the question of whether proteins are made directly from free amino acids or by using peptides or other amino acid derivatives (e.g. activated amino acids). The evidence for the participation of peptides in protein synthesis depends on three types of approach:

(a) What peptides occur in cells ?

(b) Can peptides supplied to tissues be utilised for protein synthesis ? This type of evidence demands that the peptides can pass into the cell and reach the site of utilisation without hydrolysis and it is frequently difficult to obtain conclusive proof of this.

(c) Is there any evidence of dilution of labelled amino acids by peptide pools during their incorporation into proteins ?

(a) The occurrence of peptides in cells:

Attempts have been made to demonstrate the presence of peptides in sufficient quantity and variety to allow of such synthesis. Christensen, Rothwell, Sears and Streicher (1948) were unable to demonstrate the presence of amino acids conjugates, apart from glutathione, carnosine or anserine in liver and muscle tissue. The scarcity of intermediate precursors has also been observed by Halvorson and Spiegelman (1952) working with *Saccharomyces cerivisiae*. They found that none of the amino acids was depleted from the cells when the utilisation of one of them for growth or enzyme formation was prevented by the presence of a synthetic analogue. Therefore, no appreciable quantity of intermediate precursors appeared to be involved in the synthesis of the enzyme or protein except for possible precursors already so complex as to require the entrance of the amino acid whose utilisation was blocked.

On the other hand, the presence of peptide material in tissues has been claimed by Borsook and his co-workers (1949). These workers isolated a peptide material from many sources including plasma, guinea-pig heart, kidney and spleen. In a tissue homogenate, labelled amino acids were incorporated faster into this peptide material than into protein. It has been shown that there are at least four components in this peptide material (Fels and Tiselius 1951). The biological significance of this peptide remains in doubt.

The majority of the chemical evidence thus gives no support to the concept of peptides as intermediates in the synthesis of proteins. There may well be intermediates of a peptide nature with a high turnover rate present in the tissues, but such intermediates, being transient and present in low concentration, would not be readily detected by the methods employed to date.

(b) <u>The utilisation of peptides:</u> The utilisation of peptides in place of free amino acids has not provided much more conclusive evidence of their participation in protein synthesis.

In parenteral feeding experiments on whole animals, peptides or partial hydrolysates of protein were found to be less effective than amino acids or complete hydrolysates of proteins. Thus Christensen (1950) showed that an animal could be maintained in positive nitrogen balance with amino acids injected intravenously. Peptides, however, were unable to maintain the balance and were largely excreted in the urine. On the other hand, Christensen and Rafn (1952) have demonstrated that certain peptides can actually pass through the cell membrane of the Ehrlich ascites tumour cell. This, however, cannot be taken as indicating that all cells can assimilate peptides, since, in this case, the cell under examination could hardly be called typical, being neoplastic and existing under unique circumstances in the peritoneal cavity.

Studies on animal cells carried out under <u>in vitro</u> conditions have led to some contradictory evidence. Borsook et al (1952) have shown that the presence of free amino acids stimulated the uptake of labelled amino acids into haemoglobin of rabbit reticulocytes. Using this system Nizet and Lambert (1954) were unable to show that di- or tri-peptides could act in this way to any significant extent. Nizet and Lambert concluded that the slight stimulation observed was due to the production of free amino acids from the peptides by hydrolysis which took place in plasma. Hokin (1951) has demonstrated that a mixture of free amino acids stimulates the production of α - amylase by pigeon pancreas slices. Schucher and Hokin (1954) extended this work to ribonuclease and lipase, but were unable to show that a mixture of peptides was superior to the mixture of free amino acids for the synthesis of these enzymes. On the other hand, Rychlík and Šorm (1956), using mouse pancreas slices, have obtained evidence that partial hydrolysates of chymotrypsinogen will stimulate the production of protease and amylase. They also showed that partial hydrolysates of other proteins stimulated enzyme production but the magnitude of the response was dependent on the protein from which the partial hydrolysate was prepared. Until the findings of Rychlík and Šorm can be reconciled with those of Schucher and Hokin, it is not possible to draw any firm conclusions about utilisation of peptides for enzyme synthesis by the pancreas.

Tissue culture methods have been employed in an effort to determine the role of peptides in protein synthesis. Fischer (1948) found that chicken myeloblasts grew much better in a partial than in a complete protein hydrolysate. Winnick and Winnick (1953) obtained similar results, but concluded that hydrolysis of the peptides to free amino acids was taking place. Eagle (1955) showed that a deficient medium could be made to support growth if several synthetic di-peptides were added. Once again the possibility of hydrolysis could not be eliminated.

If peptides were intermediates in protein synthesis, it is reasonable to suppose that micro-organisms with peptides as a specific requirement for growth might be found. In fact, no organism with an absolute requirement for peptides has been found. Moreover, when a peptide has been found to have growth-promoting properties, this has, in some cases, been shown to be due to hydrolysis of the peptide (Ågren, 1947; Simmonds and Fruton, 1948; Krehl and Fruton, 1948; Simmonds and Fruton, 1949[a,b]; Taylor, Simmonds and Fruton, 1950; Malin, Camien and Dunn, 1951; Nurmikko and Virtanen, 1951; Virtanen and Nurmikko, 1951). On the other hand, there is evidence that partial hydrolysates of proteins have greater growth-promoting properties than complete amino acid mixtures for certain bacteria (Sprince and Woolley (1945), Dunn and McClure (1950), Klungsøyr Sirny and Elvehjem (1951). By contrast, Danté and Thorne (1949) prepared synthetic peptides and found that these were inferior to the corresponding free amino acids as nutrients for yeast. The use of synthetic amino acid analogues has supported the view that micro-organisms can utilise peptides. Thus Marshall and Woods (1952) have shown that the inhibition of bacterial growth by 4-methyltryptophan can be reversed by tryptophan peptides in a non-competitive way. They suggest that 4-methyltryptophan inhibits growth by virtue of its ability to inhibit synthesis of tryptophan peptides; thus a supply of peptides in the medium renders the organisms insensitive to 4-methyltryptophan. Kihara, McCullough and Snell (1952) have shown that D-alanine prevents the growth of Lactobacillus arabinosis by inhibiting the assimilation of

L-alanine, but that D-alanine has no inhibiting action in the presence of alanyl peptides. These authors suggest that the D-amino acid inhibits the passage of L-alanine across the cell membranes, whereas peptides pass independently and are converted into the free amino acids within the cell. Similarly, Kihara and Snell (1955) demonstrated that, if the growth of a micro-organism is inhibited by a synthetic amino acid analogue, the reversal of this inhibition is more effectively achieved by the addition of the corresponding amino acid as a peptide component, than by the addition of the free amino acid. Rowlands, Gale, Folkes and Marrian (1957) have shown that free glutamic acid can accumulate within Staphylococcus aureus which has been incubated with peptides containing glutamic acid residues. The presence of glucose has been shown to be required for this process. Proof that peptides are absorbed as such has been obtained by showing that certain peptides e.g. γ-glutamyl-valine or γ-glutamyl-leucine give rise to a more rapid accumulation of glutamic acid within the cell than is obtained when glutamic acid itself is the external source.

It would thus appear that peptides can penetrate the cell wall of bacteria and that the rate of penetration can be faster in the case of a peptide than in the case of the free amino acid. Thus the apparent superiority of peptides over the corresponding free amino acids in bacterial nutrition may depend on their more rapid assimilation rather than their use as intermediates in protein synthesis. Similarly, the results obtained by Marshall and Woods (1952) and Kihara and Snell (1955) can be explained on the basis of quicker penetration of the cell membrane by peptides than by free amino acids.

From these various studies on peptide utilisation, it is apparent that there is no unequivocal evidence of a peptide requirement or stimulation in the case of animal tissues. In the case of bacteria, there is reason to believe that peptides penetrate into the cell, in some cases more rapidly than do the corresponding amino acids. It has also been established (Rowlands et al (1957)) that the peptides are subsequently hydrolysed in some instances at least. Thus, there is no reason for concluding that utilisation of peptides by bacteria inevitably implies a role of peptides in protein synthesis.

(c) Dilution of labelled amino acids by peptide pools: If proteins are synthesised directly from amino acids without the intervention of peptide intermediates, then, if the amino acids are labelled, the same amino acid should have the same specific activity at all loci in the protein molecule. Any pool of the unlabelled peptide intermediate will dilute the isotope and this will result in uneven distribution of the label throughout the protein molecule.

Anfinsen and Steinberg (1951) have found unequal labelling of aspartic and glutamic acid molecules in ovalbumin, synthesised *in vitro* by incubating a mince of hen oviducts in a labelled bicarbonate medium. Similar results have been obtained *in vivo*, (Steinberg and Anfinsen, (1952)) though there was much less dissymmetry in the labelling. The inequality of the labelling was greatest at the beginning and tended to grow less, the longer the duration of the experiment. These studies were extended to other proteins and the same inequality of labelling was found in insulin and ribonuclease (Vaughan and Anfinsen, 1954). These results are interpreted as indicating the presence of pools of peptides of differing size from which proteins are synthesised. It should, however, be borne in mind that these results would also be obtained if different parts of the protein molecule were synthesised at different times (Dalgliesh, 1953). There is as yet no definite information of this point.

This inequality of labelling has not been found by other workers under other experimental conditions. Muir, Neuberger and Perrone (1952) examined the terminal and non-terminal valine residues of rat and rabbit haemoglobin and found that the labelling was equal. Godin and Work (1956) carried out a more rigorous experiment and found that after the intravenous administration to a lactating goat of a mixture of lysine peptides (prepared from casein) and ^{14}C-lysine, the labelling of the lysine was equal in all parts of the casein molecule. From the results, they conclude that casein is synthesised from free amino acids and that no peptides take part as such.

A slightly different approach was adopted by Simpson and Velick (1954) and Heimberg and Velick (1954) who injected rabbits with eight different labelled amino acids and determined their specific activities in three enzymes with different turnover times in muscle tissue. In the case of all the amino acids the ratio of the specific activities of the amino acids in the three enzymes was the same. This would indicate that the three enzymes are formed from the same amino acid pool and that a given amino acid in a protein is replaced at the same rate in all the positions in which it occurs. If there were any significant amount of peptides, the ratios of the specific activities of the amino acids in the three proteins would be different.

In reviewing the evidence of dilution of labelled amino acids by peptide pools, it is apparent that the evidence obtained by Anfinsen and his co-workers is in flat contradiction to that of other investigators in this field. The negative evidence of Godin and Work depends on their lysine-containing peptides being absorbed into the mammary gland cells as such, and not being hydrolysed before entry, in which case equality of labelling in the synthesised protein would be the result. However, there is no evidence that the peptides employed for this study are suitable building blocks for the synthesis of casein molecules. While they are prepared from casein by partial hydrolysis it cannot be assumed that the peptides so formed are capable of being utilised by the cell for the formation of casein.

The evidence for the occurrence of peptide intermediates in the synthesis of protein is extremely confusing. While the existence of such intermediates has not been detected by chemical means, this may only indicate that peptides have a very rapid turnover and that the concentration at any moment is very low. The apparent inability of animal tissues to utilise peptides, as shown by lack of stimulation on providing peptides exogenously, may merely indicate that peptides are hydrolysed before entry into the cell. This explanation can also be used in the case of the experiments of Godin and Work, where the giving of peptides along with labelled free amino acids failed to cause unequal labelling of amino acid residues in casein. There is thus no positive evidence for the utilisation of peptide material for protein synthesis in mammalian cells and it would appear safer to assume that proteins are synthesised without the participation of peptides.

Other Possible Intermediates in Protein Synthesis: The conclusion that peptides may not be intermediates in protein synthesis leaves us with the question of whether the biological fabrication of proteins from free amino acids, is, in fact, a one-stage process, or whether a search for alternative intermediates is indicated. The possibility of intermediates has been strengthened by some recent experiments performed by Hultin and his colleagues.

Hultin (1956) has obtained evidence that two stages are involved in the incorporation of ^{14}C-glycine and also of ^{14}C-leucine (Hultin and Beskow 1956) into proteins of a cell-free liver preparation containing microsomes. At different periods after addition of the labelled amino acid to a microsome preparation, a large excess of unlabelled glycine or leucine was added for the purpose of isotope dilution. If the dilution was made very early in the experiment, the subsequent incorporation was considerably decreased. As the time of addition was increased, less and less effect was noticed on the degree of incorporation and indeed, if the addition was made after fifteen to twenty minutes there was no appreciable effect at all on the incorporation as compared with the controls. This has led to the conclusion that, after the first fifteen to twenty minutes, amino acid for incorporation was no longer supplied from the general amino acid pool but rather from some other intermediate, presumably originally derived from the pool but no longer in equilibrium with it. That the microsomes had no part in theformation of this intermediate was shown by the fact that, if the addition of the microsomes was delayed until after twenty minutes, dilution with unlabelled amino acid as before had no effect on the subsequent amino acid incorporation. Thus some intermediate is formed in the cell sap which is subsequently utilised by the microsomes.

These observations of Hultin find a parallel in recent studies of enzymic activation of free amino acids. In cell-free systems, it is apparent that ATP is involved in amino acid incorporation into proteins. Thus Peterson and Greenberg (1952) found that ATP accelerated amino acid incorporation into protein _in vitro_ in an enzyme system that was composed of mitochondria plus a supernatant fraction of rat liver homogenate. Siekevitz (1952) had found evidence of a soluble co-factor produced by mitochondria which stimulated amino acid incorporation _in vitro_ into rat liver microsomes. He also observed some stimulation by ATP and concluded that ATP was involved in the formation of the soluble co-factor. Zamecnik and co-workers followed up the observation that the highest rate of incorporation of labelled amino acids was into the microsome fraction of liver (Hultin (1950), Borsook (1950)) by showing that this incorporation required ATP and a heat-labile non-dialysable constituant of the supernatant fraction of liver homogenate centrifugation at 100,000 g. Keller and Zamecnik (1956) have shown that there are five essential components in an incorporation system prepared from rat liver. These are (1) microsome fraction, (2) cell supernatant, (3) ATP and usually an ATP - regenerating system, (4) GTP or GDP, (5) the labelled amino acid. The omission of any one of these components causes cessation of incorporation. Littlefield and Keller (1956) have simplified the incorporation system by using cellular fractions prepared by 0.5M sodium chloride extraction and centrifugal fractionation of distilled water lysates of Ehrlich mouse ascites tumour cells. Here it is possible to obtain good incorporation into the ribonucleoprotein particulate fraction of the microsomes in the almost complete absence of the membranous lipoprotein fraction. The role of GTP or GDP is unknown. Several other dinucleotides of guanine were tried but none was found to be effective. (Keller and Zamecnik (1956)).

Part of the enzymic requirement of the incorporation system was accounted for by enzymes which would generate ATP from a precursor such as phosphocreatine, phosphopyruvate or phosphoglycerate. It was apparent however, that after fortification of the incorporating system with the precursors and the appropriate ATP generating enzymes, heat-labile, non-dialysable components of the soluble fraction were still required.

Hoagland (1955) investigated the soluble protein fraction and found that after dialysis the soluble protein fraction catalysed an exchange of pyrophosphate (PP^{32}) with ATP which was enhanced several fold by the addition of pure L-amino acids. This suggested that the amino acids were being activated as an amino-acyl AMP compound. On the addition of hydroxylamine, the carboxyl-activated amino acid reacts to form the hydroxymate while AMP and pyrophosphate accumulate. The reaction may be formulated :-

$$E + ATP + HOOC\text{-}CHRNH_2 \longrightarrow E\text{-}AMP\text{-}CO\text{-}CHRNH_2 + PP$$

$$E\text{-}AMP\text{-}CO\text{-}CHRNH_2 + NH_2OH \longrightarrow E + HONH\text{-}CO\text{-}CHRNH_2$$

It was found that D-amino acids were inert. A fraction was obtained from the soluble protein fraction by precipitation at pH 5. This precipitate was dissolved in buffer pH 7.6 and was found to be about five times as active as the original soluble protein fraction. (Hoagland, Keller and Zamecnik, (1956)). To determine whether separate enzymes mediate the activation of individual amino acids, several iso-electrically precipitable fractions were tested for their activity towards a representative group of amino acids. In this way separate activating enzymes have been prepared for leucine and alanine. On fractionation with ammonium sulphate a methionine activating enzyme was obtained.

Cole, Coote and Work (1957) have confirmed Hoagland's findings and have examined the distribution of this activating enzyme in several mammalian tissues. They found that the enzyme was widely distributed in guinea pig tissue and that the highest concentration was in the pancreas. These workers have prepared the pH 5 enzyme from other tissues, e.g. bovine and pig pancreas and have attempted to fractionate it. Their work indicates that there is a different distribution of enzyme activity towards different amino acids and this difference is characteristic of the type of tissue from which the enzyme was prepared. A similar enzyme preparation capable of activating certain amino acids has been reported by Demoss and Novelli (1956) who were studying micro-organisms. Borsook (1955) has described an activating enzyme system which he obtained from the supernatant, after centrifugation at 100,000 g, of lysed rabbit reticulocytes.

These findings would suggest that, before being incorporated into proteins, the amino acids may be activated by the system described by Hoagland.

Holley (1957) suggested that the amino-acyl compound could undergo a reaction with another substance (X) with the production of an amino acid-X compound and the liberation of AMP and the free enzyme.

$$\text{Enzyme} + \text{AA} + \text{ATP} \rightleftharpoons \text{Enzyme} - \text{AA} - \text{AMP} + \text{PP}$$

$$\text{Enzyme} - \text{AA} - \text{AMP} + \text{X} \underset{\text{Enzymes}}{\overset{\text{Cofactors}}{\rightleftharpoons}} \text{Enzyme} + \text{AAX} + \text{AMP}$$

The method employed to determine whether, in fact, this reaction was occurring was to assume that the reaction was reversible and that if

AMP were added ATP would be formed. Holley was able to show that, on the addition of labelled AMP, radio active ATP was produced. He used the pH 5 enzyme system of Hoagland and found that, with a mixture of fifteen amino acids, he obtained a good yield of ATP from added AMP. He found that this production was prevented by the presence of ribonuclease. On further examination he showed that the activity of the amino acid mixture resided with L-alanine. This may imply that there is more than one compound X, i.e. one compound for each amino acid, and that in the course of the preparation of the system only the X-compound appropriate for L-alanine is not destroyed.

It would therefore appear that the activated amino acids undergo a further reaction with unknown compounds and that this reaction is ribonuclease sensitive, implying that RNA plays some part at this stage.

Mechanism of Amino Acid Assembly in Predetermined Sequences.

Having considered the energy requirements and the nature of the building blocks used in protein synthesis, we are now in a position to view the theories which have been proposed to account for the final assembly of amino acid residues in the specific sequences peculiar to each type of protein.

There are two main theories of protein synthesis which must be discussed:

(a) Transpeptidation theory.

(b) Template theory.

(a) Transpeptidation theory: This theory arose out of the work of Bergmann and his school on the synthetic capacities of proteolytic enzymes. Bergmann (1937) showed that intracellular proteolytic enzymes could bring about the condensation of acetyl, benzoyl or carbobenzoxy derivatives of a number of amino acids with aniline or phenylhydrazine. These reactions represented the synthesis of peptide bonds. In the same way chymotrypsin would catalyse the condensation of benzoyl tyrosine and glycine anilide to form benzoyl-tyrosyl-glycine anilide in 65% yield. (Bergmann and Fruton (1944)). This reaction may be formulated as follows:-

Bz-Tyr + Gly-NHPh ⟶ Bz-Tyr-gly-NHPh

This reaction takes place on account of the insolubility of the product and whether this insolubility factor is of importance in animal tissues is not known. There is, moreover, no evidence for occurrence of such substituted amino acids in living tissues and the use of more physiological compounds e.g. acetylated or phosphorylated amino acids has not been profitable. Thus Cohen and McGilvrey (1947) were unable to synthesise p-amino hippuric acid from p-amino benzoic and N-acetyl glycine in an enzyme system capable of effecting the synthesis with free glycine. Borsook (1953a) found that N-phosphorylated glycine and benzoyl phosphate were no more active in the synthesis of hippuric acid than glycine and benzoic acid although ATP is known to promote the synthesis under anaerobic conditions.

During the course of these studies evidence was found suggesting the occurrence of exchange reactions during hydrolysis of amide and peptide bonds. Bergman and Fraenkel-Conrat (1937) studied the reaction:-

$$Bz.gly{-}NH_2 \quad + \quad PhNH_2 \xrightarrow{papain} Bz.gly{-}NH{-}Ph \quad + \quad NH_3$$

i.e. the hydrolysis of hippurylamide by papain in the presence of aniline, and found that considerably more hippuric acid anilide was formed than could be accounted for under the circumstances by direct synthesis from hydrolysis products. This picture was firmly established by the advent of isotopic techniques when Johnston, Mycek and Fruton (1950[a]) demonstrated the replacement of amide N by isotopic N during the hydrolysis of benzoyl glycinamide by papain in the presence of N^{15} ammonium salts. Fruton, Johnston and Fried (1951) showed that lengthening of a peptide chain can be achieved by proteolytic enzymes under certain conditions. Thus if cathepsin or papain catalysed hydrolysis of benzoyl tyrosinamide in the presence of glycinamide is stopped short of the equilibrium point benzoyl-tyrosyl-glycinamide can be isolated. This reaction can be formulated :-

$$Bz{-}tyr{-}NH_2 \quad + \quad gly{-}NH_2 \longrightarrow Bz{-}tyr{-}glyNH_2 \quad + \quad NH_3$$

These reactions are all examples of "transamidation".

In the same way "transpeptidation" reactions occur in which replacement of one of the components of a peptide bond linking two amino acid residues takes place, e.g. benzoyl tyrosyl glycinamide incubated with chymotrypsin and labelled glycinamide results in the label appearing in the dipeptide (Johnston, Myeck and Fruton (1950b)). This reaction may be written :-

$$\text{Bz-tyr-glyNH}_2 \quad + \quad \text{gly}^*\text{NH}_2 \longrightarrow \text{Bz-tyr-gly}^*\text{NH}_2 \quad + \quad \text{glyNH}_2$$

Fruton (1952) demonstrated that glycyl-glycine can be substituted for the terminal residue of carbobenzoxy-glycyl-phenylalanine under the influence of papain, viz :-

$$\text{gly-gly} \quad + \quad \text{cbz-gly-phe} \xrightarrow{\text{papain}} \text{cbz-gly-gly-gly} \quad + \quad \text{phe}$$

These reactions will occur provided the necessary energy, in the form of a preformed amide or peptide bond, is available.

These findings have been made the basis of the transpeptidation theory as propounded by Fruton. He postulates that peptides or amides can serve in protein synthesis by donating amino acids to acceptors as yet unidentified. These reactions would be catalysed by the intracellular proteolytic enzymes which have been shown to be capable of either performing hydrolytic and condensation reactions or replacement reactions (transamidation or transpeptidation). The extent to which hydrolysis or replacement occurs depends on the pH of the reaction mixture and at physiological pH values replacement reactions may predominate.

However, the transpeptidation theory has some drawbacks. For a transpeptidation mechanism to be responsible for the entire synthesis of proteins would require the presence of simple peptides in cells. No substantial evidence for the occurrence of these peptides in cells is at hand to date. Furthermore, although the enzymes studied show specificity towards the acceptor molecule, e.g. a peptide, they exhibit little or no specificity for the amino acid or peptide transferred. However, the possibility that transpeptidation reactions may be involved in the final stages of protein synthesis from polypeptides should not be overlooked, and in such cases enzyme specificity may well be greater.

(b) Template Theory: Other investigators have proposed model systems based on the idea that precursors are organised into protein molecules on a template. In most of the theories the template is formed from ribonucleic acid (RNA) or ribonucleo-protein. There is abundant evidence in the literature to connect RNA with protein synthesis. A recent excellent review of this field is given by Brachet (1955). Most of the evidence is circumstantial in nature, but recent study on cell-free systems for protein synthesis have provided more direct evidence of RNA participation. Thus Gale and Folkes (1954) found that protein synthesis in the particles of a cell-free preparation of Staphylococcus aureus was abolished by treatment with ribonuclease. Furthermore, addition of either RNA or DNA prepared from the fragmented Staphylococcus aureus augmented the protein synthesis. These findings have been confirmed by Hunter and Butler (1956) working on Bacillus megatherium. Zamecnik and Keller (1954) have found that the addition of ribonuclease prevented the uptake of radio-active amino acids into the microsomes of a cell-free system prepared from rat liver. Beljanski (1954) in working with lysed Micrococcus lysodeikticus cells found that the incorporation of radio-active glycine was inhibited by treatment with ribonuclease. A role for DNA in protein synthesis has been proposed by Allfrey (1954). Calf thymus nuclei isolated in sucrose solution were found to incorporate labelled alanine into nuclear proteins in the presence of an energy source. Treatment with ribonuclease did not alter the incorporation whereas treatment with deoxyribonuclease nearly abolished incorporation entirely. It would appear that, in the nucleus, DNA participates in protein synthesis.

Dounce (1952) envisages the nucleic acid molecules as forming a master template which can either reproduce itself or produce a specific arrangement of amino acids. Dounce postulated that ATP contributes the necessary energy by means of a phosphotransferase which transfers its terminal phosphate to the phosphate of nucleic acid, the net result being the transfer of a pyrophosphate linkage from ATP to nucleic acid. The amino groups of amino acids then react to form amino-phosphate compounds on the nucleic acid, and for each amino group so combined, the phosphate which came originally from the ATP is displaced and appears as inorganic phosphate. Another enzyme links the free carboxyl group of the adjacent amino acid to the phosphate bound amino group to form a peptide linkage. The peptide so formed then leaves the template and the phosphate of the nucleic acid is then free to repeat the whole process of synthesis. One class of enzymes (P_1) mediates the attachment of the amino acid to the nucleic acid; another (P_2) effects the formation of the peptide bonds and concomitant liberation of the peptide chain from the template.

This theory has undergone modification in view of the carboxyl activation of amino acids described by Hoagland (1955) and discussed above. Thus, Borsook (1955) suggested that the amino acids are attached to the phosphates of nucleic acids by their carboxyl groups. Then, as before in Dounce's scheme, another enzyme forms the peptide bond, thereby removing the amino acids involved from the nucleic acid template. Borsook's scheme calls for some mechanism of transporting the activated amino acid to the template. For this role he suggests either the activating enzyme itself or a co-enzyme or nucleotide. On the other hand Zamecnik, Keller, Hoagland, Littlefield and Loftfield (1956) do not think that there is any chemical attachment between the amino acid and the nucleic acid template. They suggest that the activated amino acyl nucleotide compounds line up along a ribonucleoprotein template with their side chain R groups determining the sequence by their ability to fit into particular spaces occurring on the ribonucleoprotein surface. Peptide bond formation takes place between adjacent amino acid residues and the ribonucleoprotein passes on the protein or large peptide chain to other parts of the cell, particularly the membranous, lipid-rich, deoxycholate-soluble portion of the microsome fraction for transformation into a completed protein molecule or lipo protein complex.

There is as yet no positive proof that nucleic acids can act as templates in the manner invisaged by the various authors mentioned above though the participation of RNA in the early formative stage of protein synthesis is becoming much more certain. The exact mode of this participation is as yet not understood.

It would be unwise at this point in our knowledge of protein synthesis to imagine that the template theory and the transpeptidation theory are mutually exclusive. It may well be that the true picture of protein synthesis lies somewhere in the combination of these two theories.

Incorporation without net protein synthesis (" exchange reaction ").

Most of the studies on protein synthesis have employed labelled amino acids. The criterion of protein synthesis has been the incorporation of the labelled amino acid into the protein and this has been demonstrated by many workers (see review by Borsook (1955)[b] and Gale (1955)). In the majority of cases, it has been shown conclusively that the labelled amino acid has become incorporated into protein, the radio-activity of the protein not diminishing with prolonged and repeated washings with hot and cold TCA, or with solution and reprecipitation of the protein. On the other hand, in many of the experiments in which incorporation of labelled amino acids has been established, no actual net protein synthesis has been demonstrated. It is therefore, relevant at this point to consider whether incorporation of labelled amino acids is synonymous with protein synthesis.

Gale and Folkes (1955a), working with cell-free preparations of Staphylococcus aureus, have shown that in the presence of a complete amino acid mixture (Condition 1) incorporation of ^{14}C-glutamic acid proceeds linearly for some hours and is accompanied by a significant increase in protein nitrogen. On the other hand, if only one amino acid is present (Condition 2), the incorporation ceases when only a fraction of the added amino acid has been incorporated and no net synthesis of protein can be demonstrated. Moreover, if cell-free preparations of Staphylococcus aureus, rendered radio-active by previous incorporation of ^{14}C-glutamic acid, are incubated with ^{12}C-glutamic acid and an energy source there is a loss of radio-activity from the preparation due to an exchange reaction.

From this evidence, it would appear that the incorporation under Condition 2 takes place as a result of an exchange reaction between the amino acid added to the medium and corresponding residues in certain of the proteins present in the preparation. It may be that such exchange incorporation is an activity of a part or parts of the protein synthesising mechanism and that this activity can occur when total protein synthesis is not possible.

In the case of protein synthesis a labelled amino acid can thus become truly incorporated into a protein by two main methods:-
(1) exchange reactions (2) de novo protein synthesis. These two methods may well operate at one and the same time, but under special conditions, e.g. condition 2 the exchange rate predominates and no net protein synthesis occurs. It is therefore desirable when studying

protein synthesis to be able to demonstrate an actual net increase in protein content. One of the most sensitive methods of demonstrating net protein increase is to measure the amount of an enzyme present before and after incubation. Increases will be detected by this means which would not be revealed by chemical methods. Chemical methods can only give an indication of overall protein increase, whereas, enzyme assay gives information that a specific protein has been synthesised and an attempt can be made to isolate a pure product. This principle has, in fact, been applied by Gale and others to the study of protein synthesis in bacteria.

In the case of higher animals the study of enzymes also offers a means of following the course of protein synthesis and can be used as an ancillary aid in the interpretation of labelled amino acid incorporation data. However, the use of enzyme synthesis as a tool demands some cell-type in which enzyme turnover is rapid. Such a situation obtains in the digestive glands, e.g. the pancreas. We have therefore attempted an integrated study of the process of protein synthesis as it occurs in the pancreas, using not only isotopic and enzymic technique, but also corellating these with chemical changes and electron microscopy.

We selected the pancreas because the exocrine part is highly specialised for the synthesis and secretion of well known enzymes and a number of studies have already been made on it. The organ can be depleted of its enzyme content at will by the injection of pilocarpine or carbamylcholine after which intense protein synthesis must take place in order to restore its enzymic content within three to five hours.

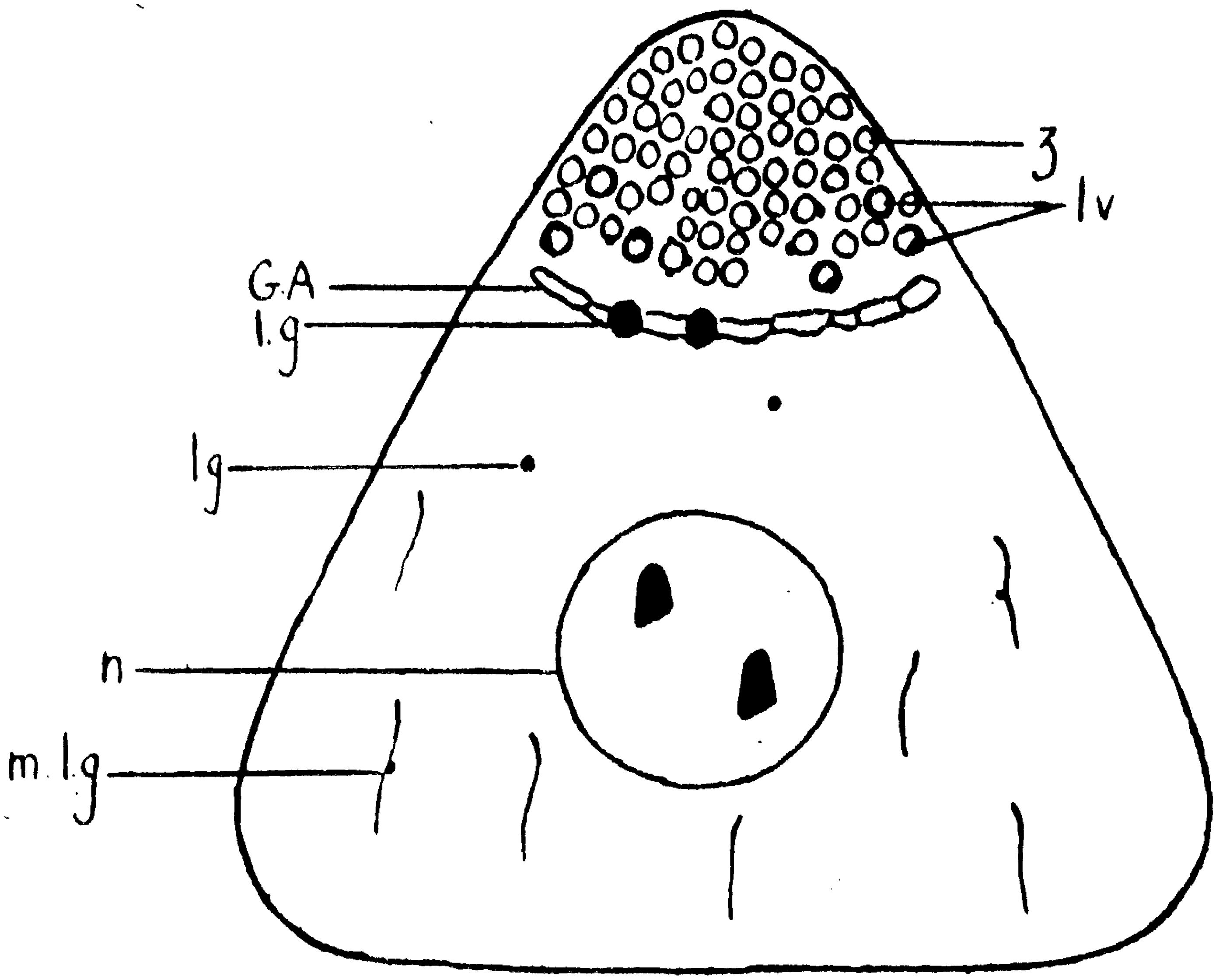


Diagram of Exocrine Pancreatic Cell.

GA = Golgi apparatus
lg = developing granule
mlg = mitochondrial origin of granule
n = nucleus
z = zymogen granule
lv = vacuolated granule

Pigeon pancreas was selected because here the organ is well defined and is relatively homogeneous. It contains very little adipose tissue and the consistency of the organ is very suitable for slicing techniques.

THE PANCREAS.

Histologically, the pancreas consists of long tubular secreting alveoli. The terminal alveoli consist of granular, somewhat conical shaped cells lining the lumen. In the apical portion of the cells are located the secretory granules. (Plate 1). These granules which contain the digestive enzymes, such as, amylase and lipase, are discharged into the alveolar lumen on receipt of a suitable stimulus, such as food. After the discharge phase, the cell manufactures the granules which slowly accumulate in the apical region. There is thus a "secretory cycle" in the pancreas during which the granularity varies with the state of functional activity. Daly and Mirsky (1952) studied the changes in enzyme activity in mouse pancreas during the cycle of secretion and synthesis. They examined protease, amylase, lipase and, in one case, carboxypeptidase, and found that the cyclical variations came at about the same time for the different enzymes. They found that the minimal enzymic activity after pilocarpine stimulation occurred one and a half hours after the stimulus, whereas, with food, the minimal enzyme activity was found after three to five hours. The loss of enzyme protein can be very large; in one case, ninety per cent of the total pancreatic enzymes were lost in the course of secretion. Fernandes and Junqueira (1955) conducted biopsy experiments on pigeons, in which they took samples of the pancreas over a period of seven hours after carbamylcholine stimulation. They found that the minimum enzyme level (60%) occurred one hour after stimulation. After four hours the enzyme level was actually above the starting value. Dineen and Robertson (1955), who were studying mouse pancreas used pilocarpine as the stimulant and found that the minimum enzyme level was at one hour. They also noted that after seven hours the enzyme level exceeded the starting value.

The chemical evidence for a substantial loss in enzyme protein as a result of secretion parallels the original microscopic observations of Heidenhain (1875) and later workers. They observed that the zymogen granules formed a considerable part of the resting cell and that almost all these granules are extruded during secretion. Even in the resting pancreas, however, (i.e. the pancreas of a fasting animal), a slow steady secretion goes on, the amount of which varies in different animals. In the rabbit, for which precise measurements are available, the total enzyme content of the constant secretion of the resting pancreas is about one third that of the actively secreting gland (Baxter, (1931)).

Analysis of cell fractions obtained from pancreas: Since enzyme activity seems to go along with the presence of granules in the pancreas, it is reasonable to ask whether the enzyme content of the gland is confined to the granules. This is best explored by differential centrifugation.

Differential centrifugation of cells has been extensively applied to the biochemical study of sub-cellular structures, and in the case of the liver, has yielded a vast amount of information. Studies on the pancreas are fewer however.

Khesin (1953) fractionated pancreatic tissue, homogonised in 0.25 M sucrose, by differential centrifugation and found considerable variation from experiment to experiment. His results were : - Nuclei 4 - 5.5%, Granules and Mitochondria 27 - 56%, Microsomes 15 - 5.5% and Cell Sap 53 - 33%. These results are expressed as a per centage of the total activity found in the whole-cell homogenate.

Hokin (1955) has carried out a slightly more elaborate fractionation of dog pancreas in 0.25 M sucrose. The results he obtained for amylase distribution are as follows : - Nuclei 1390, Granules 1730, Mitochondria 520, Microsomes 23, Cell Sap 1060. The results are expressed as Smith and Roe units per mg. N.

It is thus apparent that the amylase content of the pancreatic gland is not confined to the granules. It may be wondered whether the high cell sap enzyme content arises from granules ruptured in the course of differential centrifugation. This point will be considered when we present our own data. In this connection, it is pertinent to quote a short communication by Siebert (1955a), in which he studied the distribution of enzymes in the nucleus, mitochondria and microsomes + cell sap in several species. The trypsin and cathepsin were especially concentrated in the nucleus, amylase was uniformly distributed, whereas lipase and esterase were more concentrated in the mitochondria.

Although Siebert's data require to be examined when they eventually appear in detail, it would seem that the distribution of amylase in the cell sap is not paralleled by other enzymes.

Changes in Cell Components during the cycle of secretion and restitution.

Since there is a great loss of enzyme proteins during the discharge phase and this loss is made good within a few hours, considerable protein synthesis must take place in the recovery phase of the cycle. It would be of value to examine the pancreatic cells during this time for changes in composition which occur as a result of such protein synthesis. Quantitative changes in two components, protein and nucleic acids, have received attention in this way.

(a) Protein: Daly and Mirsky (1952) found that there was no measurable change in the total content of pancreatic protein during secretion and synthesis. There are, however, considerable changes in the content of the enzyme proteins amounting to some twenty percent loss, followed by restitution. From these data these authors conclude that, as rapidly as the enzymes are extruded, other protein is formed in the gland relatively rapidly and that this protein is gradually transformed into the enzymes destined for secretion. The absence of change in protein content during secretion was inferred from difference by adding up the non-protein constituents of the gland and subtracting from the total weight. This seems an amazingly indirect approach to a measurement of protein content and certainly does not justify the elaborate hypothesis which Daly and Mirsky build on it.

On the other hand, Farber and Sidransky (1956a) studied the effect of ethionine upon protein metabolism in the rat pancreas and found that the protein content paralleled the amylase level and the level of total enzyme activities. They extended these studies (Farber et al (1956b)) and found that, after stimulation with carbamylcholine or pilocarpine, there was a decrease in pancreas weight, protein nitrogen, and enzyme activity during depletion. The total proteolytic activities closely followed the amylase activities. They also found, in common with many other workers in this field, that the response to stimulation was quite variable from animal to animal.

(b) Nucleic acids: Brachet and Caspersson, (1941), in their original observations, correlated a high RNA content with active protein synthesis. Such an association exists in the pancreas and Caspersson (1941) using microspectrophotometric methods reported changes in the RNA content during the secretion cycle. There appeared to be an increase during the synthetic phase and a loss at secretion. However, these results were shown to be in error when more accurate spectrophotometric methods became available. Several workers, using purely chemical methods, have shown that there is no apparent increase in RNA content of the pancreas during the secretion cycle. (Rabinovitch, Valeri, Rothschild, Camara, Sesso and Junqueira (1952); Daly and Mirsky (1952); Deken-Grenson (1952) and Langer and Grassi (1955)). In agreement with this, Daly and Mirsky (1952) examined the pancreatic juice from a dog injected with pilocarpine and could not detect the presence of any nucleic acid.

Rabinovitch et al (1952) and Daly and Mirsky (1952) studied the DNA content of the pancreas during the secretion cycle. They could detect no change and it would therefore appear that no cell division is taking place during the cycle of secretion. Freed (1955), working with tadpole pancreas, was able to show by cytochemical studies that the nucleic acid content did not vary during the secretory cycle.

On the other hand, Gubernicw and Kovyrev (1949) who were studying the nucleic acid content of the dog pancreas after stimulation with secretin reported an increase of both DNA and RNA after three to four hours.

The majority of the evidence would indicate that there is no change in either RNA or DNA content of the pancreas during the secretion cycle. It does, however, confirm the general relationship between the RNA content of an organ and the intensity of protein synthesis in it.

Studies of protein synthesis by the pancreas:

Two approaches have been made in these studies. A number of investigators have employed labelled molecules. In addition a few have examined the formation of enzymes in isolated fragments of pancreas.

(a) Isotopic Studies: There is evidence that the proteins of the pancreas readily incorporate labelled molecules. Thus Allfrey, Daly and Mirsky (1953) found that the uptake of ^{15}N-glycine by the proteins of the pancreas was about twice that occurring in the liver and about four times that occurring in the kidney.

Incorporation into various parts of the pancreas cell has also been examined. Thus, Allfrey et al (1955) measured the uptake of ^{15}N-glycine by the mixed protein of the whole tissue, the microsome protein and the cell supernatant protein (after 105,000g). They found, when they compared the time-incorporation curves of the microsome protein and mixed tissue protein, that the ^{15}N- uptake by the microsome protein was higher than that of the mixed tissue protein initially and that the curves subsequently crossed. They concluded that the microsome protein probably lies on the pathway of synthesis of the general tissue protein. More recently (Daly, Allfrey and Mirsky (1955)), it has been found that the most active protein that could be obtained under these circumstances was trypsinogen and chymotrypsinogen. The uptake into these proteins was greater than that into the nucleoprotein fraction. The authors concluded that the nucleoprotein could not give rise to these enzymes. It may be commented that the determination of isotopic incorporation was made by digesting the whole protein fraction by the Kjeldahl method and the subsequent liberation of gaseous nitrogen by hypobromite. This means that all the amino acids of the protein are involved and that no consideration is given to the fact that different proteins contain different amounts of glycine, and, therefore, incorporation into mixed proteins may give rise to erroneous results, due to a variable degree of dilution of glycine by other amino acids.

The effect of stimulation on labelling of pancreas protein has also been examined. Allfrey et al (1953) studied the effect of feeding on the incorporation of ^{15}N into mixed tissue proteins of the pancreas. Mice received injections of ^{15}N-glycine every one and a half hours for eight hours. One group was fed just before the experiment began and received no more food, so that in these animals the enzyme content of the pancreas gradually increased during the period of the experiment. A second group was fed continuously throughout the experiment, providing several cycles of pancreatic synthesis and secretion. The mixed tissue proteins were examined for their ^{15}N contents two hours after the final injection of glycine. The liver proteins from the two groups showed no difference in ^{15}N content, but there was a great difference in the mixed proteins of the pancreas. There was greater incorporation of ^{15}N into the pancreatic proteins of those animals fed before the experiment. This would show that more protein was passing through the glands of the continually fed mice and therefore more synthesis occurred. Since the duration of the experiments was the same, the rate of synthesis must be accelerated by secretion. The validity of this conclusion depends on the assumption that the curve of the specific activity of free glycine is the same for fed and fasted animals. This will be commented on when we present our own data.

Allfrey et al (1953) also showed that secretion had a greater stimulant effect on the incorporation of ^{15}N-glycine into the microsome protein, but that the incorporation of ^{15}N-glycine into the RNA purines was unaffected by such stimulation. On the other hand, Fernandes and Junqueira (1955) used ^{14}C-glycine to study incorporation into protein and into RNA at the same time. They found that, after stimulation by carbamylcholine, there was an increased incorporation of labelled glycine into protein and at the same time a parallel increase in incorporation into RNA. The explanation may lie in the possible contamination of the RNA with highly active protein. The method of preparation of RNA described by these workers would support this view.

The evidence provided by the use of ^{32}P to measure RNA turnover has also proved to be conflicting. Thus, Gubernkev and Il'lina (1950) have reported an increase of ^{32}P uptake by RNA of many tissues after stimulation with secretin. The increases they obtained were 1200% by pancreas, 400% by the parotid, and 500% by the liver. It is possible that the results obtained were due to the presence of contaminating substances, such as inorganic phosphate, in their RNA preparation, which under the experimental conditions would have a very high radio-activity. However, De Deken-Grenson (1953) working with mouse pancreas, and using better analytical methods, was unable to show any increase in ^{32}P uptake by RNA after stimulation by pilocarpine.

It would appear that the lack of increased incorporation of ^{14}C-glycine or ^{32}P into RNA would agree with the quantitative data that there is no change in RNA during the secretory cycle. This aspect of pancreatic metabolism will receive further consideration in the light of our own data.

Junqueira, Hirsch and Rothschild (1955) have studied the uptake of ^{14}C-glycine by the proteins of rat pancreatic juice. They found that in rats continuously stimulated by intravenous secretin that ^{14}C-glycine does not appear in the pancreatic juice until ninety minutes after injection. On the injection of previously labelled plasma proteins they found that no radio-activity appeared in the pancreatic juice, thus showing that plasma proteins cannot serve as the precursors of pancreatic enzyme protein and that no plasma proteins are excreted as such along with the pancreatic juice.

From these various studies on isotope uptake by pancreatic tissue and cell fractions it is apparent that the incorporation rate is high and, as in other tissues (Hultin (1950)), the microsome fraction is highest at early time intervals after administration of the labelled compounds. This uptake is enhanced by stimuli causing dischange of secretion. Parallel changes in RNA metabolism following discharge are still disputed, and the reliability of the evidence favouring changes in RNA metabolism with secretion can be called in question.

(b) Enzyme Synthesis by Pancreas Slices and Homogenates: Hokin (1951a) demonstrated that pigeon pancreas slices can synthesise amylase on incubation with glucose and a complete mixture of amino acids. Later

Hokin (1951b) modified the amino acid requirement to the nine essential amino acids present in amylase plus tyrosine. During the synthesis the uptake of oxygen was increased as compared with a non-synthesising slice. The value of certain keto-acids (corresponding to certain essential amino acids) was also demonstrated. These studies in amylase synthesis were extended when Schucher and Hokin (1954) showed that other enzymes, ribonuclease and lipase, were also synthesised by pancreas slices.

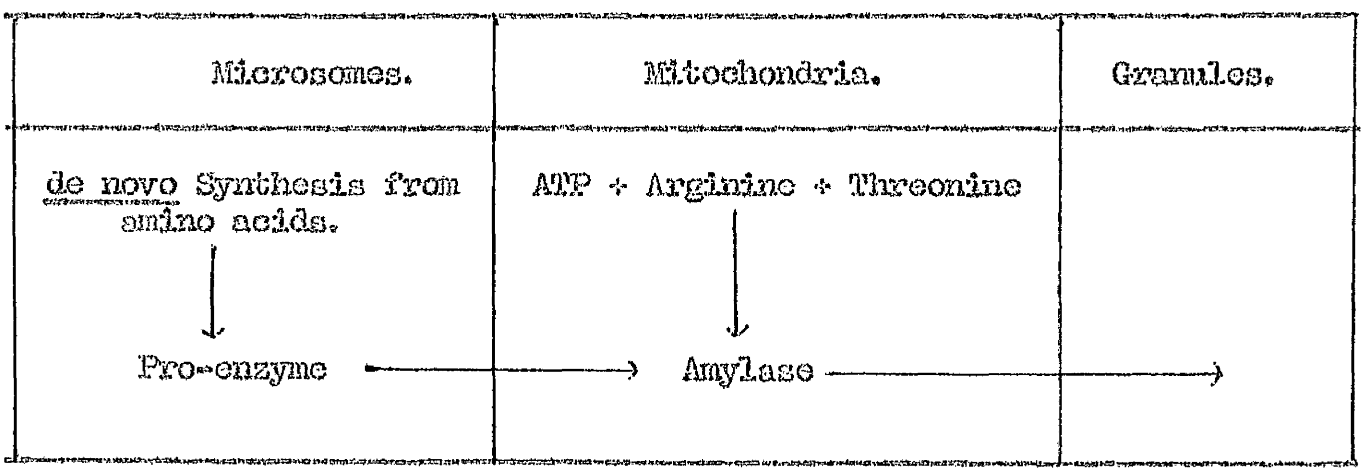
M.R.Hokin (1956) demonstrated that in mouse pancreas "slices" synthesis of amylase took place under aerobic conditions. The addition of amino acids to slices of pancreas from fed mice did not augment the production of amylase as had been demonstrated in the case of pigeon tissue (Hokin, (1951a)). However, if the mouse had been fasted for twenty four hours, then the addition of amino acids to the incubation mixture augmented the production of amylase.

Younathan and Frieden (1956) have confirmed Hokin's work on pigeon pancreas slices and have investigated the effect of energy sources other than glucose. Fructose 1:6-diphosphate and ATP were found to be effective but glucose itself was superior. Several amino acid analogues were tried and were found to be inhibitory to the increase in amylase found on incubation. The effect of various hormones, antibiotics and a mixture of purines and pyrimidines had no effect on the course of the synthesis. Straub (1957) has reported that with pancreatic slices he can obtain similar results to those described by Hokin though perhaps his increase in the presence of amino acids is not so large. At the same time he finds that radio-active tyrosine and radio-active glycine are incorporated into the proteins of the tissue. When, however, he includes in his incubation medium an inhibitor such as flurophenylalanine, he finds that, while the uptake of labelled amino acids is reduced, the production of amylase by the tissue slices is inhibited much less. This would suggest that the production of amylase in tissue slices is not entirely a _de novo_ synthesis from amino acids, but perhaps also entails the production of amylase molecules from polypeptide intermediates. Fernandes and Junqueira (1955) were, however, unable to obtain any increase in amylase content on incubation of pigeon pancreas slices in the presence of complete amino acid mixtures. In an attempt to justify their data, they point out that the rate of synthesis obtained in tissue slices by Hokin far exceeds the capacity of the pancreas to synthesise amylase _in vivo._ In their biopsy experiments on the same pigeon pancreas, they found that, following depletion, amylase restoration was far less than the factor of three times the depleted level as found by Hokin in pancreatic slices. This would appear to suggest that _in vivo_ the rate of amylase synthesis is about one quarter of that found _in vitro._ It would appear from the figures presented in their paper that the initial value of the amylase level in their tissue slices is very high as compared to the initial value quoted by Hokin

It may be mentioned at this point that our own data, to be discussed later, indicates that Fernandes and Junqueira may well have failed to secure adequate expulsion of enzymes at the beginning of their experiment.

Hokin and Hokin (1954) studied the incorporation of ^{32}P into the nucleotides of RNA in pancreas slices during enzyme synthesis and secretion. They found that the incorporation of ^{32}P was totally inhibited under anaerobic conditions. On stimulation of enzyme synthesis by the addition of a complete amino acid mixture there is an increase in the specific activity of RNAP and also an increase in respiration. Omission of tryptophan abolished the stimulating effect of the amino acids on enzyme production, but had no effect on respiration or RNAP turnover. It would appear that the increased turnover in RNAP noted was not directly related to the increased synthesis of protein, but to some effect on energy metabolism.

The synthesis of amylase by cell-free homogenates has been claimed by Straub and his co-workers (1955). They have demonstrated that, in a homogenate of pigeon pancreas or a "Mitochondrial" fraction of this homogenate, an increase in amylase content results on incubation with a complete amino acid mixture and a high concentration of ATP. He, however, points out that, while this is a "homogenate", there are still structures present in which permeability factors still operate. Consequently, he prepared a water extract of acetone-dried pancreas and demonstrated that this preparation could still synthesise amylase when incubated with ATP and amino acids. He found that the synthesis of amylase was inhibited by either Chloramphenicol or Fluorophenylalanine. Ribonuclease also appeared to interfere with this synthesis. In a later paper, Straub and his co-workers (1957c) have further investigated this system by using radio-active amino acids, and find in the homogenate and cell-free system that the uptake of radio-active amino acids and amylase synthesis do not occur in parallel. They showed that, for the synthesis of amylase, only two amino acids are required, viz. arginine and threonine. These amino acids are presumably required for the completion of the amylase molecule from precursors of a polypeptide nature. Since synthesis occurs in the "mitochondrial" preparation, he suggests that the union of the polypeptides with arginine and threonine occurs in this part of the cell. His scheme of synthesis is shown diagramatically thus:-



From the data on pancreatic slices it is apparent that enzyme formation *in vitro* can be demonstrated. The claim that it can occur in cell-free systems has also been made, and this will be evaluated in a later section of this thesis.

From this survey of the literature on enzyme formation by the pancreas, there are still a number of fundamental points in need of elucidation and there are several features in dispute by different investigators. In order to use pancreatic enzyme synthesis to throw light on the mechanism of protein synthesis, it has therefore been necessary to attempt a complete biochemical examination of the changes occurring in the process of enzyme formation by this gland. The data have been assembled in the following sections, which are finally brought together in a general discussion.

1. The Composition of the Pancreas at rest and during secretion.

2. The Uptake of ^{14}C-2-glycine by the proteins of different cell fractions of Pigeon Pancreas.

3. The Metabolism of RNA during the secretory cycle.

4. *In vitro* Synthesis of Amylase.

5. The Location of Bound Amylase in the Pancreatic Cell.

6. The role of the Microsomes in Enzyme formation.

SECTION I.

The Composition of the Pancreas at rest and during secretion.

INTRODUCTION.

Studies on the composition of the pancreas reported in the literature are not very numerous and have already been enumerated in the introduction to this thesis. The results that have been obtained by other workers have tended to be contradictory or not very complete. In order to clarify the position, we undertook the analysis of the pancreas at rest and in active secretion. The previous state of knowledge may be briefly recapitulated.

Resting gland: Khesvin (1953) has fractionated normal pigeon pancreas and has found that the amylase distribution is very variable. He reports the distribution, as follows:- Nuclei etc. 4-3.5%, Granules and mitochondria 27 - 56%, Microsomes 15 - 3.3%, Cell Sap 53 - 33%. Hokin (1955) fractionated resting dog pancreas and found that the highest concentration of amylase per mg. of nitrogen was to be found in the granules. The microsomes were very low indeed; as discussed later, this may be due to the lack of accessibility of the amylase in the microsomes.

Secreting gland: Studies on the secreting gland are not particularly numerous either, and once again, considerable variation is encountered. It is generally agreed that about thirty to sixty minutes after stimulation, the enzyme content drops considerably. The magnitude of the drop varies widely and there may be a loss of more than half of the amylase content. (Daly and Mirsky (1952), Fernandes and Junqueira (1955).). The weight of the gland has been reported to drop after stimulation (Farber and Sidransky (1956b)), but the wide variation found in individual weights of pancreases tends to diminish the usefulness of such findings. The total protein content of the pancreas has been investigated by Farber et al who demonstrated that the total protein content fell during secretion. This finding does not agree with the conclusions of Daly and Mirsky (1952) who were unable to detect any change. Although the protein content of the whole gland has been investigated there is no information about its distribution in the various subcellular fractions.

To date, there do not appear to have been any studies of the changes in composition and enzyme content of different subcellular fractions during secretion and restitution. In view of the contradictory findings on the resting gland, and absence of data on the actively secreting gland, we carried out studies on the weight, total nitrogen, protein and amylase content of different fractions of the pigeon pancreas at rest and following stimulation.

EXPERIMENTAL.

Birds: In this series of experiments, analysis of whole pancreases and pancreatic cell fractions were made from pigeons which had undergone different treatments. All birds were fed *ad libitum* with corn and maize. Birds were selected at random and grouped as follows:-

(a) Untreated pigeons. - Normal pigeons which had received no special treatment.

(b) Depleted pigeons. - These birds were injected intramuscularly with 0.07 mg of carbamylcholine 45 minutes before sacrifice. The period of forty-five minutes was selected because, after this time interval the level of enzymes in the pancreas is at its lowest (Hokin (1952); Fernandes and Junqueira (1955)). Salivation and prostration occurred in these birds to a variable degree. Only in birds which had salivated, could it be safely assumed that the pancreas had discharged its enzyme content.

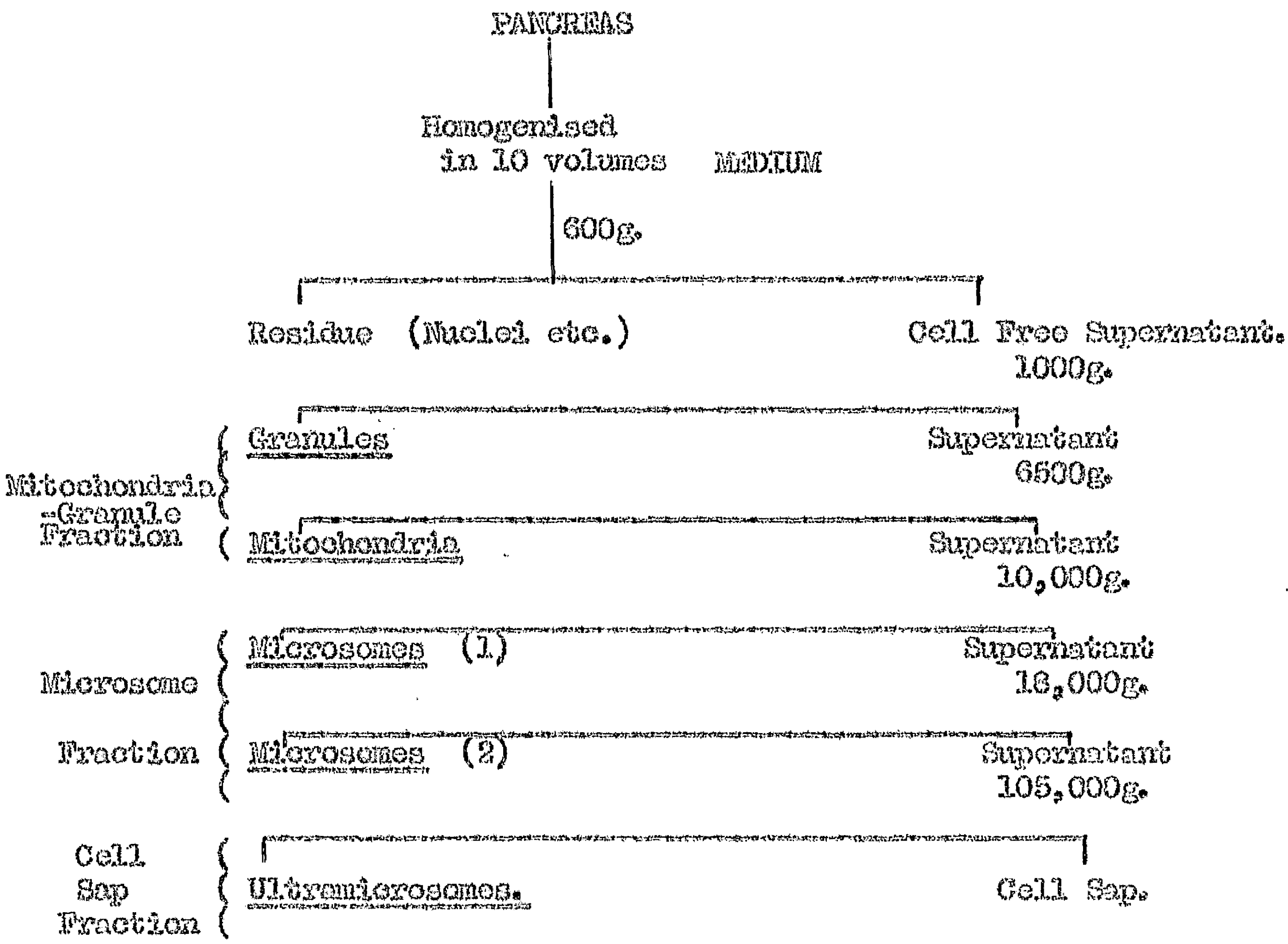
(c) Recovering Pigeons: - These birds were injected with carbamylcholine as before, but in this group there was a time interval of one hundred and sixty five minutes before sacrifice. This interval was chosen as representing the recovering pancreas on the basis of evidence provided by Fernandes and Junqueira (1955). In this group, the pancreas had been depleted but was now partially restored in its enzyme content.

The birds were killed by decapitation. The abdominal wall was cleared of feathers and opened with a post-sternal incision. The duodenum was located and the pancreas removed with scissors. In most experiments the pancreas was weighed immediately.

Preparation of Homogenate. - The pancreas was finely minced with scissors and transferred to a Potter-type perspex-glass homogeniser.

TABLE I.

Scheme of Cell Fractionation Carried out on Pigeon Pancreas Homogenate.



This represents the complete scheme. In certain cases modifications were made and certain fractions were not separated, being obtained with the next fraction. e.g. Granules and Mitochondria; Microsomes (1) and (2); Ultramicrosomes and Cell Sap.

The homogenising medium was either 0.25 M sucrose or 0.25 M sucrose in 0.2 M phosphate buffer, pH 7.2. The quantity of homogenising medium was 10 mls per gm. of pancreas. The homogenisation was carried out at 0°C.

Fractionation of the Homogenate: - This was carried out by differential centrifugation at 0° as outlined in the flow sheet (Table 1). Each fraction was re-homogenised in a known volume of distilled water and estimations carried out as described.

Estimation of Amylase: - The estimation of amylase was carried out by the method of Smith and Roe (1949), with the modification suggested by Hokin (1951b). It is not sufficient to estimate the amylase content of some subcellular fractions without further treatment, since not all of the amylase is in a free form. Some authors have used butanol to free the amylase from subcellular particles (Khessin (1953)) (Straub (1955)), but we have disintegrated particulate material with Ballotini beads for reasons given in a later section. The details of the actual amylase estimation are given in the appendix.

Estimation of Nitrogen: - The micro-Kjeldahl method was used. Full details will be found in the appendix.

Protein Nitrogen: - The nitrogen content of trichloroacetic acid (TCA) precipitable material was determined by the micro-Kjeldahl method.

TABLE 2.

Effect of different Homogenising Media on the distribution of amylase and nitrogen in different cell fractions.

FRACTIONS	AMYLASE		NITROGEN		AMYLASE/mg. TN.	
	Sucrose.	Sucrose-PO_4	Sucrose.	Sucrose-PO_4	Sucrose.	Sucrose-PO_4
	%	%	%	%	Smith & Roe Units.	
Mitochondria and granules	18.5	8.5	17.5	8.2	815	1260
Microsomes	11.6	11.3	37.8	22.0	237	690
Cell Sap	70.0	80.4	44.5	70.6	1205	1405

The data give the distribution of the amount of amylase and nitrogen recovered in each fraction as a percentage of the total amount in the whole homogenate.

RESULTS AND DISCUSSION.

The object of these experiments was to provide a more complete picture of the composition of the pancreas at rest and during enzyme secretion, and especially to evaluate the changes in different subcellular fractions. The first step was to study the effect of different homogenising media on the results obtained in different fractions of the cell. Following this, the chemical anatomy of the resting gland was investigated and finally, changes in composition during depletion and recovery were studied.

(a) The effect of different homogenising media on amylase and nitrogen distribution in the subcellular fractions. As mentioned in the section dealing with the preparation of the homogenates, two different homogenising media were used. 0.25 M sucrose is commonly used in cell fractionation studies. However, we were interested eventually in thepreparation of a cell-free system which would synthesise amylase and therefore, several possible homogenising media were considered. In order to eliminate the pH changes which are said to occur during the homogenising process (Siebert (1955b)), we tried 0.25 M sucrose buffered with 0.2 M phosphate buffer pH 7.2. The final molarity of this solution is 0.45 and this affected the cell fractionation procedure. Table 2 shows the results obtained with the two homogenising media, i.e. 0.25 M sucrose and 0.25 M sucrose buffered with 0.2 M phosphate. The results in each case are expressed as a percentage of the total content of the homogenate. The effect of the sucrose-PO_4 is to reduce the yield in the heavier fractions and to keep the material in suspension so that there is a higher proportion of constituents found in the cell sap. The alteration in composition of the cell fractions, due to different homogenising media, emphasises the need for conditions of cell fractionation to be closely defined. In a cell homogenate there exists a spectrum of particles of all sizes and the exact point of cut of the fractions is arbitrary. Nevertheless, certain well defined particles are known to exist, but the only certain way of preparing homogeneous fractions is to examine each fraction with the electron microscope. This is, as yet, not a practical proposition for our studies. We generally prefer the sucrose medium because when we studied labelled amino acid uptake into subcellular fractions we found that sucrose gave us results for the pancreas which were comparable to those found for other tissues. The use of sucrose-PO_4 led to some anomalous results with ^{14}C-glycine uptake. (Section 2).

(b) Distribution of constituents in the resting pancreas. The distribution of amylase in different subcellular fractions has been reported by Khesšin (1953) and Hokin (1955). Khessin made use of butanol to liberate the total enzyme content, but in our experience, this leads to partial inactivation of amylase (also found by Hokin(1955)). Hokin (1955) did not submit the subcellular fractions to any form of treatment, and this may explain his very low microsomal amylase content,

TABLE 3.

Fractionation of Pancreatic Tissue in 0.25M Sucrose.

Fraction.	Amylase Smith & Roe Units.	Total Nitrogen mgs.	Amylase per mg. TN.
Granules	1880 (6.5%)	1.06 (5.0%)	1,770
Mitochondria	2667 (9.5%)	1.98 (9.3%)	1,350
Microsomes	4558 (16.5%)	7.74 (36.6%)	595
Ultramicrosomes	389 (1.4%)	2.56 (12.0%)	152
Cell Sap	18,200 (66%)	7.80 (37.0%)	2,340

The data were obtained in two independent experiments, in each of which two pigeons were sacrificed. The birds were untreated. In each fraction the data for the amylase and total nitrogen content are expressed as the total amount per gland. The percentage of the total pancreatic content represented by each fraction is given in parenthesis.

since we have found the amount of non-available (bound) amylase to be highest in this fraction. Our data on amylase and total nitrogen distribution are given in Table 3, for a rather wider range of particles than either of these authors presents. Furthermore, the amylase content is probably an accurate estimate of the total enzyme content in each fraction, since Ballotini beads were used to effect its complete liberation. (See later Section 4). The results are expressed as the total content of enzyme and nitrogen in the individual fractions of the gland. It will be noted that the highest quantity of amylase is found in the cell sap. The microsomes are next, containing more amylase than the mitochondria or granules. The ultramicrosomes are very poor in amylase. The distribution of nitrogen shows that the granules contain, only, 5% of the total nitrogen. When the concentration of amylase per mg of nitrogen is calculated it is seen that the cell sap is highest followed by the granules. This finding would indicate that the cell sap enzyme could not originate from burst granules of the type isolated in the cell fractionation procedure since, inevitably, this would result in the cell sap having a lower concentration of enzyme per mg of nitrogen than the granules. It could be the case that the granules arose from the enzymes of the cell sap by a process of aggregation. Another possibility is that there is a population of richer and more fragile granules occurring in the cell, and it is those granules being ruptured during the homogenising process that give the cell sap its high amylase content. Further consideration will be given to this problem later in this section.

There are two points which should be mentioned at this juncture in the calculation of data of the type presented in Table 3. First, when a gland such as pancreas is homogenised, some unbroken cells always remain. These are removed with the nuclear fraction and from a practical point of view the estimation of amylase in such material is unsatisfactory. We also considered that the preparation of clean nuclei was beset with many pitfalls and not worth the technical effort. Accordingly, we estimated the nitrogen content of the residue, i.e. nuclei and unbroken cells, and allowed for this in the calculation of distribution of components per pancreas. Secondly, the washing of cell fractions is a vexed question in all fractionation studies. On washing, unfortunately, soluble material tends to be removed and hence, wrong values for certain components may be obtained. Our experience was that contamination of cell fractions was not very high in respect of amylase at least. This is evident if we consider that the ultramicrosome fraction, which had an amylase content of 389 S. & R. units, was sedimented from a solution which contained 18,000 S. & R. units of amylase.

(c) <u>Changes in composition of the whole gland during secretion and recovery</u>: From data published by Fernandes et al (1955) it would appear that maximal depletion of the pancreas occurs thirty to sixty minutes after stimulation with carbamylcholine and that after a further

TABLE 4.

Changes in Whole Pancreas during The Secretory Cycle.

	Weight. (g.)	Total N (mg.)	Amylase (Smith & Roe Units.)
Untreated	0.917	28.7	33,170
Depleted	0.854	25.5	18,520
Recovering	0.876	30.3	46,240
Birds per Group	8	6	6

two hours the pancreatic enzyme content is almost completely restored. Accordingly, we selected these as the most appropriate times to examine the composition of the pancreas and Table 4 shows the results obtained in the whole gland. On the average, the normal weight of the pigeon pancreas is around one gm. and on depletion there is a tendency for the weight to decrease. On recovering the weight tends to rise back to the normal level. These differences. however, are not statistically significant, the variations in the groups being so wide. The total nitrogen of the whole pancreas shows the same tendency as was found for the total weight, but no significant change was found. On the other hand, the amylase content shows a drop of 50% on depletion and on recovery actually exceeds the control value. Such an observation has been reported by other workers (Rabinovitch et al (1952)), (Dineen and Robertson (1955)). Our data indicate therefore, that during the two hours of recovery, the amylase content of the pancreas has risen by a factor of 2.5. It would appear that the factor of 3 obtained by Hokin for pancreatic slices incubated in the presence of amino acids is not so impossibly large as Fernandes et al seem to consider.

From these data it is apparent that the depletion and restoration of enzyme content is not accompanied by a corresponding change in weight or total nitrogen. Hence the change occurring during the secretory cycle must be essentially confined to expulsion and resynthesis of the secretory products.

TABLE 5.

Changes in Nitrogen Distribution in different Cell Fractions during Secretory Cycle.

Fraction	Total Nitrogen mgs.			Protein N mgs.			Non-Protein N mgs.		
	U.	D.	R.	U.	D.	R.	U.	D.	R.
Mitochondria + Granules.	3.08	2.88	3.40	2.35	2.12	1.95	0.32	0.47	1.26
Microsomes (1) + (2).	7.10	5.47	6.45	4.00	2.96	3.35	2.12	1.02	2.61
Cell Sap + Ultramicrosomes	16.6	16.1	20.4	10.66	9.54	10.35	4.94	5.42	9.47

U = Untreated. D= depleted. R = Recovering.

The above data represent the changes found in the distribution of Total Nitrogen, Protein Nitrogen and Non-Protein Nitrogen in the cell fractions of pigeon pancreas during the secretory cycle. The homogenising medium used was sucrose-PO_4. Non-Protein N = Total N - (PrN + RNA Nitrogen)

TABLE 6.

Distribution of Amylase in different Cell Fractions of Pigeon Pancreas During the Secretory Cycle.

Fraction	Total Amylase per Fraction			Amylase per mg. P.N.		
	Untreated	Depleted	Recovering	Untreated	Depleted	Recoveri[ng]
Mitochondria + granules.	3490	2630 (-23%)	4220 (+20%)	1510	1240 (-18%)	2110 (+39%)
Microsome (1) +(2)	4570	3020 (-34%)	4460 (-2%)	1140	1086 (-5%)	1413 (+23%)
Cell Sap + Ultra-microsomes	33,900	15,700 (-54%)	52,000 (+54%)	3280	1860 (-43%)	5020 (+53%)

The data presented above represent the mean of 6 birds, each group.

The amylase units are those of Smith & Roe and the figures in parenthesis represent the changes from the level of the untreated bird.

Homogenising medium used was Sucrose-PO_4.

(d) Changes in composition of individual cell fractions during secretion and recovery. Table 5 shows the changes which have occurred in the total nitrogen, protein nitrogen and non-protein nitrogen during the secretory cycle. The total nitrogen figures do not reflect changes of any great importance. When the protein nitrogen figures are examined, however, it is seen that the most striking change occurs in the microsome fraction in which, on depletion, there is a loss of 30%. The loss in the other two fractions, at the same time, is only around 10%. Recovery in most fractions is marked by a return to the normal level.

The non-protein nitrogen data tend to favour an accumulation during the recovery phase at a time, when presumably, free amino acids would be required for protein synthesis.

Table 6 shows the changes which have occurred in total amylase per fraction (based on theoretical recovery from the whole gland) and concentration of amylase per mg. of protein-nitrogen. It would appear that the greatest loss in amylase occurs in the cell sap (55%), whereas the mitochondria and microsomes lose about 30%. On recovery, the cell sap exceeds the normal value by about 50%. Only in the microsome fraction is the recovering value less than the normal resting level. When the concentration of amylase per mg. of protein-nitrogen is considered it is seen that in the microsomes the concentration is fairly steady during the secretory cycle. On the other hand, the mitochondria and cell sap show both a considerable loss per mg. of protein-nitrogen during depletion and a correspondingly large increase in the recovery process.

The data provided by these studies would indicate that the cell fractions behave differently in relation to the secretory cycle. Thus, on depletion the microsome fraction appears to lose enzyme and protein in parallel and this parallelism is still evident in the recovery phase. The mitochondria and granule fraction shows on depletion a greater loss of amylase than of protein and a greater gain of amylase in the recovery process. The cell sap shows the biggest loss in amylase per mg. of protein and exhibits the greatest gain on recovery. This would suggest that the bulk of the protein of the mitochondrial fraction and cell sap is unconnected with the secretory process.

CONCLUSIONS.

It is generally held that the enzymes of the pancreas are located in the granules and that, on discharge, these granules are extruded through the cell wall into the lumen of the acinus. The problem of the formation of these enzyme-containing granules is thus intimately associated with the process of protein synthesis. It is commonly believed that the microsomes are involved in protein synthesis and we may now proceed to consider how far this might be applied to granule formation.

Firstly it is apparent that the microsomes have a low amylase content compared with the granules and to the cell sap (Table 5); this would suggest that more of the microsome protein is non-enzymic in nature. Yet, during depletion by carbamylcholine, the microsomes lose protein at the same rate as they lose their amylase. This suggests that complete microsomal particles are involved in the disappearance of enzyme from this fraction during depletion, presumably by becoming larger structures on the way to granule formation. If the microsomes were secreting amylase, one would expect them to lose amylase to a much greater extent than protein. In other fractions of the cell, changes in amylase content and protein content do not show this parallelism because, unlike the microsome fraction, these cellular elements are mainly unconnected with protein synthesis.

We would suggest that there are two main possibilities for the formation of the zymogen granules from microsomes.

(a) Secretion of enzyme by microsomes into the cell sap, followed by subsequent aggregation to form granules. The possibility that the enzyme is secreted directly into the cell sap by the microsomes would account for the high concentration of amylase found there. The formation of granules would then take place by the aggregation of the enzyme molecules. Such an aggregation process would result initially in the formation of smaller particles which would sediment with the ultramicrosomes as well as with other cell fractions, depending on the size of the aggregate. The association of highly active enzyme particles with the ultramicrosome fraction would result in this fraction possessing a high amylase content. Table 5 showed that the ultramicrosome fraction has a very low amylase content and the possibility that aggregation of enzyme molecules occurs in the formation of granules does not seem very likely.

(b) Granule formation by detachment of a particle from the endoplasmic reticulum followed by maturation. The particles are formed in the endoplasmic reticulum (microsomes) and gradually their enzyme content increases. At the same time the particles become larger and more fragile. The most mature particles are highly fragile and are ruptured during the process of homogenisation, resulting in the very high amylase content of the cell sap noted above. The particles which we isolate in the granular fraction are not the most mature and hence are not ruptured in the homogenisation.

We shall attempt to substantiate our ideas on the formation of the secretory granules of the pancreas in the later sections of this thesis.

SECTION 2.

Uptake of ^{14}C-glycine by the proteins of different cell fractions of Pigeon Pancreas.

INTRODUCTION.

In this series of experiments the uptake of ^{14}C-glycine into the proteins of pancreatic cell fractions was measured at various times after administration of the isotope. In view of certain contradictory findings about the labelling of RNA during the secretory cycle, it was decided to investigate this problem at the same time; the results from this study will be reported in section 3 of this thesis. We used either sucrose or sucrose-PO_4 as the homogenising medium and the cell fractionation was carried out as described in section 1. The glycine was measured as the DNP-derivative.

<u>Preparation of Birds:</u> The birds were fed <u>ad libitum</u> with corn and maize. Selection was at random and the birds were divided into the following groups:-

(a) <u>Control birds:</u> these birds were unstimulated and only received an intramuscular injection of ^{14}C-2-glycine.

(b) <u>Stimulated birds:</u> these birds received carbamylcholine 45 minutes before the ^{14}C-glycine. In these birds the pancreas had been depleted of enzymes before the administration of the isotope which would then be available to the pancreas for protein synthesis.

<u>Administration of Isotope.</u> ^{14}C-2-glycine was injected intramuscularly at a dose of 40 μ c per bird.

The birds were killed by decapitation at various time intervals after the injection of the isotope. The pancreases were removed and finely minced with scissors. The mince was divided into two equal portions.

(1) For estimation of free glycine: The tissue was homogenised in 20 ml ice-cold 10% TCA in a Nelco blender. The protein precipitate was removed by centrifugation at 0^{o} and washed twice with ice-cold 10% TCA. The TCA was extracted by ether and free glycine estimated by the DNP method as described in the appendix.

(2) For cell fractionation: The tissue was homogenised in 10 mls. of medium and the subcellular fractions obtained by differential centrifugation as described in Section 1. The protein in each fraction was precipitated with ice-cold 10% TCA. After lipid extraction, the proteins were hydrolysed with 6N hydrochloric acid for eighteen hours in a closed tube. The hydrochloric acid was removed *in vacuo* and the glycine estimated as the DNP derivative as described in the appendix.

RESULTS.

Experiments using Sucrose-PO_4 as the Homogenising Medium: In the first series of experiments in this section we used the pancreases of resting birds and of birds stimulated by injection. The stimulated birds were sacrificed on one day and the control birds on another occasion. Time-activity curves were constructed for both these series of birds.

TABLE 7.

Specific Activities of Glycine in Amino Acid Pool and Proteins of Pigeon Pancreas.

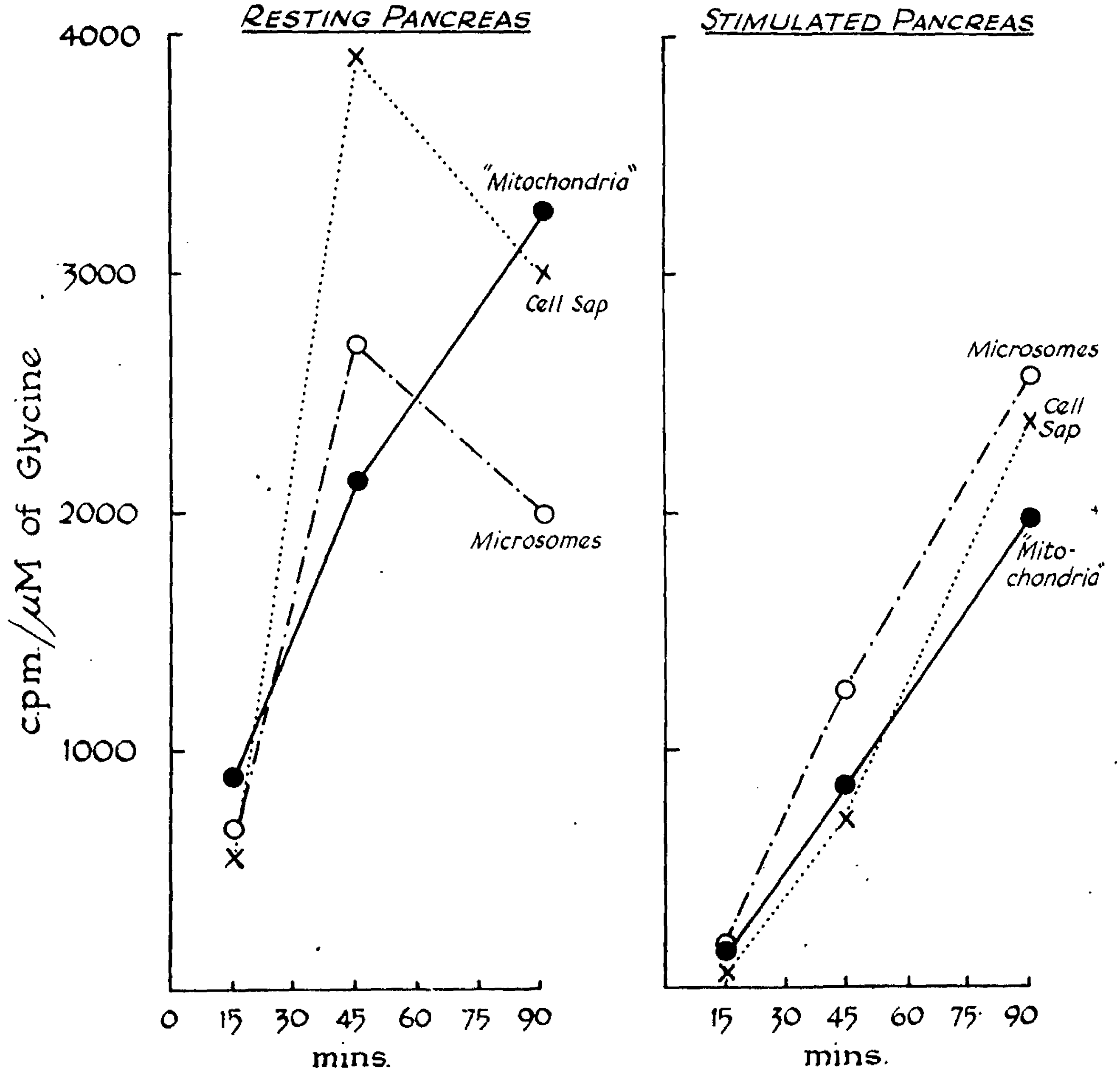
Time after Glycine injected. minutes.	Free Glycine		Mitochondria		Microsomes		Cell Sap	
	C.	I.	C.	I.	C.	I.	C.	I.
15	16,950	13,000	875	142	680	179	574	55
45	16,500	15,900	2105	870	2700	1270	5900	740
90	7,500	11,300	3260	1985	1980	2590	2990	2410

C = Control.   I = Injection.

The data are expressed as counts per minute per μ M glycine. The medium used for homogenising was sucrose.

Fig. 1.

Specific Activities of Protein Glycine in Different Cell Fractions Isolated in Sucrose-Phosphate Medium.
(as c.p.m. per μM glycine).



The results are shown in Figure 1 and Table 7. The specific activity of the free glycine of the tissue (Table 7) shows a somewhat different time course in these two series. The stimulated birds exhibit a slower but more sustained rise which may account for the generally lower level of activity found in the tissue protein of this group (Figure 1). In addition to this difference in general level of uptake by the cell proteins of the stimulated and resting birds, the pattern within the sub-cellular fractions differs in the two series (Figure 1). In the case of the resting pancreas, both the microsome and cell sap protein attain their maximum activity at 45 minutes and thereafter fall off sharply, whereas the mitochondrial fraction continues to show a steady increment. In the case of the stimulated birds, all three fractions continue to rise during the ninety minutes of the experiment.

We were surprised to note in these experiments that, at short time intervals after injection of the labelled amino acid, the uptake into the microsome fraction was not notably higher than into the other cell fractions as one might anticipate on the basis of studies made on other tissues (Hultin (1950)) and also the studies of Mirsky and his co-workers (1955) on the pancreas, where they found that the microsome pellet had a higher incorporation of ^{15}N-glycine for first sixty minutes as compared to the other proteins of the cell. This made us suspicious that our microsome

TABLE 8.

Effect of different homogenising media on the distribution
Eff
of uptake of ^{14}C-glycine by proteins
of cell fractions of Pigeon Pancreas.
of cell fractions of Pigeon Pancreas.

	Pigeon No. 1 15 minutes after glycine injection.		Pigeon No. 2 30 minutes after glycine injection.	
	Sucrose	Sucrose-PO_4	Sucrose	Sucrose-PO_4
Mitochondria	790	695	1555	1315
Microsomes	1920	1395	2710	1230
Cell Supernatant (Sap)	1100	1235	1360	2220

The data are expressed as counts per minute per μ M glycine.

fraction, as prepared in sucrose-PO_4, was contaminated with other particles less intimately connected with the process of protein synthesis. This suspicion was strengthened by our previous experience with sucrose-PO_4 in which, as noted before (Table 5), we found that the substitution of sucrose for sucrose-PO_4 as the homogenising medium led to a redistribution of amylase activity in the different cell fractions. It was therefore decided to perform an experiment in which birds were injected with ^{14}C-glycine and killed fifteen and thirty minutes thereafter. Their pancreases were divided into two portions, one of which was homogenised in sucrose, the other in sucrose-PO_4. Differential centrifugation was then performed and the protein isolated from the two series of fractions gave the analysis shown in table 6. In the case of the sucrose medium the microsome fraction exhibited the highest uptake at both fifteen and thirty minutes. In the case of the sucrose-PO_4, the microsome fraction shows a little more activity than the cell sap at fifteen minutes, but is appreciably lower at thirty minutes. It is therefore evident that the nature of the homogenising medium profoundly affects the observed radio-activity and that the microsome fraction isolated in sucrose-PO_4 suffers dilution with less radio-active material. Presumably the sucrose-PO_4 causes suspension of the lighter mitochondria and granules so that they pass into the microsome fraction; correspondingly, the lighter microsomes will find their way into the cell sap fraction. For these reasons the

TABLE 9.

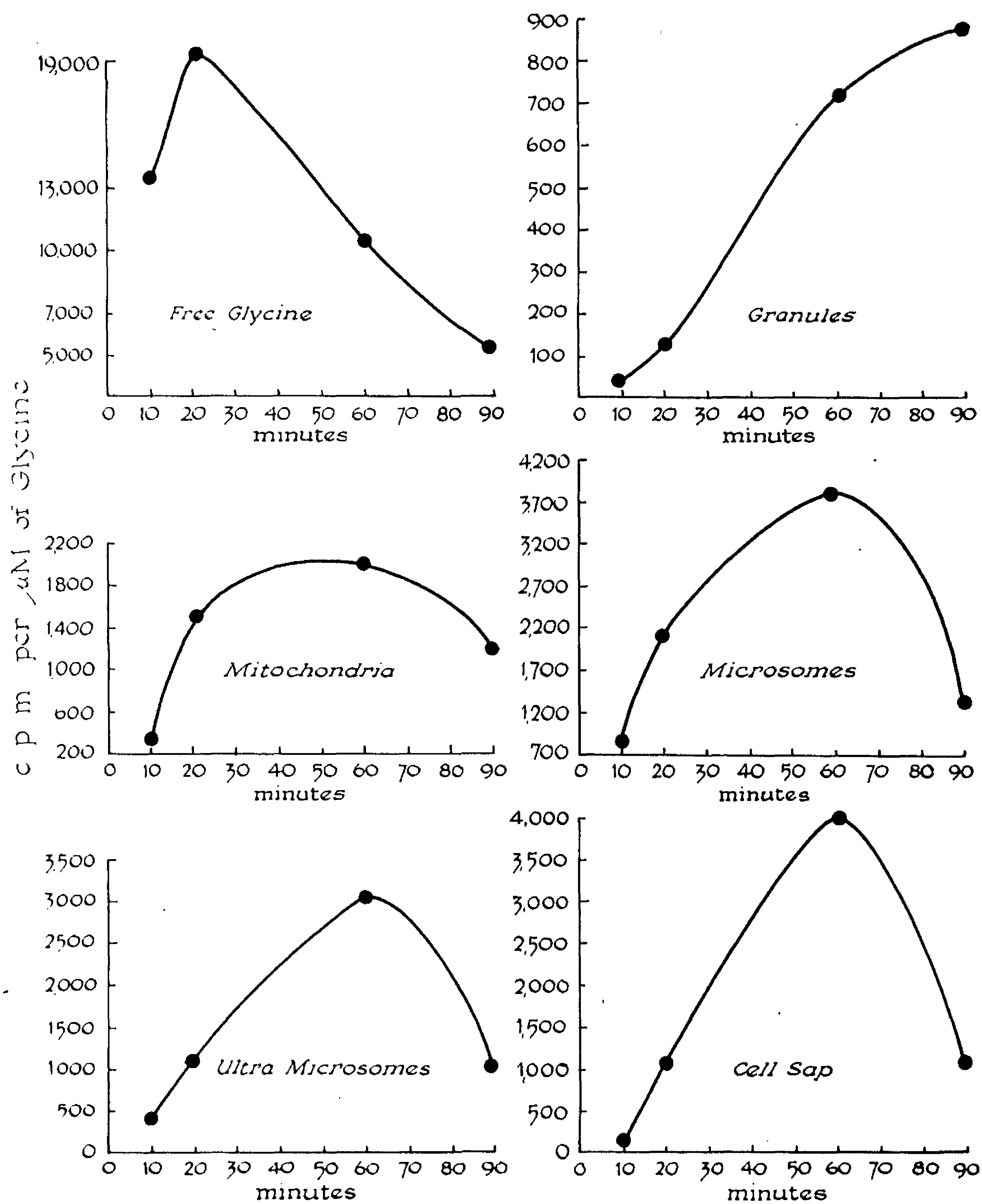
Specific Activity of Glycine in Amino Acid Pool and Proteins of Pigeon Pancreas.

Time after Glycine Injection. minutes.	Free Glycine	Granules	Mitochondria	Microsomes	Ultra-microsome	Cell Sap.
15	18,150	930	1650	2525	1780	1005
30	11,700	2160	2045	4510	3390	2400

The above data are expressed as counts per minute per μ M glycine.

0.25 M sucrose was used as the homogenising medium.

Specific Activities of Protein Glycine in Different Cell Fractions of Pancreas Isolated in 0·25 μM Sucrose. *(as cpm per μM of glycine)*



microsome radio-activity will suffer a reduction through dilution with mitochondria and through loss of active particles into the cell sap. In view of these findings and their implications, we decided that a repetition of the data using sucrose as the homogenising medium was desirable. In the first instance only studies on the resting pancreas were attempted.

<u>Experiments using 0.25 M Sucrose as the Homogenising Medium:</u> In these experiments a more ambitious scheme of cell fractionation was carried through (Table 1), Granules, Mitochondria, Microsomes, Ultramicrosomes and Cell Sap being prepared. The results are shown graphically in Figure 2. At the first two time intervals the microsome fraction exhibits by far the highest activity and attains a maximum at sixty minutes, followed by a decline. This picture is also shown by the ultramicrosome fraction which has an appreciably lower uptake of glycine at all time intervals and also by the cell sap which equals the microsome activity at sixty minutes. The mitochondrial fraction similarly attains a peak of activity at sixty minutes (Figure 2). The only fraction to continue to gain radio-activity during the ninety minutes studied is the granular fraction. It is also the fraction with the lowest radio-activity (Figure 2).

A confirmatory experiment on birds killed fifteen and thirty minutes after ^{14}C-glycine injection was carried out and the results support the picture described above. They are presented in detail in table 9.

DISCUSSION.

Our radio-activity data may now be considered in relation to the special function of the exocrine pancreas, namely the formation of granules and secretion of enzymes. As discussed in Section 1, if we assume that the enzyme proteins in the pancreatic granules are formed primarily by the microsomes, then we may conceive of two alternative routes. Either fragments of microsomal material undergo a maturation process to form granules, or alternatively the enzyme molecules are secreted into the cell sap and thereafter aggregate to form granules. We may now consider how far our radio-activity data go towards resolving this question. In so doing, it must be remembered that the radio-activity data do not distinguish between the formation of amylase and of other proteins, including proteins not involved in pancreatic secretion. The correlation of the uptake of ^{14}C-glycine and enzyme secretion must therefore be made with due caution.

The view that the microsomes are the primary site of amino acid incorporation in the pancreas (for which much evidence has been accumulated in other tissues) is supported by our radio-activity data. Using sucrose as the medium for isolation (Figure 2) the microsomes show initially a much higher uptake than any other cell structure. Since the mitochondrial fraction shows a lesser activity than the microsomal fraction, and at ninety minutes after isotope injection is about to attain the activity of the microsomes, the data are compatible with the presence in the mitochondrial fraction of particles (small granules) derived from the microsomes. Similarly, the radio-active curves (Figure 2) are compatible with the formation of the mature granules from the smaller granules present in the mitochondrial fraction since these two curves (i.e. mitochondria and granules) bear a product-procursor relationship. It is especially noteworthy that the granule fraction (presumably the only pure secretory enzyme fraction in the cell) shows the lowest uptake of ^{14}C-glycine and is still accumulating activity after ninety minutes. This picture is confirmed by reference to the data on resting pancreas in the earlier experiment using sucrose-PO_4 (Figure 1), in which the so-called mitochondrial fraction (almost certainly mainly composed of granules) shows the same picture, namely rising activity at a time when the lighter fractions are falling off in activity.

As regards the status of the cell-sap in granule formation, the radio-active data do not provide a conclusive answer. In this fraction, we are presumably dealing with a very heterogeneous collection of protein molecules having quite diverse functional activities. The amylase content of the sap may represent enzyme secreted by the microsomes and subsequently aggregated into granules, or it may represent mature granules which have ruptured into the sap. If we regard the radio-activity of the mitochondrial fraction as being due to the presence of small granules, this fraction shows a much greater ^{14}C-uptake than the sap at ten and twenty minutes after isotope injection; this could, of course, be explained on the grounds that amylase and other secretory enzymes present in the sap have their radio-activity diluted by other cell sap proteins. On the other hand, the cell sap has at all time intervals a considerably higher radio-activity than the mature granules, so that the activity of the sap cannot be due, to any significant extent, to rupture of granules into it.

The picture of isotope uptake following expulsion of the granules and enzyme resynthesis was only obtained in the case of fractions isolated in Sucrose-PO_4 (Figure 1). Nevertheless, it indicates considerable problems in interpretation. Instead of a much higher radio-activity (Fernandes and Junquiera (1955), the stimulated birds had a greatly diminished ^{14}C-glycine uptake into the proteins of all cell fractions. This is probably due to the distortion of the free glycine curve by alterations in vascularity following carbamylcholine stimulation. There is an obvious engorgement of the gland under these circumstances and the free glycine data (Table 7) reveal a lower specific activity at early time intervals and a higher activity at ninety minutes. This displacement of the peak of free glycine activity presumably also explains the finding (Figure 1) that the specific activities of the microsome and cell sap fractions of stimulated birds are still rising at ninety minutes after isotopic injection. It is thus not possible to draw, from these data, any firm conclusions regarding changes in the pattern of protein synthesis in the repleting pancreas.

Section 3.

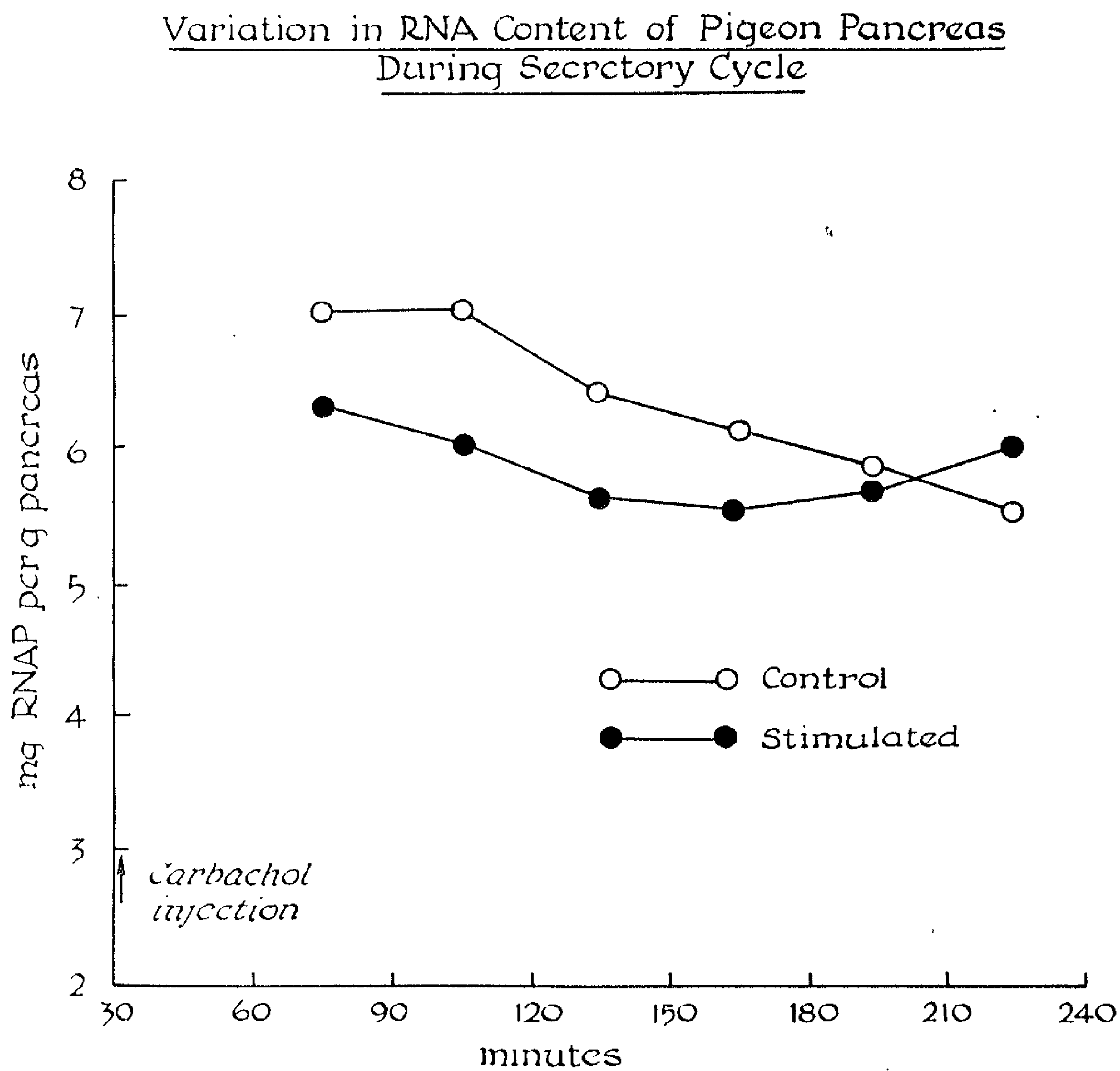
Metabolism of RNA during the Secretory Cycle in the Pancreas.

INTRODUCTION.

As outlined in the general introduction to this thesis, the results obtained by many workers are in conflict on the participation of RNA in the secretory cycle in the pancreas. Thus, by purely chemical methods, it seems to be established that there is no change in the total RNA content of the pancreas during the secretory cycle. (Rabinowitch et al (1952). On the other hand, the isotope evidence provided by the use of ^{14}C-glycine and ^{32}P would indicate that there is an increased turnover of pancreatic RNA in an animal whose pancreas has been stimulated to secrete by the use of carbamylcholine etc. Thus Fernandes and Junquiera (1955) obtained evidence that the incorporation of ^{14}C-glycine into pancreatic RNA was increased after stimulation. This evidence, however, is a flat contradiction of that provided by Allfrey and Daly (1953) who found that stimulation to secrete (feeding in their case), did not augment the incorporation of ^{15}N-glycine into the pancreatic RNA. The uptake of ^{32}P by pancreatic RNA under the conditions of stimulation has been increased 1200% according to Gubernicv and Il'lina (1950) but De Deken Grenson (1953) did not obtain any increase in uptake working with mouse pancreas under similar conditions.

In order to clarify the position, we have attempted to examine the changes in RNA during the secretory cycle in the pigeon by several techniques, namely, quantitative examination and isotopic measurements

Fig. 3.



Variation in RNA Content of Pigeon Pancreas During Secretory Cycle
mg RNAP per g pancreas
8
7
6
5
4
3
2
30
60
90
120
150
180
210
240
minutes
Control
Stimulated
Carbachol injection

using ^{14}C-glycine and ^{32}P.

Preparation of Birds. The birds were fed ad libitum with corn and maize, as usual.

Quantitative determination of RNA. The control method, as described in the appendix was used.

Isotopic techniques.

(a) ^{32}P: The dose of ^{32}P varied from 40 μc - 500 μc per bird and was given intramuscularly. The RNA was extracted from the TCA precipitated protein with 10% sodium chloride. The DNA was removed and the nucleotides were separated by ionophoresis. The specific activity of the individual nucleotides was determined and the average of the four obtained. Phosphorus determinations were by the Allen (1940) method. For details see appendix.

(b) ^{14}C-2-glycine. 40 μc of ^{14}C-glycine was given intramuscularly to each bird. The separate nucleotides were prepared as before and the free bases isolated as described in the appendix. Aliquots of these bases were estimated for radio-activity and the concentration determined spectrophotometrically as described in the appendix.

RESULTS.

Quantitative changes in RNA during the Secretory Cycle. The results which we obtained on the quantitative changes in RNA during the pancreatic secretory cycle are shown in Fig. 5. The pancreas had been stimulated to discharge by the injection of carbamylcholine, and the estimations were carried out at the times indicated after the stimulant injection. The controls are birds which were sacrificed at the same time but had received no carbamylcholine. The results are given as mgs. of Ribose P per gm. of pancreas. It will be seen that the general level of the controls is higher than that of the stimulated birds. However, the difference is probably due to the presense of more blood in the stimulated pancreas, which would tend to increase the weight of the gland and consequently decrease the concentration of RNA found. To obviate this difficulty the possibility of estimating DNA was considered. Unfortunately the blood of the pigeon contains nucleated erythrocytes, so that the increased amount of blood would lead to an increased DNA content of the gland. This would complicate our interpretation of the data once again.

From these studies we were, therefore, unable to draw any firm conclusions about the metabolism of RNA during the secretory cycle. Accordingly, we adopted a different approach, that of measuring the turnover of RNA using isotopic tracers.

Uptake of ^{32}P into the nucleotides of RNA during the secretory cycle.

In the first experiments in this series, we used doses of 40 μc ^{32}P injected intramuscularly into stimulated and unstimulated birds. The stimulated birds had received carbamylcholine forty five minutes before the ^{32}P, hence at the time of isotope injection the pancreas was depleted of enzymes. During the period when rapid enzyme synthesis was occurring ^{32}P was available for incorporation into the nucleotides if required. The birds were sacrificed at various time intervals after the ^{32}P injection. With doses of 40 μc per bird

TABLE 10.

The uptake of ^{32}P into the nucleotides of RNA of stimulated and unstimulated pigeon pancreas.

Time after ^{32}P Injection. mins.	Dose of ^{32}P per bird.	Specific Activity of Tissue Inorganic P. U	S	Average S.A. of Nucleotides U	S	R.S.A. of Nucleotides U	S
120		16,005	22,530	8.7	27.5	0.29	0.61
150	250 μc	24,615	15,520	52.6	32.0	0.66	1.03
180		32,375	22,735	19.6	19.8	0.29	0.44
120		39,300	61,500	138	180	0.35	0.29
150	500 μc	29,850	59,600	75	126	0.25	0.21
180		73,000	57,400	275	544	0.38	0.69

The stimulated birds had received carbamylcholine 45 minutes before the ^{32}P injection.

The Specific Activity of the nucleotides is the mean figure for all four nucleotides expressed as counts per minute per 100 μg. P.

Tissue Inorganic Phosphate is expressed as counts per minute per 100 μg. P.

TABLE 11.

The uptake of ^{14}C-glycine into the Nucleotides of RNA of Pigeon Pancreas.

	Free Glycine		Specific Activity				Relative Specific Activity			
			Adenine		Guanine		Adenine		Guanine	
Time after Isotope Injection. (mins)	C.	I.	C.	I.	C.	I.	C.	I.	C.	I.
15	23,700	17,300	2	170	52	276	0.01	0.92	0.22	1.6
45	23,200	21,200	3	7	74	170	0.01	0.034	0.32	0.8
90	10,000	15,100	5	18	46	268	0.05	0.11	0.46	1.8

C=Control, I = Injected.

Free glycine data expressed as counts per minute per μM glycine.

Adenine and Guanine data are expressed as counts per minute per μ M.

Relative Specific Activity = $\dfrac{\text{specific activity}}{\text{free glycine activity}}$ X 100.

we could obtain no significant labelling of the ribonucleotides. Accordingly, we repeated the experiment using 250 μc per bird and latterly 500 μc ^{32}P per bird. The results from these two experiments are shown in Table 10. It will be seen that, even in the experiment in which 500 μc ^{32}P were used, the level of labelling attained in the ribonucleotides is not very great. The interpretation of these figures, therefore, requires to be made with this fact in mind. It can, however, be said, that there is no evidence for a more rapid turnover of RNA under the conditions of the experiment.

In an attempt to confirm this rather negative finding, we decided to use as a precursor of purine synthesis, ^{14}C-2-glycine.

Uptake of ^{14}C-2-glycine into the nucleotides of RNA during the Pancreatic Secretory Cycle. The conditions for the use of the isotope were the same as for the experiments in ^{32}P uptake just described. 40 μc ^{14}C-2-glycine per bird were injected intramuscularly and the individual nucleotides were obtained by ionophoresis. The result of this experiment is shown in Table 11. The quantities of the free bases recovered in this type of experiment are very small, hence the significance of the figures must be in doubt, though no great degree of labelling is apparent either in the stimulated or unstimulated birds.

DISCUSSION.

The data which are presented above would indicate that there is no increase in the metabolism of pancreatic RNA during the secretory cycle. The quantitative data are inconclusive but the failure to obtain any increase in incorporation of precursors into RNA would tend to indicate no change in RNA metabolism during the secretory cycle. The low incorporation of precursors into the RNA of pancreatic tissues has been noted by other workers (Allfrey et al (1953)); De Deken Grenson (1955); Kihara, Amano, Ikemoto and Sibatani (1955). It has been attributed by Kihara et al to the high concentration of RNA which is present in pancreatic tissue. It does not indicate that the absolute rate of synthesis of pancreatic RNA is any smaller than that found in other tissues, but that this passes into a larger pool of RNA and is correspondingly diluted.

Section 4.

In vitro Synthesis of Amylase.

INTRODUCTION.

In the study of a highly complex process like synthesis of proteins, it is desirable to simplify the system in which synthesis is occurring. Thus, although extensive use has been made of whole animals in the study of protein synthesis, such an approach does not permit one to break down the mechanism into its component parts. The use of tissue slices has some advantages over the whole animal in that metabolites can be added at will to the tissue under investigation and different treatments carried out in parallel on samples of the same tissue. These are important considerations, but there remains the question of availability of the metabolites to the cell interior. The permeability barrier imposed by the intact cell membrane is a factor which must be borne in mind when assessing the results obtained in tissue slice experiments. The elimination of the cell membrane would therefore be highly desirable, but it may well be that there are other membrane barriers present in the cell interior just as important as the cell membrane. Thus, from the electron microscope studies of Palade and Siekevitz (1956), it would appear that there are vesicle-like structures (endoplasmic reticulum) present in the intact cell. These vesicle-like structures give rise to the "microsome" fraction of cell fractionation studies, and whether any permeability barriers are associated with these is not known.

In view of the desirability of obtaining a cell-free system which continues to exhibit protein synthesis, it was of interest to note that Khesin (1953) and later Straub (1955) described systems, prepared from pancreas, capable of amylase formation.

The following section describes our own attempts to demonstrate amylase synthesis *in vitro*. In the first place, we compared the effect of different forms of medium on the capacity of slices of pancreas to synthesise amylase and to take up labelled amino acids. We then prepared homogenates of pancreas in an attempt to achieve *in vitro* synthesis of amylase in a cell-free system. While we were actively engaged in these studies, we received a communication from Professor F.B.Straub of Budapest indicating that he had been successful in preparing, from acetone-dried pancreatic tissue, a system capable of synthesising amylase. We therefore also tried to reproduce this system.

(A) TISSUE SLICE STUDIES.

It had been demonstrated by Hokin (1951) that slices of pigeon pancreas can synthesise amylase when incubated in glucose saline, and that, in the presence of a complete amino acid mixture the synthesis is enhanced. The amino-acid requirements of this system were limited to ten (Hokin, 1951b) and it was shown that the omission of any one of these seriously interfered with the amylase production. We have used tissue slices under Hokin's conditions, in order to study in parallel the synthesis of amylase and the uptake of isotopically labelled amino acids into the protein of the pancreas. In these experiments, single essential amino acids were omitted from the medium and the effect on isotope incorporation and amylase formation compared.

METHODS.

Preparation of Birds: The preparation of birds and conditions of slice production and incubation followed the recommendations of Hokin, (1951). Mature pigeons were selected at random and were injected intramuscularly with 0.07 mg carbamylcholine. Salivation occurred in all cases though the severity of the response was variable. Forty five minutes after the injection the birds were killed by decapitation and the pancreases removed and placed in ice-cold Krebs-Ringer Bicarbonate (KRB).

Slicing Technique. The pancreas was dried by placing on filter paper and as much connective tissue removed as possible. The tissue was sliced either by the Stadie-Riggs microtome (1944) or by the McIlwain tissue slicer (1953). In either case, the slicing platform was chilled by placing in ice.

Incubation Procedure. Samples of the sliced tissue were weighed in a torsion balance and quantities around 75 mgs. were used in each incubation flask. The incubations were carried out in Krebs-Ringer

Bicarbonate containing glucose at a final concentration of 0.2%. Amino acids when used were either a mixture of synthetic amino acids or Casamino acids (Difco), which is an acid hydrolysate of casein. The final concentration of the amino acids was 0.4%. ^{14}C-2-glycine or ^{35}S-methionine were added at a final concentration of 1 μ c per ml. The flasks were gassed with oxygen-carbon dioxide mixture and the incubations were carried out for two hours at 37°C in a water bath with constant shaking.

Estimations - Amylase: After incubation, the flasks were chilled on ice. The contents were either

(a) ground with sand and centrifuged and the amylase content of the supernatant estimated (as recommended by Hokin, (1951)), or

(b) homogenised with a Potter-type homogeniser and amylase estimated in the homogenate.

The actual estimation of amylase was carried out as described in the Appendix.

Radio-Activity of Protein: The entire contents of the flasks were homogenised in ice-cold 10% TCA and the protein removed by centrifugation at 0° and lipid extraction performed in the usual way as described in the Appendix. The protein was then treated with 10% TCA at 90° for fifteen minutes, in order to remove nucleic acids. (Schneider, 1945). This was repeated twice, after which the protein was dried and finely ground in an agate mortar. The powder was counted at infinite thickness with a mica end-window counter.

TABLE 12.

Production of Amylase and uptake of labelled amino acids by Slices of Pigeon Pancreas Homogenate.

Source of AMINO ACIDS.	Casein Hydrolysate		Synthetic Mixture.	
	Amylase	Uptake of L.A.A.	Amylase	Uptake of L.A.A.
Z.T.C.	20.1	0.8	28.5	0
K.R.B.	40.0	-	44.7	-
Incomplete Amino Acid Mixture	46.1	246	45	61.9
Complete Amino Acid	41.1	277	49.3	99.3
No. of Observations	5	3	5	3

The AMYLASE is expressed in Smith and Roe units per mg. dry weight.

The UPTAKE is expressed as counts ~~per mg.~~ per planchet at infinite thickness.

Time of incubation was 2 hours.

Z.T.C. Zero Time control
K.R.B. Krebs Ringer Bicarbonate
L.A.A. Labelled Amino Acids

Dry Weight. The dry weight of the tissue used was obtained by drying a known "wet weight" of tissue overnight at 110°.

RESULTS.

In these experiments we used two different amino acid sources, i.e. synthetic amino acid mixture or Casamino acids (Difco). In both cases, tryptophan was added. Table12 shows that there is no difference in the response produced by either of the amino acid sources. In the presence of saline there is an apparent synthesis of amylase of the order of 50-100% of the initial level. The addition of amino acids has little or no effect on the production of amylase. The omission of an essential amino acid, e.g. tryptophan, for the mixture has no effect on the production of amylase. The radio-active data, on the other hand, demonstrate that the omission of an essential amino acid considerably decreases the uptake of both labelled amino acids into the protein.

DISCUSSION.

In the tissue slice experiments we have found that the production of amylase is, on the average, uninfluenced by the presence of amino acids (Table 12). Our results with amino acids have tended to be variable, some experiments being suggestive of an effect. The general lack of response to amino acid mixtures may be due to the presence in the tissue slices of a sufficiently large amino acid pool adequate for the synthetic requirements of the tissue slices. The variability of results in this field has been noted by other workers. Thus M.R.Hokin (1956) found that in the slices of pancreas from well-fed mice, amino acids produced no increase in amylase formation. However, if the slices were from pancreases of starved mice, the usual effect of amino acid stimulation was noted. This was attributed to the presence in slices from well-fed animals of a supply of amino acids sufficiently large to allow of protein synthesis.

The radio-active data show, on the other hand, a consistent response to the omission of an essential amino acid. This would suggest that general tissue protein synthesis and specific enzyme synthesis do not go hand in hand. Since our experiments were performed, Straub (1957b) has reported experiments with inhibitors such as fluorophenylalanine or chloramphenicol and drew the same conclusion; these inhibitors had a more potent effect on incorporation of ^{14}C-glycine and ^{14}C-tyrosine into protein than on amylase formation in pancreatic slices.

This suggests that we may be dealing with a two-step process in amylase formation, namely an initial linking of free amino acids to form a non-enzymic polypeptide or protein, followed by differentiation into amylase and other secretory enzymes. This would explain why factors affecting incorporation of labelled amino acids into the proteins of the tissue slice can be divorced from changes in amylase production. In other words, lack of an essential amino acid may inhibit the first stage of protein formation without influencing the subsequent differentiation of protein, already formed, into enzyme.

(B) Cell-free Formation of Amylase.

The method of exploring possible conditions for protein synthesis in cell-free preparations has been largely limited to labelled amino acid incorporation studies. (Zamecnik and Keller 1954; Gale and Folkes, 1955[a]). Under these circumstances, it is generally necessary to provide energy, usually in the form of ATP and some substance capable of regenerating ATP. The most extensive details for such a system in mammalian preparations has been provided by Zamecnik (1956), working on liver. Here there are five essential components of the cell-free incorporation system, i.e. (1) the microsome fraction, (2) the pH 5 precipitable enzyme fraction, (3) ATP (and usually an ATP regenerating system), (4) GTP or GDP and (5) the labelled amino acid. However, incorporation of a labelled amino acid is not necessarily synonymous with complete protein synthesis. This latter is most readily demonstrated by increase in some specific enzyme. Such an approach has been applied to cell-free systems by Gale and Folkes, (1955[b]), using disrupted staphylococcal cells. In the case of amylase, the formation of new enzyme by cell-free preparations has been claimed by both Khesin (1953) and Straub (1955). The system described by Khesin was prepared from pigeon pancreas and contained cell particles notably microsomes. Synthesis was dependent on ATP and all essential amino acids except methionine; if α - keto-glutarate was added the synthesis was augmented. The system described by Straub was a water extract of acetone dried pancreas and required the presence of casein hydrolysate, ATP and ascorbic acid, though latterly this factor was considered unnecessary.

Our first experiments slightly antedate reports by Straub. Our objective was firstly, to demonstrate cell-free amylase synthesis. Although amylase appeared to increase in cytoplasmic preparations, we became suspicious that our observations represented liberation of preformed enzyme rather than true synthesis. This suspicion was strengthened by the finding that certain sub-cellular elements, notably the microsomes, contained amylase in a form not readily estimated without special treatment. Furthermore, the increments in amylase content of cytoplasmic preparations during incubation were not accompanied by uptake of labelled amino acids. These data will now be presented.

METHODS.

Preparation of Birds: Birds were fed *ad libitum* as usual. An intramuscular injection of 0.07 mg. carbamylcholine was given forty five minutes before sacrifice. The birds were killed by decapitation and the pancreases removed.

Preparation of Homogenates: The pancreas was chopped with scissors and placed in 10 ml. 0.25 M sucrose in 0.2 M phosphate buffer pH 7.2. The buffer was included in order to minimise the pH changes said to occur during the homogenising process (Siebert 1955). Included also in this homogenising medium was ATP (Sodium) at a final concentration of 2.5 - 7.5 mgs. per ml. It was suggested that the presence of the ATP during the actual homogenising process would reduce the effect of the powerful ATP ase present in the pancreatic homogenate (Straub, 1955). The homogenisation was carried out in a Perspex-glass homogeniser of the Potter type, kept at 0° during the process. Nuclei and unbroken cells were removed by centrifugation at 600 g for ten minutes at 0°. The supernatant cytoplasm was removed and this is referred to as the "cell-free system".

Incubation Procedure. Glucose was added at a final concentration of 0.2%. A complete amino acid mixture (Casamino acids "Difco" supplemented with tryptophan) was added in certain cases at a final concentration of 0.4%. The use of a complete amino acid mixture was thought to be more desirable since it would prevent the possibility of "non-essential" amino acids becoming limiting factors in amylase synthesis due to the inability of the cell-free system to synthesise its requirements of "non-essential" amino acids. The flasks were gassed with oxygen-carbon dioxide mixture before the incubation which was carried out at 37°C in a water bath with constant shaking.

Addition of Alternative Enzyme-yielding Substrates: In some experiments 3-Phosphoglyceric Acid (3-PGA), Hexose Diphosphate (HDP), Sodium pyruvate, Sodium citrate, sodium α-ketoglutarate, sodium succinate and sodium fumarate were used as alternative cources of energy. Each substrate was used at a final concentration of 0.003 M.

Inhibitors of Proteolytic Action: The specific trypsin inhibitor, as described by Kunitz (1946), was prepared from soya bean meal. The potency of the inhibitor was established by assaying tryptic digestion of haemoglobin by Anson's method, with and without the presence of the inhibitor. The inhibitor was included in the incubation mixture in one case.

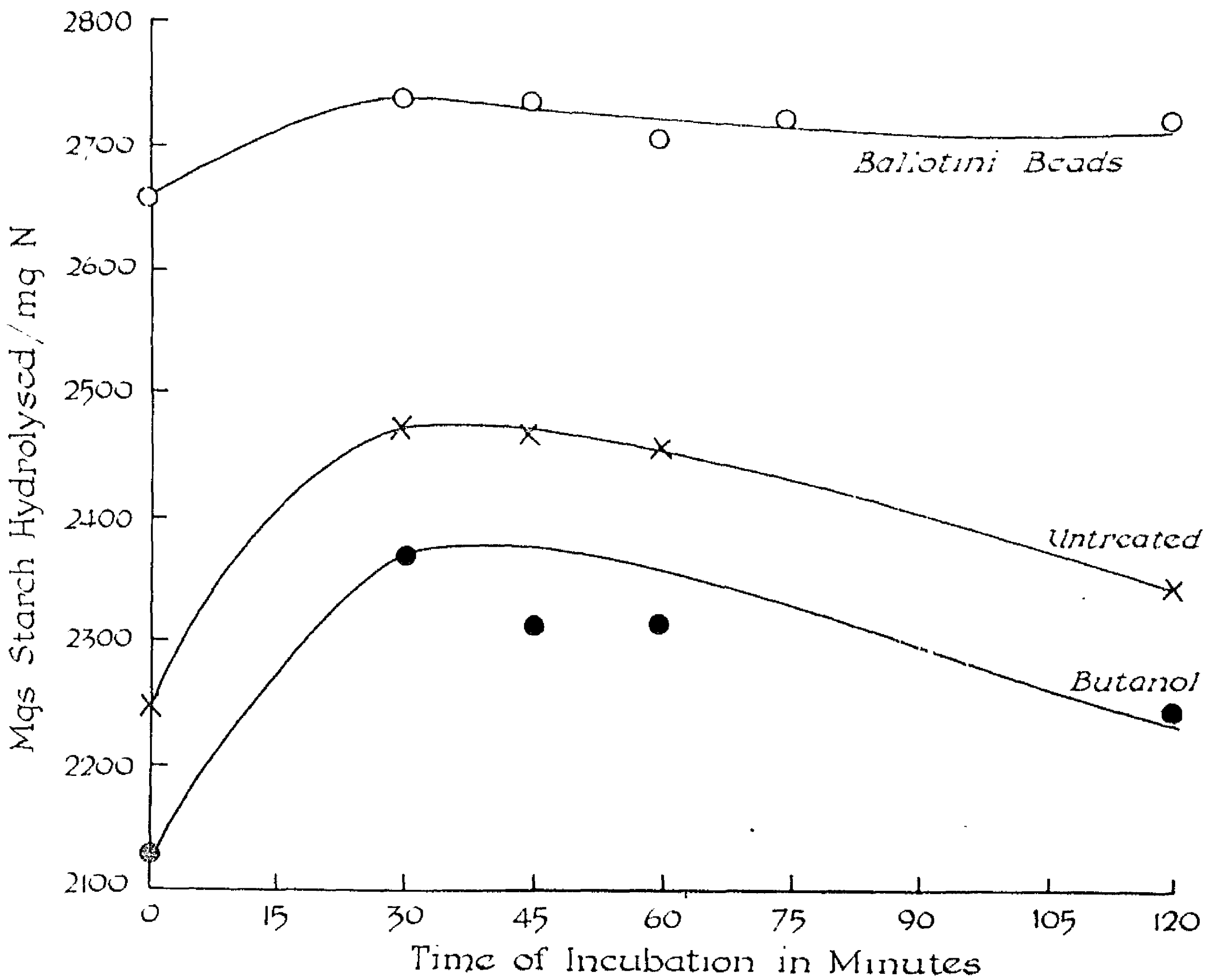
Addition of Purified RNA. Yeast RNA was purified by the method described by Schucher and Hokin (1954). This material was added to the incubation mixture at a final concentration of 2 mgs. per ml.

Addition of Radio-active Amino Acids. ^{14}C-2-glycine was added to the incubation mixture at a final concentration of 5 μc per ml.

Preparation of Acetone extracted pancreas. This was carried out according to the directions given by Straub (1955). Five pigeons were injected with carbamylcholine in the usual way and after forty five minutes the pancreases were removed and homogenised in ten volumes of pure acetone at -10°C. The acetone was filtered off and the homogenisation repeated again with ten volumes of acetone. The cake was allowed to drain free of acetone on the filter paper. Last traces of acetone were removed *in vacuo*. The powder weighed 865 mgs.

Fig. 4.

Effect of Butanol and Ballotini Beads on the Apparent Amylase Content of Cell Free Homogenate of Pigeon Pancreas.



and was extracted with 15 mls. ice-cold distilled water. The mixture was centrifuged at 600 g for five minutes at 0° and the clear supernatant removed. 0.6 ml of 0.2 M acetate buffer pH 5.0 was added to the supernatant and a whitish precipitate obtained. This precipitate was centrifuged at 0° and the supernatant discarded. The precipitate was then suspended in 2 ml ice-cold distilled water. 75 mg ATP (Sodium salt) 24 mg Casamino acids and tryptophan, 10 mgs. Ascorbic acid were dissolved in 4 ml of water and added to the 2 ml prepared above. Incubations were carried out for two hours, after which the amylase content was assayed before and after Ballotini bead treatment.

Estimations on Samples.

Amylase. This was carried out as described in the Appendix.

Radio-active Uptake. Protein was precipitated with TCA, lipid extracted and dried. The material was finely ground and counted at infinite thickness with a mica end-window counter. For fuller details of preparation of protein for counting, see appendix.

RESULTS.

Amylase Synthesis in Cytoplasmic Homogenates.

Fig. 4 shows the results obtained on incubating the cell-free homogenate of pancreas with a complete amino acid mixture and glucose. In the untreated samples there is an apparent increase in amylase content of around 20%, maximal at fifteen minutes incubation, after which the amylase content falls. An increase in amylase content of similar magnitude has been noted by Khesin (1953) using a somewhat similar pancreatic homogenate. The decrease after fifteen minutes was attributed by him to proteolytic activity effected by enzymes in the homogenate.

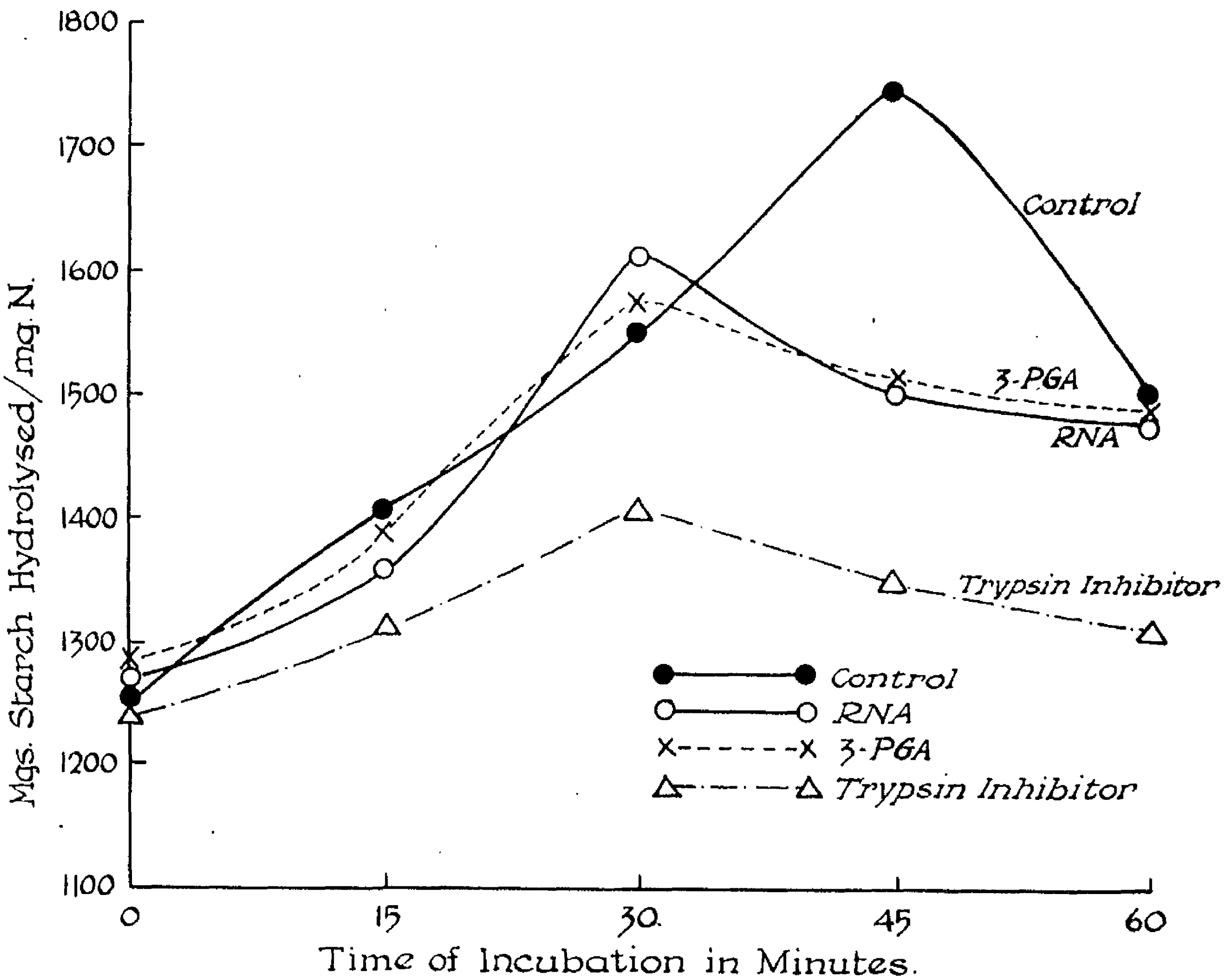
In evaluating this rise, consideration must be given to the possibility of liberation of enzyme during the incubation. To investigate this possibility, samples were treated with butanol (Morton (1950)). The effect of the butanol was apparently to inactivate some of the amylase so that a lower amylase content was found for each sample treated. It did not however, alter the apparent synthesis of amylase, since the butanol graph was parallel to, but a little lower than, the untreated graph. This result is contrary to that described by Khesin (1953), who apparently obtained an increase of 2-3 times in amylase activity, on treating samples with butanol. On the other hand Hokin (1955), in working with amylase from dog pancreas, records a loss of amylase activity of the order of 25% after butanol treatment. Morton (1954), working with alkaline phosphatase, has found it necessary, for quantitative recoveries of enzyme, to re-extract the butanol layer with sodium bicarbonate. No such procedure was carried out by Khesin. During the course of butanol treatment, we were unable to demonstrate the presence of amylase in the butanol layer, but we did observe a whitish layer at the interface of the butanol and water layer. This layer could represent de-natured protein and may account for the apparent loss in enzyme activity. In our opinion, butanol treatment is not satisfactory in the case of amylase when quantitative recoveries are required.

Other methods of liberating enzymes were considered and, after trying several procedures, we found the greatest increments following treatment with Ballotini beads which increased the assayable enzyme content and tended to flatten out the curve. (Fig. 4). It would still appear from Fig. 4 that there is evidence of a small increase during the first fifteen minutes of the incubation but the significance of this must be in doubt. Such an increase, at the best, represents only a fraction of the synthetic ability of the pancreas _in vivo_ (Table 4) or _in vitro_ with slices (Table 12). Accordingly attempts were made to provide conditions under which the increase obtained in the homogenate would be greater.

Effect of Trypsin Inhibitor. There are several proteolytic enzymes present in the pancreas, any one of which could adversely affect the course of amylase synthesis, either by acting on the amylase itself or by destroying the enzyme system required for the synthesis of amylase. Trypsin is one such enzyme and there has been described (Kunitz, 1946) a specific inhibitor for this enzyme. We prepared a specimen of this inhibitor and added this to our original incubation

Fig. 5.

Effect of the Addition of RNA, 3-PGA and Trypsin Inhibitor on Amylase Production by Pigeon Pancreas Homogenate. No treatment with Ballotini Beads was carried out.



mixture. Fig. 5 shows the results we obtained, and it will be seen that, compared to the control, no improved formation was evident. No treatment with Ballotini beads was carried out.

Effect of RNA. All the evidence goes to show that there apparently is an association between the concentration of RNA and the intensity of protein synthesis. (Brachet (1941), Caspersson (1941)). Pancreatic tissue contains a high concentration of ribonuclease, which, in the homogenate, would tend to lower the RNA content and so adversely affect the course of protein synthesis. Since there are no known specific inhibitors of ribonuclease, the addition of pure RNA may have the effect of conserving the RNA of the pancreas by acting as an alternative substrate for the ribonuclease. In Fig. 5 the results obtained with this addition would indicate that there is no appreciable effect as compared to the control. No treatment with Ballotini beads was carried out.

Effect of 3-Phosphoglyceric Acid. The synthesis of proteins from free amino acids is a reaction which requires energy which may be provided in the form of ATP. In the pancreatic homogenate, according to Straub (1955), there is present a powerful ATP ase which rapidly lowers the effective ATP concentration and this effect will limit endothermic synthetic reactions of which protein synthesis is an example. Zamecnik and Keller (1954) have shown that the provision of 3-PGA as an energy source has a stimulating effect on the incorporation of labelled amino acids into the protein of rat-liver microsomes. In an attempt to stimulate the production of amylase by a similar mechanism, 5-FGA was included in the incubation mixture and the results are shown in Fig. 5. where no appreciable effect on amylase production was obtained.

It will then be seen that none of the additions to the incubation mixture has altered the rate or the intensity of the synthesis of amylase by pancreatic homogenates compared to the control samples. These experiments were all carried out without any special treatment of specimens to liberate bound amylase, since they antedate our use of Ballotini beads. However, there is no evidence of a notable tendency towards greater accumulation of amylase with the addition of these reagents, and we may assume that the use of Ballotini beads would not reveal anything of interest.

Radio-Active Amino Acid Studies on Homogenates.

In the previous experiments, the criterion of protein synthesis has been the increase in enzyme content. This is certainly one of the most sensitive methods of detecting protein synthesis, but it suffers from the disadvantage that enzymic activity will depend on the production of "complete" protein molecules. In other words, polypeptide synthesis may quite well be occurring as a preliminary to enzyme formation, but by the enzyme method would go undetected. Radio-active amino acids offer the solution to that problem however,

TABLE 13.

Effect of incubation on the amylase content and ^{14}C-glycine uptake of pigeon pancreas homogenates.

Time of Incubation. Minutes	Experiment 1 Amylase Activity	^{14}C-glycine Uptake	Experiment 2. Amylase Activity	^{14}C-glycine Uptake
0	12,600	0.5	8,400	2.3
15	11,900	1.3	8,420	2.5
30	11,700	1.3	8,650	6.7

Ballotini beads were used and Amylase activity is expressed as mg starch hydrolysed per mg N.

^{14}C-glycine uptake expressed as counts per minute per planchet at infinite thickness.

Sucrose-PO_4 the homogenising medium.

and their use in our studies has provided some useful results.

Comparison of Amylase "Synthesis" and ^{14}C-glycine Incorporation.

Table 13 shows the results obtained in experiments in which both amylase production and amino acid incorporation into protein were measured in the same pancreatic homogenate. It will be seen that in neither case is there any significant increase in amylase content after Ballotini bead treatment and the radio-active data do not indicate any significant uptake of isotope into protein. The uptake of isotopically labelled amino acids in homogenates should be compared with that obtained *in vitro* with tissue slices. (Table 12). The isotopic data would provide further evidence that the increase in amylase content noted in untreated samples of pancreatic homogenate is not due to *de novo* amylase synthesis.

The use of 3-Phosphoglyceric acid in pigeon liver and pancreas. Attempts were made to stimulate greater uptake of labelled amino acids using conditions which have proved successful in rat liver. Zamecnik and Keller (1954) have described a system in which 3-PGA stimulated the uptake of labelled amino acids into the protein of rat liver microsomes, and in which the homogenising medium used was Sucrose 0.35 M, Magnesium Chloride 0.01 M, 0.02 M Potassium phosphate buffer pH 7.8 and Potassium chloride 0.025 M. In the experiments, described above in this section, the homogenising medium was 0.25 M sucrose in 0.2 M phosphate buffer pH 7.2. To reproduce the conditions for the apparent activation

TABLE 14.

The effect of the addition of 3-Phosphoglyceric Acid in the synthesis of Amylase and incorporation of ^{14}C-glycine into protein of cell-free homogenate of Pigeon Pancreas.

Additions to flasks.	Time of Incubation Minutes	Amylase Activity	^{14}C-glycine Uptake
None	0	5080	1.9
	15	5250	1.5
	30	5540	3.3
3-PGA	0	5080	1.0
	15	5000	3.9
	30	5260	2.7
+ 3-PGA + Amino Acids.	0	4570	0.6
	15	4170	1.7
	30	4400	2.2

The homogenising medium was that described by Zamecnik and Keller (1954)[4]. For details see text.

Amylase activity as mg starch hydrolysed per mg. N after Ballotini bead treatment.

^{14}C-Glycine uptake as counts per minute per planchet at infinite thickness.

TABLE 15.

The effect of the addition of 3-Phosphoglyceric acid and ATP on the incorporation of ^{14}C-glycine into proteins of rat liver homogenate.

Additions.	^{14}C-glycine Uptake. Time of Incubation (hours)		
		0	1
None	Exp. 1	7	41
	Exp. 2	4	21
	Mean	6	31
ATP/PGA	Exp. 1	5	71
	Exp. 2	3	34
	Mean	4	53

^{14}C-glycine uptake expressed as counts per minute per planchet at infinite thickness.

Medium used was that of Zamecnik and Keller (1954).

of amino acids described by Zamecnik and Keller (1954) we used their homogenising medium. Table 14 shows the results obtained in an experiment in which the effect of 3-PGA alone and in combination with amino acids was studied on the production of amylase and incorporation of labelled amino acids in parallel. It will be seen that there is no evidence of either amylase synthesis or labelled amino acid incorporation into protein in the pancreatic homogenate and that 3-PGA has produced no effect in this case.

The failure of 3-PGA to stimulate the uptake of ^{14}C-glycine into proteins is contrary to the results obtained by Zamecnik and Keller (1954) when using rat-liver homogenates. To confirm that our conditions were correct for liver, an experiment was conducted following the conditions laid down in the original paper using rat liver cytoplasm. The results we obtained are shown in Table 15 and it will be seen that the addition of 3-PGA has resulted in a doubling of the uptake of the labelled amino acid into rat-liver proteins. These results are similar to those described in the original paper, hence we have successfully reproduced the conditions described.

These results mean that 3-PGA does not behave in the same way with respect to uptake of ^{14}C-glycine in the rat liver and pigeon pancreas homogenates. This may be due either to a difference in species or in organ or in both. Samples of pigeon liver and pigeon pancreas homogenates were therefore prepared and incubations of both carried out with and without ATP and PGA. The results are shown in

TABLE 16.

The effect of the addition of 3-Phosphoglyceric Acid and ATP in the incorporation of ^{14}C-glycine into proteins of pigeon liver and pancreas homogenates.

TREATMENT	LIVER		PANCREAS	
	Before	After	Before	After
	Incubation		Incubation	
None	7	137	4	19
+ ATP/PGA	2	38	5	10

^{14}C-Glycine uptake is expressed as counts per minute per planchet at infinite thickness.
Medium used was that of Zamecnik and Keller (1954).

TABLE 17.

The effect of the addition of 5-FGA on the uptake of ^{14}C-glycine by rat and pigeon liver homogenate.

Treatment.	Rat Liver	Pigeon Liver.
None	21	35
+ ATP	42	51
+ FGA	80	19
+ ATP/FGA	86	19

^{14}C-glycine uptake as counts per minute per planchet at infinite thickness.

The incubation medium was that described by Zamecnik and Keller (1954) and the incubation time was 2 hours. There were no amino acids present.

TABLE 19.

The effect of various substrates on the incorporation of ^{14}C-2-glycine into homogenates of pigeon liver.

Additions.	Counts per minute per planchet.
None	42
Glucose-6-PO_4	23
α-Ketoglutarate	29
fumarate	38
S-PGA	40
Citric Acid	53
Succinic Acid	54
Pyruvate	61
HDP	95

The homogenising medium was that described by Zamecnik and Keller (1954) and ATP was present in all cases.

Incubation time 2 hours.

table 16. The pigeon liver actively incorporates the ^{14}C-glycine and on addition of the 3-PGA and ATP, the incorporation falls very rapidly. In the case of the pancreas, the degree of incorporation is not so great as in the liver, but, once again the incorporation falls rapidly when 3-PGA and ATP are added to the incubation mixture. It would appear from these results that the cause of the differing effect of 3-PGA and ATP is one of species rather than organ.

A confirmatory experiment was conducted in which samples of rat liver and pigeon liver homogenate were prepared. To each was added 3-PGA and ATP, in combination and alone. Table 17 shows the results obtained. The rat liver has responded as before (Table 15) i.e. ATP/3-PGA stimulated the incorporation of ^{14}C-glycine by a factor of at least 2. In the pigeon liver, however, (Table 17) neither ATP nor 3-PGA, nor the combination of them, has any stimulating effect. On the contrary, 3-PGA has a marked depressing effect, the incorporation being reduced to about half of that in the control sample.

<u>The use of alternative energy sources.</u> Since 3-PGA does not seem to be a suitable stimulant for the incorporation of radio-active amino acids into homogenates of pigeon tissue, an attempt was made to find an alternative energy source. Pigeon liver was homogenised in the usual way and samples were taken and various possible energy sources added. The results are shown in Table 18. The most effective addition appears to be hexose diphosphate. Four

TABLE 19.

Comparison of the effect of the addition of 3-PGA and HDP on the uptake of ^{14}C-2-glycine by homogenates of pigeon liver and pancreas.

Addition.	Liver.	Pancreas.
None	6	5
HDP	12	5
3-PGA	6	4

^{14}C-glycine uptake expressed as counts per minute per planchet at infinite thickness.

Time of incubation 2 hours.

Homogenising medium was that of Zamecnik and Keller (1954).

TABLE 20.

The use of Hexose Diphosphate as an energy source and the effect on the Amylase production and ^{14}C-2-glycine uptake by pigeon pancreas homogenates.

Time Mins.	Treatment	Amylase Content		
		I	II	III*
0	-	12,000	4,650	-
	+ HDP	11,900	4,625	6,500 (4.9)
30	-	11,100	4,475	-
	+ HDP	11,400	4,525	6,375 (4.3)
60	-	11,150 (10.5)	4,550 (11.9)	
	+ HDP	11,050 (8.2)	4,850 (8.1)	6,450 (1.9)

In experiments 1 and 2 the homogenising medium was that of Zamecnik and Keller (1954), whereas in experiment 3 sucrose phosphate was used.

Amylase activity is expressed as mgs. starch hydrolysed per mg of nitrogen after treatment with Ballotini beads.

The figures in parenthesis represent ^{14}C-2-glycine uptake expressed as counts per minute per planchet at infinite thickness.

TABLE 21.

The results of incubation of extracts of acetone-dried pigeon pancreas. (Straub system).

Time.	Treatment	Amylase Units per mg. N.
0	None	340
	Ballotini beads	325
2 hours	None	150
	Ballotini beads	165

The results are the mean of 2 samples for each figure.

Amylase Units are Smith and Roe Units.

The results are the mean of 2 samples for each figure.

Amylase Units are Smith and Roe Units.

substrates (Glucose-6-PO_4, α-ketoglutarate, fumarate and 3-PGA) apparently have a depressing effect on the uptake of labelled amino acids, but no attempt was made to confirm these.

The effect of hexose-diphosphate was again demonstrated on the pigeon liver in an experiment in which pigeon liver and pancreas homogenates were run in parallel. Table 19 shows the results and, once again, the stimulating effect of HDP is noted and also the lack of stimulant effect of 3-PGA. With the pancreas, however, HDP apparently did not stimulate the uptake of ^{14}C-glycine and 3-PGA exhibited the usual depression.

Hexose-diphosphate as an energy source. Several experiments were carried out with pigeon pancreas homogenate, using HDP as an energy source in which amylase production and labelled amino acid incorporation were measured in parallel. Table 20 shows the results of such experiments and it will be seen that there is no evidence of either increased ^{14}C-glycine uptake or increased amylase synthesis.

The use of Acetone-extracted Pancreas for Amylase Synthesis in vitro.

We prepared the system as described by Straub (1955) and Table 21 shows the results we obtained. It would appear that no synthesis of amylase has occurred in this case. The reason for the failure to demonstrate amylase synthesis is not known but Straub himself reports that he finds on occasion that, for some unknown reason, no synthesis can be obtained. Perhaps further work on this system may enable more constant results to be produced.

DISCUSSION.

On incubating a homogenate of pigeon pancreas there is an apparent increase in amylase content of the order of 20% when the enzyme is assayed without special treatment. (Fig. 4). This increase takes place during the first fifteen to thirty minutes after which the amylase level drops steadily until, after two hours, the level is less than the initial level. This latter decrease could be due to the action of proteolytic enzymes, though trypsin inhibitor did not appear to have any effect (Fig. 5). There are, of course, other proteolytic enzymes present which could destroy the amylase.

The initial increase observed is of more interest; even after butanol treatment the increase was still evident although the amylase level was about 5% lower than in the untreated samples (Fig. 4). A similar effect on amylase activity has been recorded by Hokin (1955) but Khesin (1953), on the other hand, claims an increase of two to three times in amylase activity after butanol treatment. We can offer no explanation of these apparently contradictory data.

On treatment of the samples with Ballotini beads, however, it is found that there is an increase in the amylase activity of all samples, but especially at the beginning, so that no great subsequent rise is noted (Fig. 4). Thus, it would appear that the amylase is present all the time but is not in an active form and is not estimated under ordinary conditions. After vigorous disintegration or on incubation it is liberated and this accounts for most of the increase observed. Isotopic evidence obtained would support the view that little or no actual synthesis of protein is taking place (Table 15). The system described by Straub (1955) was also prepared, but no evidence of synthesis was obtained.

The desirability of obtaining a cell-free system has been emphasised before, but there are many difficulties to be overcome in achieving this ideal. Our attempts towards this goal have not met with any real success. On homogenising a tissue the cell membrane is ruptured and if the homogenising is very vigorous then the cell contents are broken up. The endoplasmic reticulum in the intact cell consists of a vesicle-like structure on the outside of which little particles of RNA protein are located. These structures may be seen quite readily in electron micrographs of pancreas (Palade and Siekovitz (1956b)). After homogenisation the endoplasmic reticulum may be recovered as the microsome fraction and on examination of this fraction with the electron microscope, vesicular structures are still to be seen, but in much fewer numbers (Palade and Siekovitz (1956b)). Considerable disorganisation of this structure has obviously taken place and the relationship of the endoplasmic reticulum, mitochondria, nucleus etc.

has all been disturbed. A more gentle procedure, such as that adopted by Gale (1955) is perhaps better. Here the Staphylococci are disintegrated by ultrasonic means and the internal damage is not so extensive. The integrity of the endoplasmic reticulum is probably of importance in the initial incorporation of amino acids into protein. Zamecnik and Keller (1954) limited their time of homogenising to twenty seconds in their cell-free incorporation studies and record that times in excess of this very short period result in falling off of the incorporation noted.

The results obtained by Straub (1955) would appear to be at variance with the idea of the necessity for integrity in cellular architecture. However, recently Straub (1957 a, b, and c) has published a series of papers in which he demonstrates that in his system he is not getting a de novo synthesis from amino acids but rather a completion of the amylase molecule from polypeptides already present in the cell. In other words, the vast majority of the work of arranging the amino acids has been accomplished in the unbroken cell when the cellular architecture is intact.

In our studies we have demonstrated that, located in the pancreatic homogenate, is amylase in a form not readily accessible. This amylase appears to be tightly bound and to be freed only after vigorous treatment. The exact nature, location and significance of this material will be the subject of the next section of this thesis.

SECTION 5.

The location of Bound Amylase in The Pancreatic Cell.

INTRODUCTION.

We have shown in the previous section of this thesis that, when a cell-free homogenate of pigeon pancreas is incubated in the presence of amino acids and glucose, an apparent synthesis of amylase occurs. If however, the homogenate is treated in a way designed to break up any particulate material, we find that our apparent synthesis becomes negligible (Fig. 4). This implies that, during incubation, there is a release of preformed amylase which apparently could not be detected as the beginning. Thus, in fact, no *de novo* synthesis of amylase has taken place. The location of this apparently bound amylase was investigated to ascertain whether this, in fact, was confined to one fraction of the cell or was a general property of all fractions.

METHODS.

The birds were prepared as usual and the pancreases were homogenised in 0.25 M sucrose. The various cell fractions were prepared as outlined in Table 1 and the fractions were either suspended in Krebs-Ringer Bicarbonate or 0.25 M sucrose.

Freeze-Drying. The contents of the flask were frozen by placing the flask in solid CO_2-alcohol mixture. The flask was then put into a vacuum desiccator (with P_2O_5 as desiccant), evacuated, and left overnight in the cold. The material in the flask, when absolutely dry, was made up to a known volume and amylase extraction carried out as described.

Freezing and Thawing. The contents of the flask were frozen by being placed in solid CO_2-alcohol mixture and then thawed by allowing the temperature to rise to around 5°. This process was repeated several times as indicated.

Ballotini bead Treatment. This was carried out by the method described in the appendix.

Butanol Treatment. For details of use see appendix.

Amylase Estimation. The method of Smith and Roe (1949) was used. For details see appendix.

Lipase Estimation. The method of Seligman and Nachlas (1950) was used. In this method β-naphthyl laurate is hydrolysed by lipase to give β-naphthol which is converted to a purple azo dye by the coupling of two molecules with tetrazotized diorthoanisidine. This pigment is then extracted with ethyl acetate and the optical density measured colorimetrically. For details of actual estimation, see appendix.

RESULTS and DISCUSSION.

Our original observations, which suggested that some of the amylase in the pancreas occurs in bound form, came from incubation studies (Fig. 4). It was therefore decided to start by studying which subcellular fractions were responsible for this

TABLE 22.

The effect of Incubation for 2 hours in Krebs-Ringer Bicarbonate On the apparent amylase content of cytoplasmic fractions of pigeon pancreas.

Cell Fraction.	Apparent Enzyme Content.		Change.
	Before Incubation	After Incubation	
Mitochondria	8630	9415	+ 9%
Microsomes	1176	3065	+ 160%
Cell Sap	7940	7760	- 5%

The cell fractions were prepared in the usual way and were suspended in K.R.B. Incubation was for 2 hours at 37°.

Results expressed as mgs. starch hydrolysed per mg N.

Mean of 5 observations.

TABLE 23.

The effect of freeze-drying on apparent amylase content of pigeon pancreas microsomes.

	UNTREATED.	FREEZE-DRIED.
Before Incubation.	1490	5512
After Incubation.	4706	5890

The microsomes were prepared as usual and suspended in K.R.B.

Results expressed as mgs. starch hydrolysed per mg. N.

Mean of 4 observations.

TABLE 24.

The effect of repeated Freeze Drying on apparent amylase content of pigeon pancreas microsomes.

Treatment.	Amylase Content.
None.	1800
Freeze Drying x 1	5715
Freeze Drying x 2	5950

The microsomes were prepared as usual and suspended in K.R.B.

Results expressed as mgs. starch hydrolysed per mg. N.

Mean of 3 observations.

TABLE 25.

The comparison of Freezing and Thawing with Freeze-drying and with incubation on apparent amylase content of microsomes.

Treatment	Amylase Content.
None	1783
Freezing 20 times	4222
Freeze-drying	5230
Incubation (2 hours).	4445

The microsomes were prepared as usual and were suspended in K.R.B.

Results expressed as mg. starch hydrolysed per mg. N.

Mean of two observations.

increase on incubation. The usual cell fractions, mitochondria, microsomes and cell sap were prepared from a 0.25 M sucrose homogenate of pigeon pancreas. The fractions were rehomogenised in KRB and incubated for two hours at 37°. The results are shown in Table 22. Here it will be seen that the mitochondria and cell sap show little change in incubation, but the microsomes exhibit an increase of 160% in amylase content. This increase in microsomal content may represent true amylase synthesis, but, since no energy source or amino acids were provided by the medium, this does not seem likely. The alternative explanation for this change in microsomal content with incubation is that the amylase exists in the fresh preparation in a form which cannot be estimated and that incubation releases this bound amylase. If this hypothesis is correct, other methods of liberating amylase, e.g. by disintegration of the microsomes, should lead to an increase similar to that produced on incubation. Our first method was freeze drying. Table 23 shows that incubation of microsomes in a medium containing no ATP or amino acids led to the usual striking increase in apparent amylase content, but freeze drying was equally effective and, moreover, the effect of incubation on enzyme content assayed in this way was negligible. Repeated freeze drying (Table 24) showed that slightly more enzymes could apparently be liberated after two such treatments. The superiority of freeze drying over other methods tried was shown by comparing incubation and freeze drying with freezing and thawing repeatedly. Table 25 shows

TABLE 26.

Effect of Freeze-Drying from different media on apparent amylase content of pigeon pancreas microsomes.

TREATMENT.	Krebs-Ringer Bicarbonate.	0.25 M Sucrose.
None	1600	1900
Freeze Drying	4240	2160
Incubated 2 Hours.	2490	1815
Incubated for 2 Hours and then Freeze Dried.	3780	1760

The microsomes were isolated as usual and were suspended in either 0.25 M sucrose or Krebs-Ringer Bicarbonate.

Results expressed as mgs. starch hydrolysed per mg. N. Mean of two observations.

TABLE 27.

The effect of homogenising with Ballotini beads, butanol, or freeze drying on apparent amylase content of various fractions of the pancreatic acinar cell.

Fraction.	Expt.	Treatment.					
		None	Freeze Dried.	HOMOGENIZED 5 min.	HOMOGENIZED 10 min.	HOMOGENIZED 20 min.	Butanol
Mitochondria	45	100	100	96	100.	95	77
	47	100	102	108	110	109	76
	Mean	100	101	103	105	102	76.5
Microsomes	44	100	106	161	148	146	129
	48	100	99	155	143	146	94
	Mean	100	103	158	146	146	112
Cell Sap	46	100	94	102	115	110	94
	49	100	100	108	105	106	72
	Mean	100	97	105	110	108	83

The homogenising medium used was sucrose-PO_4.

the results which reveal that freeze drying is the most effective procedure. Thus, disintegration of the particles has an action similar to that of incubation and strongly suggests that incubation is merely releasing amylase already present, but not in an active or available form. Since, at the time these experiments were being performed, we were engaged in studies of cell-free synthesis of amylase, it was desirable to use particles suspended in 0.25 M sucrose. Accordingly, some experiments were carried out to determine whether the particles could still be made to yield up their full amylase content after preparation in this material. Table 26 shows that 0.25 M sucrose had a protective effect and full liberation of the enzyme content could not be achieved. When sucrose solutions are subjected to freeze drying they become very viscous and presumably this prevents rupture of the particles.

We thus were forced to explore alternative means of releasing bound amylase from sucrose containing media. Attention was drawn to the fact that Ballotini beads had been effective in securing rupture of very resistant bacteria (Lamanna and Mallette (1954)). We therefore tried this method and the results we obtained are shown in Table 27. In this table the amylase content of the untreated sample is taken as 100 and the values obtained by the various methods are calculated in relation to this figure. As usual, freeze drying is not effective in sucrose media. However, the use of Ballotini beads has increased the content of the microsomes by 58% after five

TABLE 28.

The effect of Various Procedures on apparent lipase content of various fractions of the pancreas.

Fraction.	TREATMENT					Butanol.
	None	Freeze Dried	Homogenized 5 Min.	10 Min.	20 Min.	
Mitochondria	100	100	77	73	54	2
Microsomes	100	95	72	66	67	5
Cell Sap	100	105	78	87	93	4

Fractions were prepared as usual (Table No.1) and were re-suspended in 0.25 M sucrose. Lipase was estimated according to the method described in the appendix.

TABLE 29.

Distribution of Bound Amylase in Cell Fraction of Pigeon Pancreas Isolated in 0.25 M Sucrose.

Fraction.	% Increase after Ballotini bead Treatment. 1.	2.	Mean.
Granules	±0	+3	+1.5
Mitochondria	-3%	+3	±0
Microsomes (1)	+24 (Microsomes (1) and (2) together)	+27	+46 (Microsomes (1) and (2) together)
Microsomes (2)		+40	
Ultramicrosomes	+10	(lost)	-
Cell Sap	+5	-1	+2

The fractions were prepared according to Table No.1 and were re-suspended in 0.25 M sucrose. Ballotini beads were used in accordance with the method described in the appendix.

minutes treatment. On longer treatment enzyme losses are apparent. The mitochondria and cell sap show increments after homogenisation with Ballotini beads which are consistent with some contamination with microsomes. Butanol was also tried at the same time and we confirmed the results we had obtained previously, namely, that butanol is not a suitable means of releasing amylase (Fig. 5).

To investigate whether this binding was limited to amylase we examined lipase under the same conditions. Table 28 shows the results we obtained with this enzyme and from these it will be seen that lipase is being inactivated during the procedures employed. Particularly notable is the effect of butanol which has resulted in complete inactivation of the lipase.

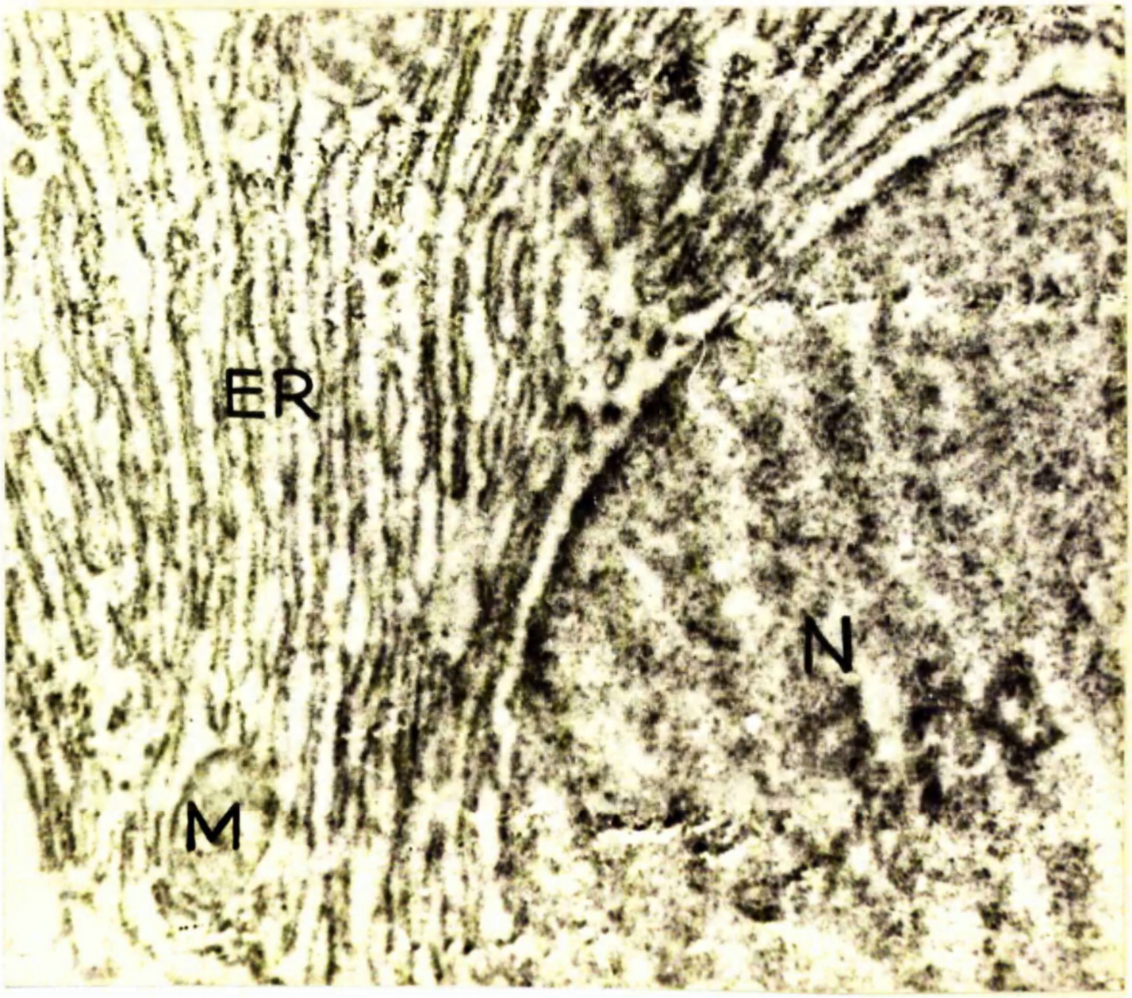
The most effective method we had used for the liberation of amylase from particles suspended in 0.25 M sucrose was homogenisation with Ballotini beads. Using this procedure to locate bound amylase a more extensive scheme of cell fractionation was carried out in 0.25 M sucrose (Table 1) and each of the fractions was suspended in 0.25 M sucrose. The amylase content of each fraction was assayed before and after Ballotini bead treatment. Table 29 shows the results we obtained in two separate experiments. It will be seen that the greatest amount of bound amylase is found in the microsome fraction as before (Table 22). On subfractionation of the microsome fraction the lighter microsomes (i.e. sedimenting between 10,000 and 18,000 g) contain a greater amount of bound amylase than the heavier microsomes. The ultramicrosomes are the only other fraction to show any significant bound amylase. This may be due to the contamination of this fraction with the microsome fraction.

CONCLUSION.

From the data presented above it would appear that amylase present in microsomes freshly isolated at 18,000 g takes a somewhat different form from the enzyme present in the rest of the cell. In view of the possibility, already mooted, that amylase is formed by the microsomes, further investigation of the nature of the bound enzyme will be considered in our next section.

Section 6.

The role of the microsomes in enzyme formation.



<u>Plate 2.</u> Survey section of pancreatic exocrine cell. Magnification x 30,000.

N = nucleus

M = mitochondria

ER = endoplasmic reticulum

INTRODUCTION.

It is convenient at this point to consider the structure in the living cell which is identified with the fractions isolated by the technique of differential centrifugation. The ordinary light microscope has enabled much detail to be observed in relation to the larger structures of the cell. However, many of the important cell particles are beyond the limits of resolution of the ordinary light microscope and so the electron microscope has been used for biological material in cell structure studies. The electron microscope offers many advantages particularly as to resolving power and, as a result, the preparation of specimens for examination must be very carefully done. Special techniques of fixing, mounting and cutting the sections have had to be devised and these are all very time-consuming.

As a preliminary to studying isolated microsome fractions, we made some electron micrographs of whole pancreas cells, and these may now be considered. In our study small pieces of pancreas were removed as rapidly as possible after death and placed in osmic acid buffered to pH 7.4. After dehydration the tissue was embedded in n-butyl methacrylate and sections cut. Plate 2 shows a general survey section of normal pigeon pancreas. The magnification is x 30,000. In the section can be seen the nucleus (N) which is situated at the base of the cell. The cytoplasm of the

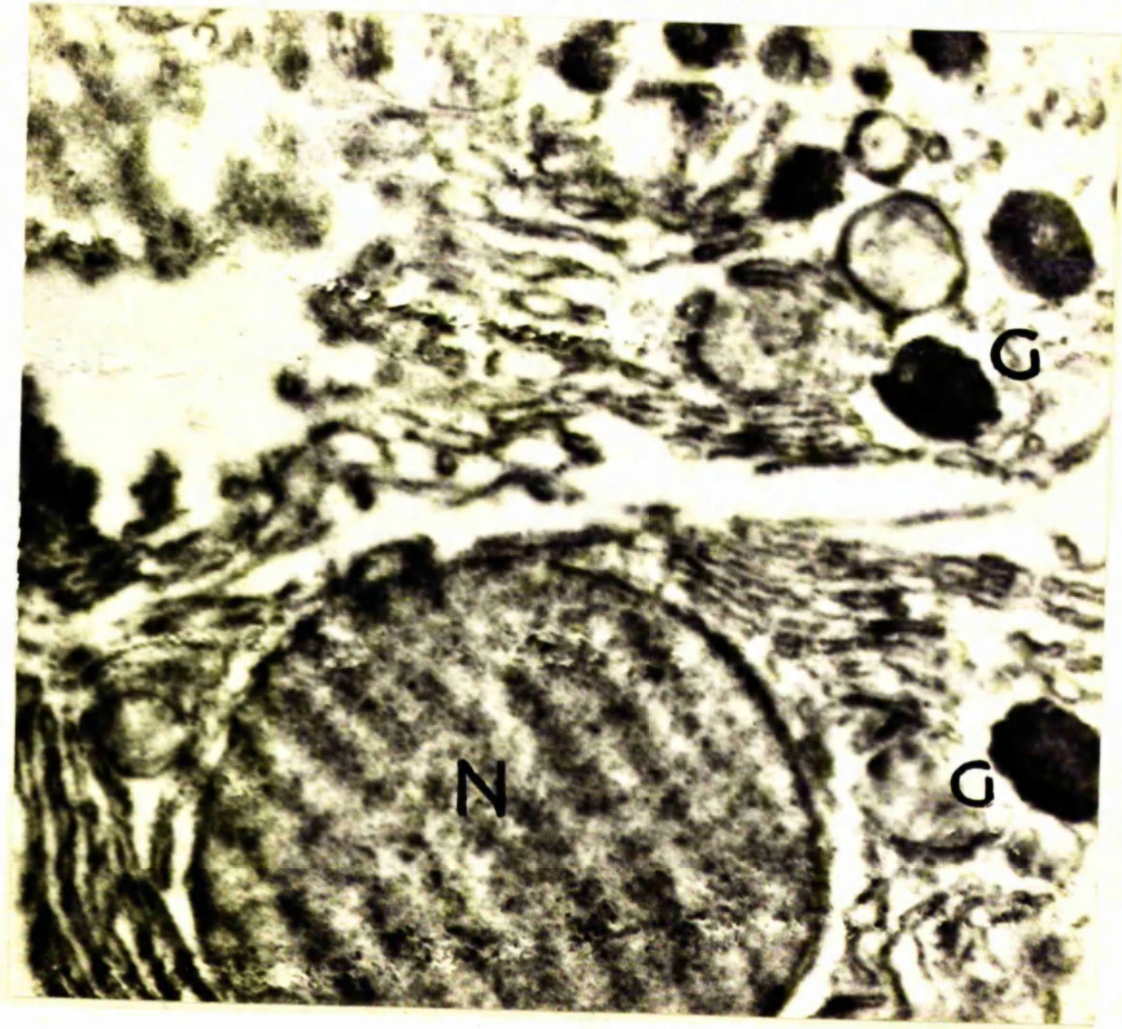


Plate 3. Survey section of pancreatic cell.

Magnification x 30,000.

N = nucleus

G = zymogen granules

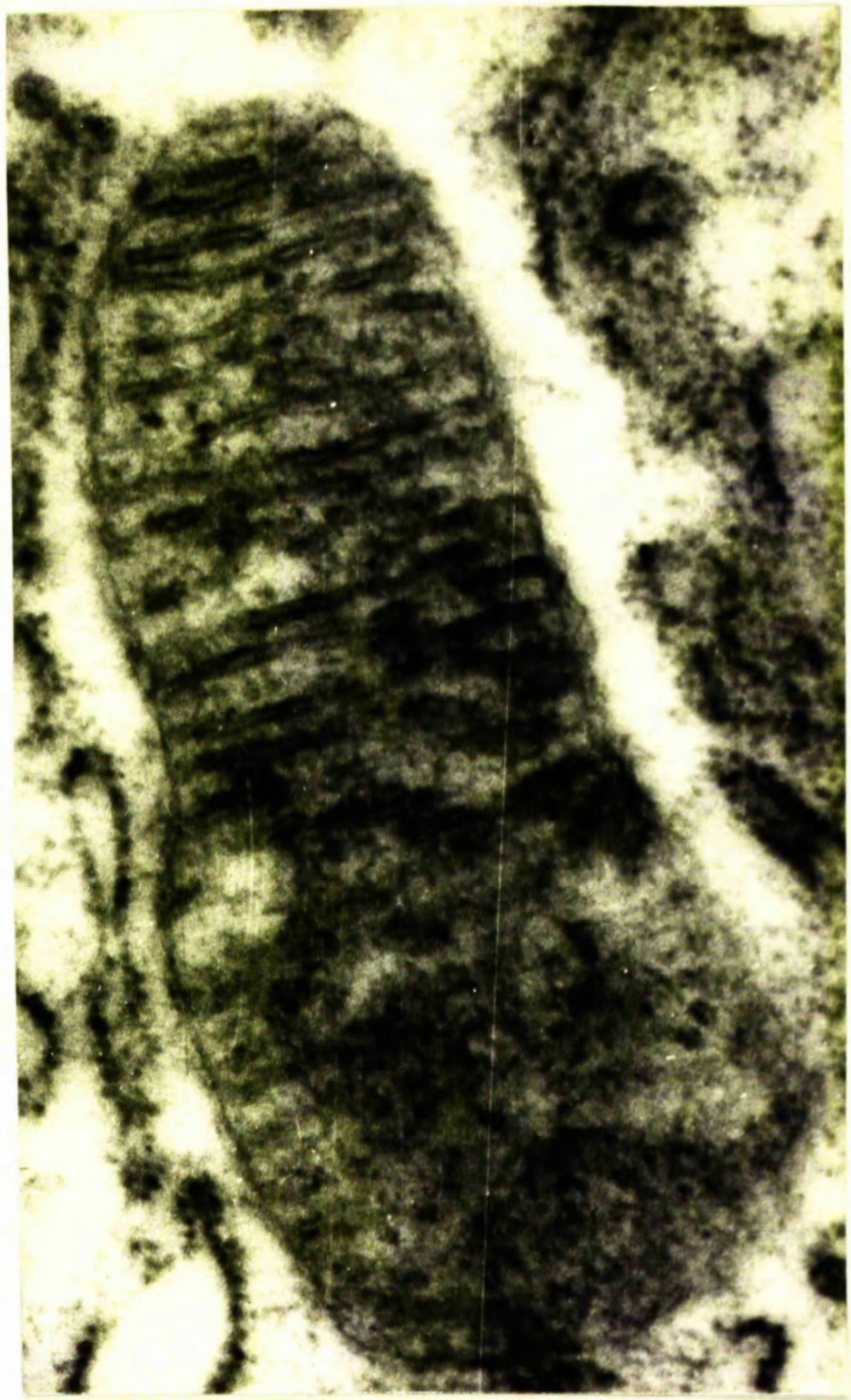


Plate 4. Mitochondrion of pancreatic cell showing the characteristic cristae. Magnification x 37,000.

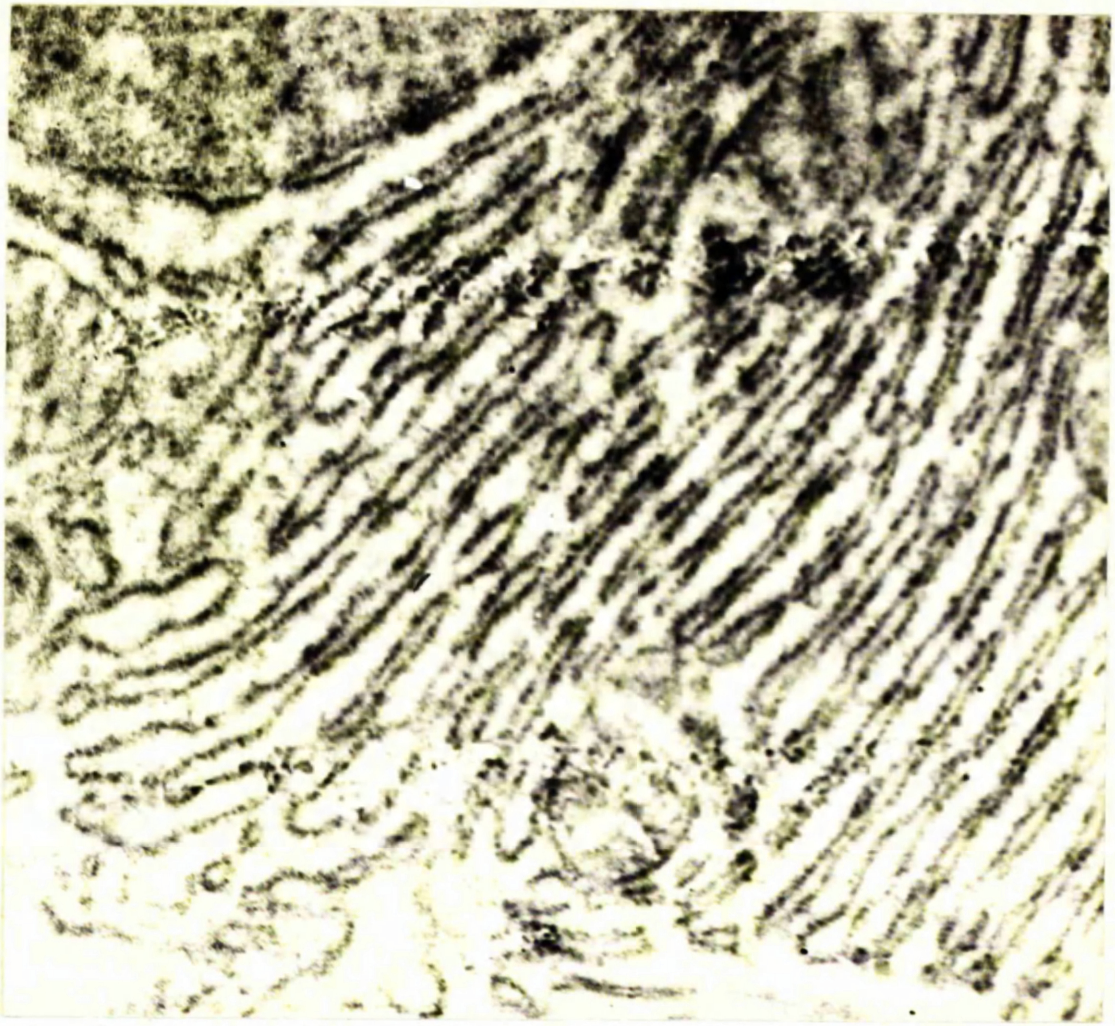


Plate 5. Endoplasmic reticulum of pancreatic cell showing the vesicular and particulate (RNA-rich) components. Magnification x 37,000.

exocrine pancreatic cell contains several mitochondria (M), a well developed endoplasmic reticulum (E.R.), small dense particles and in some cases large electron dense structures, the granules (G). In section the endoplasmic reticulum is represented by numerous profiles of circular, oval, elongated or irregular shape. In the pancreas the elongate form predominate. They are bounded by a thin homogeneous membrane and, in most cases, appear to have no contents. Attached to the outer surface of these profiles are small dense particles which comprise mainly RNA. Plate 3 shows another section of pancreas in which some secretory granules (G) are present. These findings are in accord with the studies of Palade and Siekevitz (1956, a & b) on liver and pancreas tissue. Plate 4, taken at a higher magnification (x 37,000), shows more structural detail of a mitochondrion in which the transverse cristae, characteristic of mitochondria, are well defined. Plate 5 is the one which is of greatest interest from our immediate point of view. This shows the structure of the endoplasmic reticulum in greater detail. In this picture the endoplasmic reticulum is seen in greater detail and the presence of the RNA-containing particles is evident on the outside of some of the vesicles. Occurring free in the cytoplasm are small dense particles which may be RNA particles liberated from the vesicles in the course of the preparation of the tissue. These free particles will give rise to the ultramicrosome fraction sedimenting at 105,000 g in 0.25 M sucrose. It has been demonstrated by Palade and Siekevitz (1956) that the endoplasmic reticulum gives rise to the microsome fraction sedimenting at 105,000 *g* from 0.88 M sucrose or at 18,000 *g* from 0.25 M sucrose (Schneider, 1948).

Those who have associated protein synthesis with microsomal particles have not made any clear distinction between the heavier microsomes and ultramicrosomes in their studies. However, it is apparent that both our isotopic incorporation data (Fig. 2) and our bound amylase studies (Table 29) indicate a difference between the microsome vesicles (18,000 *g*) and the ultramicrosomes (105,000 *g*), the initial incorporation of ^{14}C-glycine and the percentage of bound amylase being higher in the heavier microsome fraction.

In order to try to evaluate the role of the microsomes in pancreatic enzyme synthesis with greater precision, we therefore conducted further experiments on (1) the nature of bound amylase and (2) on ^{14}C-2-glycine uptake by this part of the sub-cellular architecture.

(1) <u>The Nature of Bound Amylase in the Microsome Fraction.</u>

INTRODUCTION.

We have already described the evidence for the existance, in association with the microsomal fraction, of amylase which is not estimated under ordinary conditions. This bound amylase is associated almost entirely with the microsome fraction. The nature of this amylase and the form in which it occurs are of interest in view of the alleged association of the microsome fraction with protein synthesis. We must guard against the possibility of this amylase being merely trapped among aggregates of microsome particles. We must also try to establish whether it occurs in a structure which sediments along with the microsomes during differential centrifugation. In this section we will attempt to show the true relationship between the bound amylase and the microsome fraction.

METHODS.

Samples of microsomes were prepared in the usual way from 0.25 M sucrose homogenates as described in Table 1.

Amylase Estimations were carried out by the method of Smith and Roe (1949) as described in the appendix.

RNA was estimated by the orcinol method - for details see appendix.

Nitrogen estimation - micro-Kjeldahl method as usual.

RESULTS and DISCUSSION.

Our first experiments were designed to eliminate the possibility that the bound amylase was mechanically trapped between microsomal particles which had aggregated during the isolation of this fraction.

TABLE 30.

The effect of Washing at 0^{o} on the Amylase Content of Microsomes.

Fraction	Untreated	Ballotini Beads.	
Microsomes	362	445	(+ 83)
Washed Microsomes	282	360	(+ 78)

The amylase data are expressed as Smith and Roe units per ml of solution.

At the same time we desired to investigate the significance of the bound amylase in relation to enzyme synthesis and to these ends we undertook several experiments:-

(a) The microsome fraction was washed to determine whether the bound amylase could be freed.

(b) The effect of ageing of the microsome preparation on the amount of bound amylase.

(a) The effect of Washing on Microsomal Amylase. A sample of microsomes was prepared in the usual way and re-suspended in 0.25 M sucrose with light homogenisation. One portion of this was re-centrifuged at 18,000 g for sixty minutes, the supernatant decanted and these washed microsomes re-suspended in 0.25 M sucrose. Amylase estimations were carried out before and after Ballotini bead treatment of each fraction. Total nitrogen determinations were also made on the two microsome preparations. The results obtained are shown in Table 30. The amylase content of the microsomes drops by 22% on washing. This loss most likely represents amylase contaminating the microsome fraction, probably from cell sap. On treatment of both the samples with Ballotini beads an increase of approximately the same magnitude, i.e. 80 Smith and Roe units per ml, is found. Thus the effect of washing appears to be confined to the removal of the free amylase and does not apparently affect the bound amylase. If the bound amylase were, in fact, trapped between aggregates of microsomes, one might anticipate that

TABLE 31.

Effect of Washing at 37° on the Amylase Content of Microsomes.

Microsomes	Untreated	Ballotini Beads.
Unwashed	359	550 (+ 191)
Washed at 37°	218	403 (+ 189)

The amylase data are Smith and Roe units per ml of solution.

re-suspension would lead to liberation of a considerable portion. In an attempt to give the particles a more vigorous washing treatment in order to dislodge amylase trapped in aggregates, we carried out the following experiment. Microsomes, prepared in the same way as before, were re-suspended in 0.25 M sucrose with light homogenisation and a portion was shaken vigorously in a water bath at 37° for fifteen minutes. After this period, the microsomes were centrifuged at 18,000 g for sixty minutes, harvested and suspended in 0.25 M sucrose. Amylase estimations were carried out on the untreated and washed microsomes before and after Ballotini bead treatment. Table 51 shows the results which were obtained. It will be seen that following this treatment the microsomes have lost approximately 40% of their amylase, but that on treatment of both samples by Ballotini beads the same total amount of amylase is apparently liberated, i.e. 190 Smith and Roe units per ml. Thus the effect of washing at 37° appears to be essentially the same as washing at 0°, i.e. only on the contaminating amylase and not on the bound material.

(b) Changes in Amylase Content of Microsomes on Ageing. We had previously observed that the amylase content of microsomes apparently increased on standing in water in the cold for twenty four hours or more. To investigate this fact and to relate it if possible to the question of bound amylase, a sample of microsomes was made up in water and allowed to stand in the cold for three days. After this time,

TABLE 32.

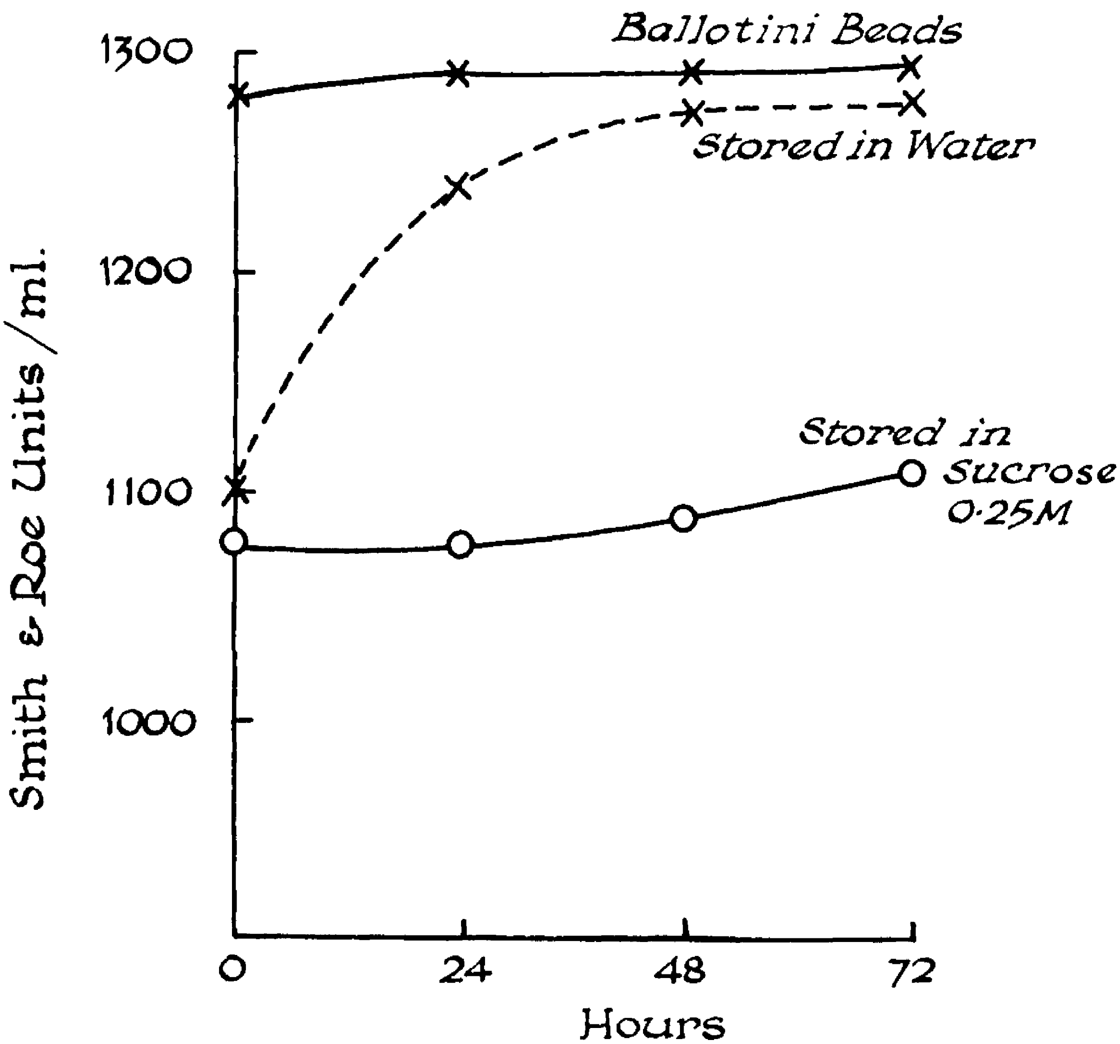
The effect on the Amylase content of Microsomes of storage in water or in 0.25 M Sucrose.

Storage in	Treatment	Hours of Storage.			
		Experiment 1.		Experiment 2.	
		0	72	0	72
Water	None	200	588	157	562
	Ballotini beads.	448	465	325	357
0.25 M Sucrose	None	200	215	157	170

The amylase data are Smith and Roe units per ml of solution.

Fig. 6.

The Effect on the Apparent Amylase Content of Ageing Pigeon Pancreas Microsomes in Water compared to 0·25 M Sucrose.



amylase estimations were carried out and compared to the starting value. A sample of microsomes treated with Ballotini beads was aged in the same way. A further sample of the same microsomes was stored in 0.25 M sucrose in the cold and also re-estimated after three days. The results of two such experiments are presented in Table 32. In both experiments we found that, on standing in water, the apparent amylase content of the microsome suspension had risen and was approaching the level of the Ballotini bead-treated specimen which had remained unaltered during the three days in the cold. On the other hand, the amylase content of the specimen stored in 0.25 M sucrose remained essentially similar to the original value. A further experiment confirming these findings is shown in Fig. 6. It can therefore be concluded that suspension in water causes some special change resulting in the bound enzyme becoming assayable.

The question thus arises, does this amylase become detached from the microsomal particles when it is made assayable by ageing, or do the particles undergo some change which results in exposure of the substrate to the enzyme. This problem was investigated by separating the microsomes from the suspending fluid at various times during the ageing process, and determining how much of the amylase had migrated into the surrounding fluid. With these data, we have also correlated changes in electron micrographs of the microsomal particles, using the technique elaborated by Palade and Siekevitz (1956) for examining isolated cell fractions.

TABLE 55.

Effect of ageing in Water on the Amylase and Ribose Content of Washed and Unwashed Microsomes.

Fraction.	Time of Incubation (Hours).			
	0	24	48	72
Microsomes	1100	1240	1260	1250
Microsomes *	1280	1290	1280	1290
Supernatant	790	790	800	760
Washed Microsomes	408	480	864	468
Washed Microsomes *	588	576	584	528
Supernatant	24	74	48	56

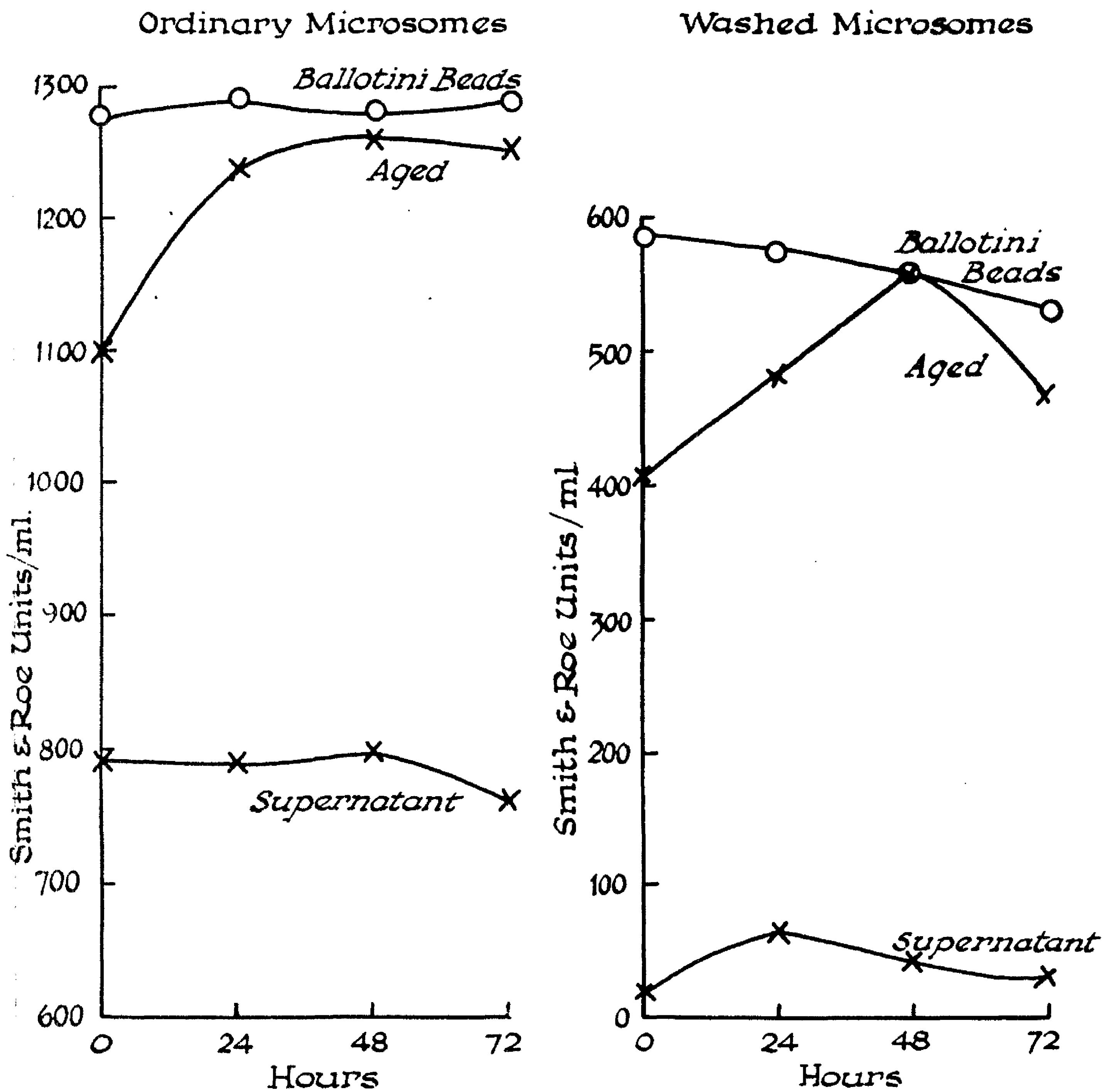
Fraction	mg. N per ml.	mgs. Ribose in Pellet.	
		0 hours.	72 hours.
Microsomes	0.750	1.48	0.955
Washed Microsomes	0.415	0.77	0.478

* Ballotini bead treated.

Amylase data are Smith and Roe units per ml of original solution.

Fig. 7.

The Effect of Ageing in Water on Amylase Content of Ordinary and Washed Microsomes.



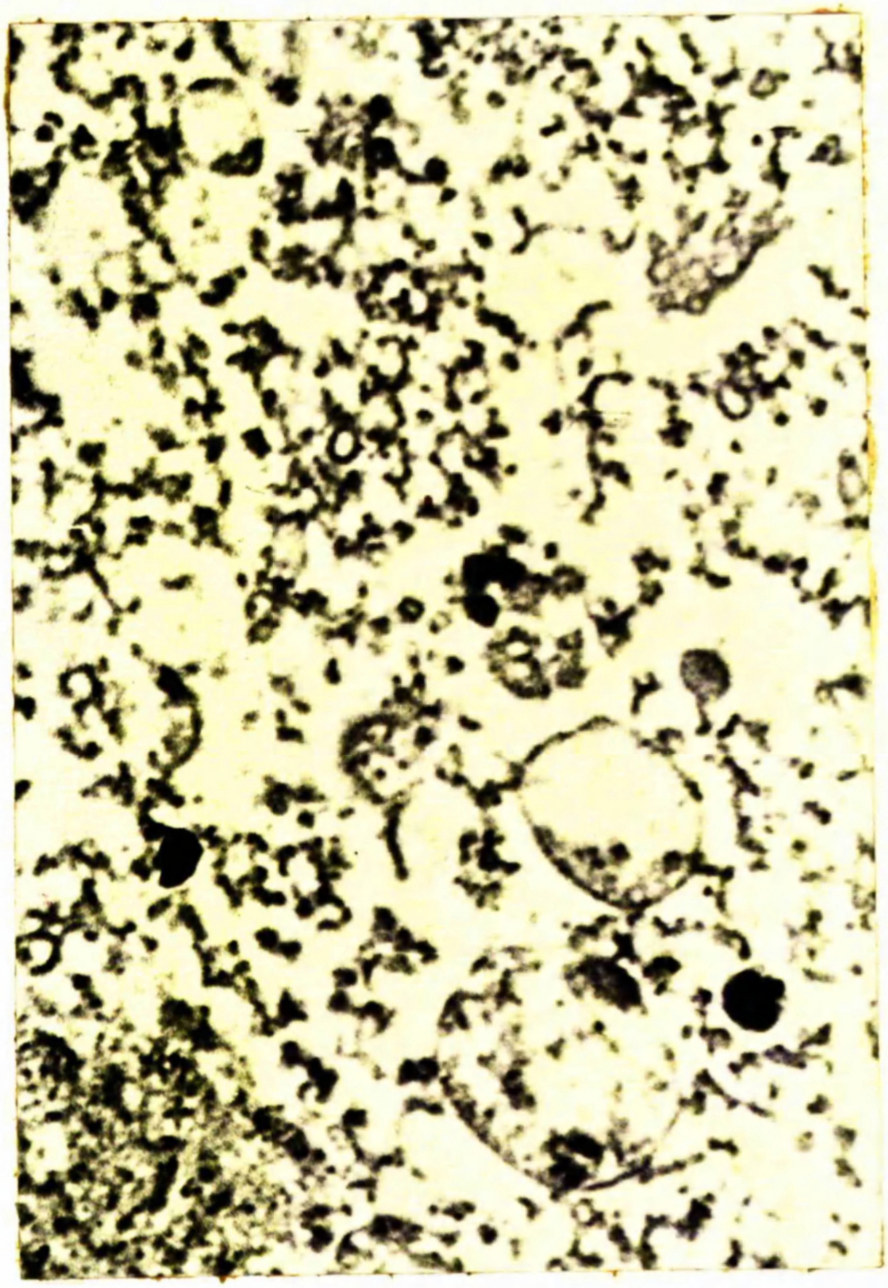


Plate 6.

Ordinary Microsomes: Electron Micrograph of pancreatic microsomes isolated in 0.25 M Sucrose. Note the presence of the vesicular structures. (V).

Magnification x 14,000.

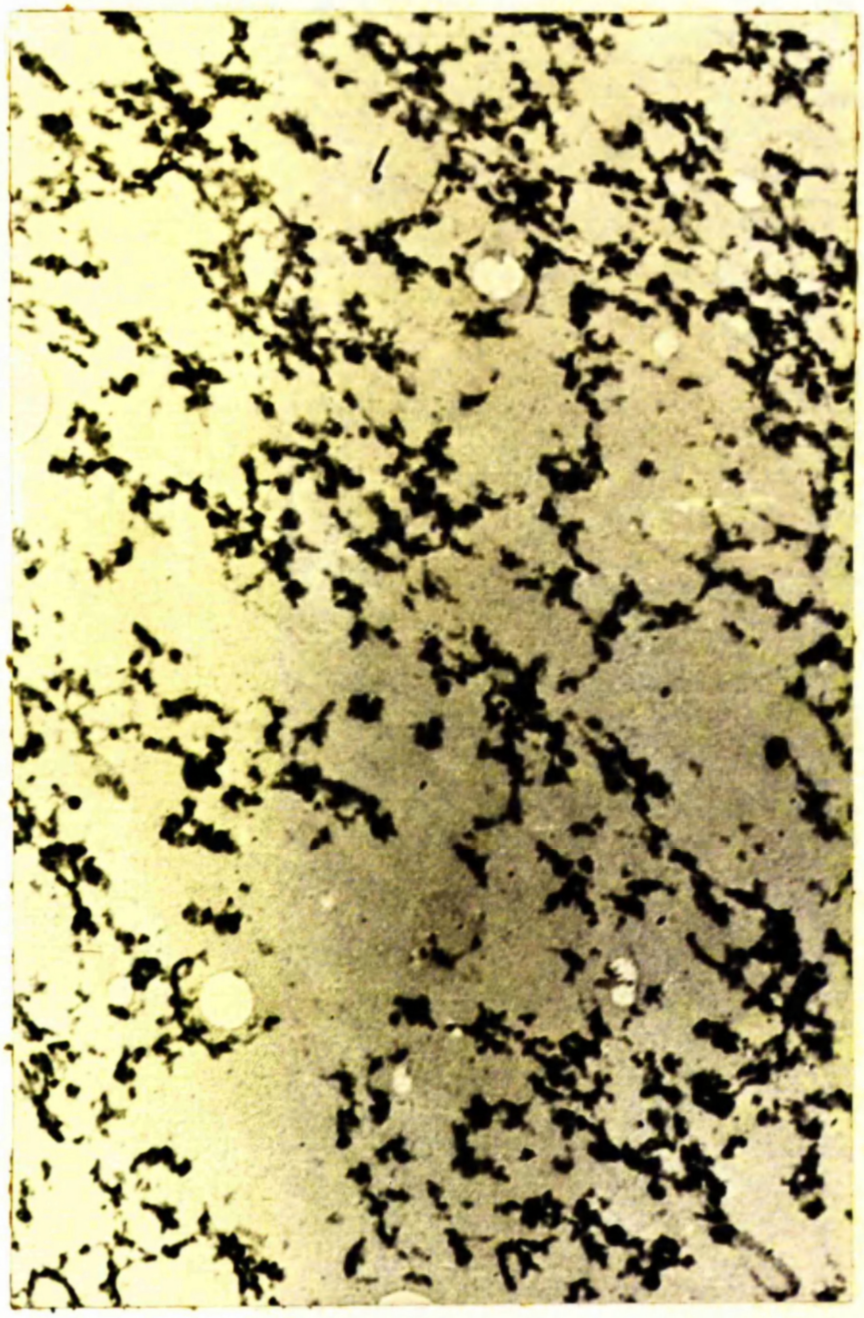


Plate 7.

Washed Microsomes. Electron Micrograph of washed pancreatic microsomes shows the absence of the vesicular structures noted in Plate 6. Magnification x 20,300.

The microsomal fraction was prepared in the usual manner and a sample taken of the original pellet for electron microscopy. (Plate 6). The remainder was suspended in sucrose and the amylase content of this material is shown in Table 33 and Fig. 7. It yielded an extra 180 amylase units per ml of microsome suspension when treated with Ballotini beads (bound amylase). A portion of the fraction was washed in 0.25 M sucrose at 37° for fifteen minutes. The pellet spun down from this material (washed microsomes) was submitted to electron microscopy (Plate 7) and also assay for free and bound amylase. From Table 33 it will be seen that, whereas the free amylase content had gone down by more than a half, as a result of the washing procedure, the bound amylase still stands at 180 units per ml. of original microsome preparation. In other words, washing has removed only free amylase contaminating the preparation. This confirms the findings recorded in Table 31.

The effect of ageing on the bound amylase of the freshly prepared and of the washed microsomes was then investigated. A portion of each pellet was re-suspended in water and stored at 0°. At 0 hours, 24 hours/48 hours and 72 hours samples were taken and spun at 18,000 *g* and the amylase content of the supernatant fluid estimated. (Table 33 and Fig. 7). Irrespective of whether the microsomes had been washed or freshly prepared, there was a slow conversion of bound amylase to free amylase, so that after seventy two hours storage in water the amylase content assayed directly agreed with that obtained

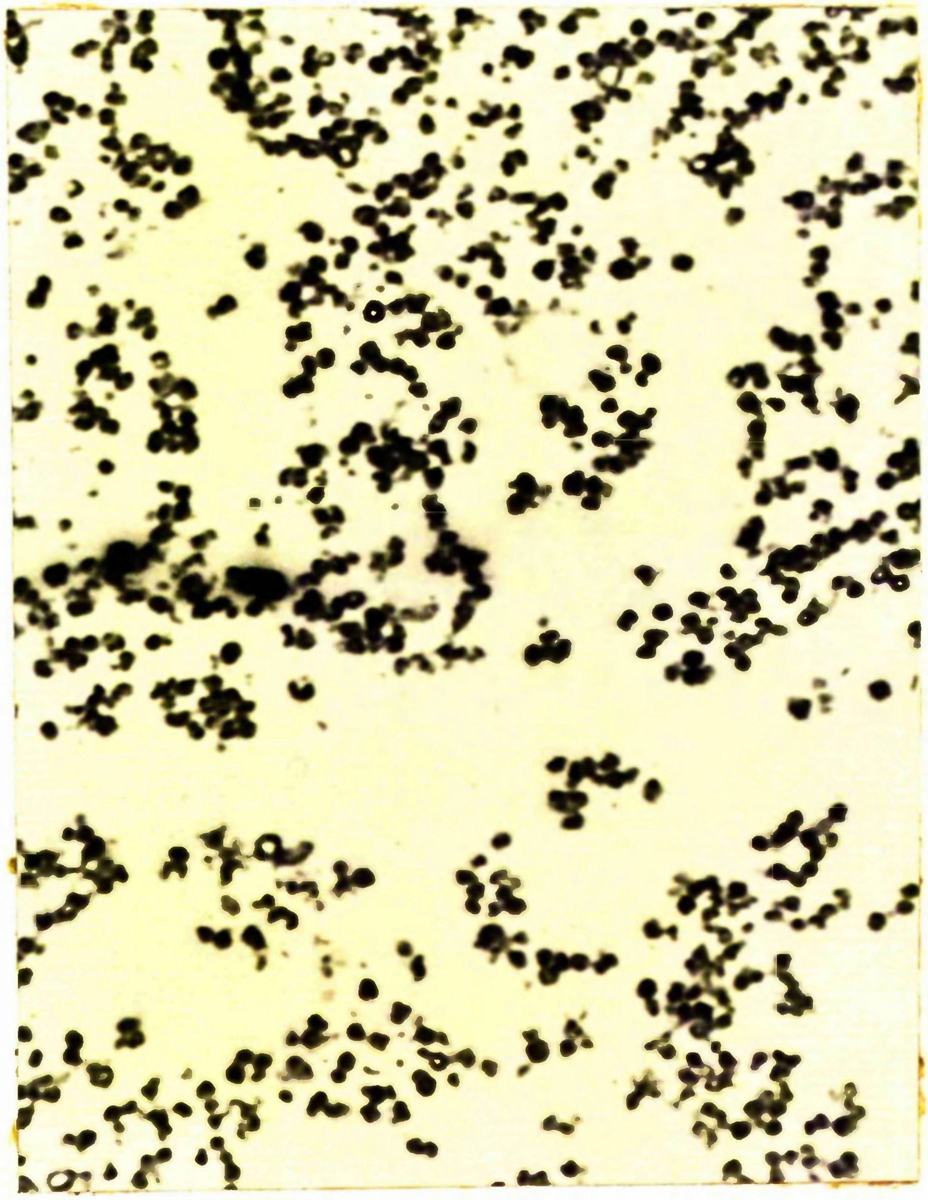


Plate 8.

Aged microsomes. Electron Micrograph of pancreatic microsomes aged in water for 72 hours. Note the swollen vacuolated structures which are now present. Magnification x 19,000.

following Ballotini bead disruption. This confirms the data given in Table 32 and Fig. 6, namely that storage in water leads to liberation of bound amylase. At each time-interval, the suspension of microsomes in water was spun at 18,000 g for sixty minutes, and the amylase remaining in the supernatant fluid assayed. The data show that the liberation of bound amylase in the ageing process was not accompanied by the appearance of a corresponding amount free from the sedimented particles (Table 33. Fig. 7). In other words, the amylase, though now capable of being fully assayed, was still attached to the particles. At this stage, a sample of the microsomal deposit was examined by electron microscopy (Plate 8).

The electron micrographs corresponding to these changes are of some interest. Plate 6 is a typical example of the untreated microsome fraction, magnification about x 21,000. There appear to be present in this fraction large thin walled vesicular structures, approximately 1,000 - 5,000 mμ in diameter, with rather diffuse contents. These structures, in view of their content do not appear to be endoplasmic vesicles, which in the intact cell are empty, and must represent either mitochondria or mature granules which have undergone partial disintegration. They are very similar to degenerated mitochondria found on isolation in 0.25 M sucrose by Birbeck and Reid (1956). Scattered throughout the rest of the field are numerous small dense particles of average size 30 mμ in diameter. The origin of these structures is probably from the vesicles of the endoplasmic reticulum. It is unlikely that they represent the RNA particles alone since these latter structures are much smaller (10 - 15 mμ in size) according to Palade and Siekevitz (1956) and Plate 5 of the whole pancreas. These dense particles, in some cases, appear to be arranged in circles reminiscent of a network, similar to that of the endoplasmic reticulum (Plate 5).

The picture obtained with washed microsomes is somewhat different as is shown in Plate 7. The most obvious difference is the absence of the large, thin-walled vesicles which have been eliminated by the washing procedure. The dense particles seen in the unwashed microsomes now appear to be a little larger than 50 mμ in diameter and, moreover, appear to be more evenly distributed throughout the field, probably due to the loss of the fine debris. Thus, in this preparation, we have noted the apparent loss of the vesicular structures, fine debris and most important, the increase in size of the dense particles.

On ageing the microsomes, the picture obtained is quite different from that of the other two preparations. Plate 8 shows that there is a complete absence of the fine ground material and that the particles are now quite discrete. The size of these particles has increased considerably to over 100 mμ in diameter. In many cases there is the suggestion of a vacuole or a less dense area in the centre.

In addition to the changes in amylase content which have been discussed above, we measured the changes in nitrogen and RNA content of the microsomes during the washing procedure. Table 53 shows the results and it is apparent that about 50% of both of these components has been removed by washing. The changes which have occurred in the RNA content of the microsomes during the ageing process are also recorded in Table 53. After seventy two hours it will be seen that the sedimentable material has, in both washed and unwashed microsomes, lost about 30% of the original RNA of the pellet. Since the magnitude of the change in both the untreated and the washed microsomes is the same, the possibility of action by degradative enzymes e.g. ribonuclease is the most likely explanation.

CONCLUSIONS.

The experiments which we have carried out to establish the nature of the association of bound amylase and the microsome fraction have been described above. From the data obtained it would appear to be established that re-suspension and vigorous washing do not alter the availability of the bound amylase, which renders unlikely the possibility that the binding is merely trapping. Moreover, electron micrographs of the isolated fractions have demonstrated that the changes occurring with washing comprise the removal of mitochondrial and other debris, which as we know from previous studies (Table 29) do not contain any bound amylase. On the other hand, the ageing of the microsomes in water for seventy-two hours has the effect of unmasking the amylase but not of liberating it into the supernatant fluid (Table 53 and Fig. 7). It is thus still attached to particles. Electron micrographs of the microsomes in this condition after ageing (Plate 8) demonstrated that a considerable amount of alteration had taken place in the structure of the particles in the microsome fraction. Whereas in the unwashed microsomes these particles were dense and of the order of 30 mμ in diameter, they had now the appearance of swollen, slightly vacuolated particles of about 100 mμ in diameter.

It would appear from the series of micrographs presented that the particles which are undergoing the change represent fragments of endoplasmic reticulum to which are still attached the RNA-rich bodies described by Palade and Siekevitz (1956) and which, when free from the reticulum, appear to constitute the ultramicrosome fraction. The changes in chemical composition of this fraction during washing and ageing provide further evidence on the nature of the binding of amylase. During the washing procedure, some of the protein and RNA is lost along with the amylase from these microsomes. When the washed microsomes are stored in water, no further leakage of amylase into the supernatant fluid occurs, (Table 55), but the loss of RNA proceeds, presumably because of the continuing action of ribonuclease. Although only a part of the RNA is thus decomposed, all of the bound amylase is liberated. It is therefore unlikely that the liberation of amylase is dependent on its detachment from an RNA-template. In support of this contention it will be noted that, following storage in water, the RNA content of the washed microsome pellet was half that of the pellet derived from the untreated microsomes. In spite of this difference in RNA content it will be observed from Table 33 that the pellet from the untreated microsomes retained the same amount of total amylase (1290 in whole specimen less 760 in supernatant = 530 units in pellet) as did the washed microsome pellet (528 units), and, more important, that in both cases the amylase was now all in assayable form, despite the very different degrees of RNA loss. Thus the unmasking of the amylase by ageing would not appear to be dependent on loss of RNA.

(2) ^{14}C-2-glycine uptake into Microsomal Sub-fractions.

INTRODUCTION.

We have in Section 2 of this thesis presented evidence that the pancreas is similar to other tissues, in so far as the highest uptake of radio-activity shortly after administration of ^{14}C-glycine is located in the microsomes. From the previous description of the structure of the microsomes it has been shown that there are two distinct parts i.e. a membranous part and the RNA-rich particles and it would be important to know how much each part contributes to this high ^{14}C- uptake. The separation of these two parts has been achieved by the use of sodium deoxycholate (Littlefield, Keller, Gross and Zamecnik (1955)) and they have shown that the highest labelling is to be found in the RNA-protein part. We accordingly attempted to separate the two fractions of pancreatic microsomes and to examine the distribution of the labelled amino acid. As mentioned earlier the ultramicrosome fraction consists of particles of RNA-protein (Palade and Siekevitz (1956)) and we have already demonstrated that this fraction has a lower uptake than the microsome fraction (Fig. 2). By the use of sodium deoxycholate in this fraction, we considered that it might be possible to prepare an RNA-protein fraction of the ultramicrosomes with the same activity as that from the microsomes.

Methods.

Pigeons were injected with ^{14}C-2-glycine ten minutes before sacrifice and the pancreases were homogenised in 0.25 M sucrose. Microsomes and ultramicrosomes were prepared in the usual way. (Table 1).

<u>Treatment with Deoxycholate.</u> The fractions were treated with sodium deoxycholate as described by Littlefield et al (1955) with a slight modification in the buffer used. The approximate weight of the fraction was determined and three quarters as much sodium deoxycholate, in the form of a freshly prepared, cold 5% solution in Tris buffer 0.2 M at pH 8 was added directly to the pellet. The pellet was

homogenised with a cold pestle until no gross particles remained. After standing on ice a few minutes, the tube was filled with ice-cold water, mixed and centrifuged at 0° for one hour at 105,000 *g*. The supernatant and precipitate were separated by decantation and the protein in each precipitated with TCA. Lipid extraction was performed as usual (see appendix) and the dry protein was hydrolysed for eighteen hours with 6N Hydrochloric acid in a closed tube. The hydrochloric acid was removed *in vacuo* and the glycine estimated as the DNP derivative (see appendix for details).

In their original paper Littlefield et al (1955) recommend the use of glycyl-glycine buffer for the addition of the deoxycholate. As we were interested in the specific activity of the glycine in the protein fractions isolated this buffer was not suitable. We adopted the use of Tris buffer at the same molarity and pH.

Treatment with Trichloracetic Acid. Under the conditions of our experiments, we were hydrolysing protein samples with 6N Hydrochloric acid for eighteen hours. These protein samples were known to contain differing amounts of RNA which, under the conditions of hydrolysis, may give rise to a variable amount of glycine from the purine nucleus (Munro and Naismith - private communication). As this glycine would not be labelled to any extent (Table 11) this would result in a varying dilution of the radio-activity of the protein glycine. We, therefore, in most cases, treated the proteins twice with 10% TCA for fifteen minutes at 90° to remove the RNA (Schneider (1945)), before

TABLE 34.

Sub-fractionation of the Microsome and Ultramicrosome fraction of Pigeon Pancreas with sodium deoxycholate.

Cell Fraction	Sub-fraction	EXPERIMENT 1	2	3		
		No TCA	TCA	No TCA	TCA	% Loss
Microsomes	Whole	655	590	498	299	40
	RNA protein	915	238	441	258	46
	Soluble protein	525	854	513	421	18
Ultramicrosomes	Whole	394	328	295	222	25
	RNA protein	283	(lost)	313	189	40
	Soluble protein	450	422	399	300	25

The pancreases were removed 10 minutes after the injection of ^{14}C-glycine and the microsomes prepared as usual (Table 1).

TCA treatment was carried out according to the method described by Schneider (1945).

The radio-active data are expressed as counts per minute per μM glycine.

hydrolysing with Hydrochloric acid.

RESULTS and DISCUSSION.

The results of these experiments are presented in Table 34. It will be noted that the radio-activity in the ultramicrosome fraction in all cases is lower than that in the microsome fraction. This finding of lower uptake in the ultramicrosome fraction, as compared to the microsomes, has been noted before (Fig. 2). On treatment of either the microsomes or ultramicrosomes with deoxycholate, it is seen in almost every case in the microsomes and in every case in the ultramicrosomes that the highest activity is to be found in the soluble protein. In other words the RNA protein has less activity than the vesicular protein. In one case, Table 34, experiment 1, the RNA protein has a greater activity than the vesicular protein in the case of the microsomes. The difficulty of controlling this separation is the most likely reason for the apparent contradiction in these results. There are several factors beyond control at the moment in the separation procedure, which must be considered as fairly crude. In the original paper Littlefield et al (1955) discuss the optimum conditions for rat liver microsomes and demonstrated that different separations could be achieved with slight variations in conditions. More recently, Palade and Siekevitz (1956b) in discussing pancreas microsomes mention that they seem to be more difficult to fractionate with deoxycholate than are rat liver microsomes.

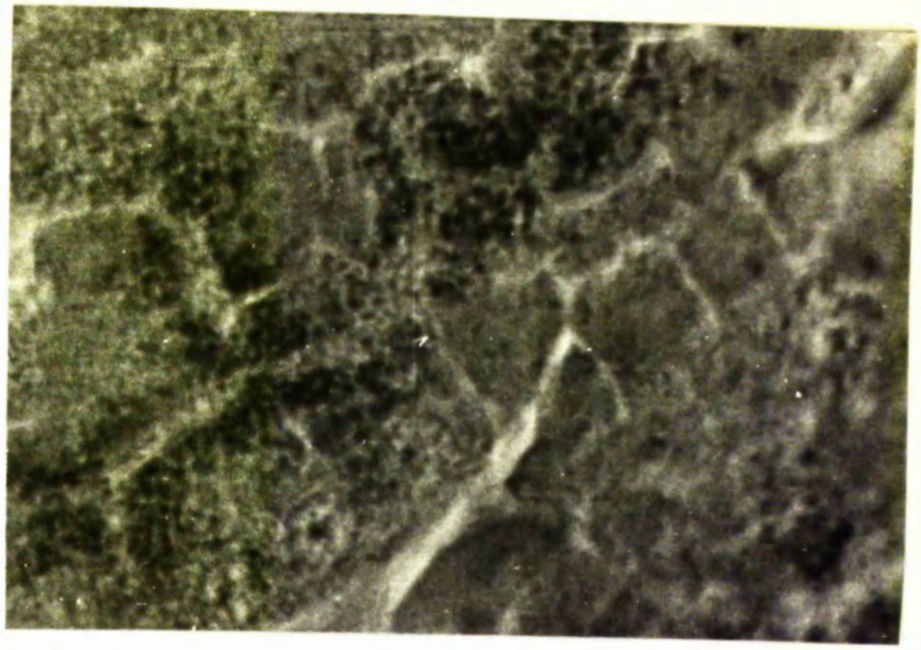
Some of the above experiments were carried out on proteins without removal of attached RNA. Since the purine nucleus can undergo slight degradation to glycine under the conditions for protein hydrolysis, we thought it advisable to run some experiments in which the RNA was removed with hot TCA. The objection to this, as a routine procedure, is the loss of protein from an already small sample. In one case, (Table 34, experiment 3), the treated and untreated specimens came from the same sample of pancreas. In the other comparison, different pancreases were used. Since pancreatic RNA purines are very poorly labelled by ^{14}C-2-glycine (see Section 3), we had expected that any degradation to glycine would dilute the apparent uptake into protein glycine. Moreover, since the ultramicrosomes are richer in RNA than the microsomal vesicles (Palade and Siekevitz (1956)), this source of weakly labelled glycine might be a technical explanation of the lower labelling of glycine obtained in this fraction. (Table 34). However, Table 34 shows that even in TCA treated specimens the ultramicrosomes are still less highly labelled with ^{14}C- in their protein glycine. Comparison of the effect of TCA treatment on the protein associated with the RNA and the soluble protein reveals an interesting finding (Table 34, experiment 3). Whereas the soluble protein loses some 18-28% in its specific activity as a result of this treatment, the fraction containing RNA loses 40 - 46% in activity. This is apparent in both the microsome and ultramicrosome portions. This suggests that removal of RNA with TCA also results in loss of a protein containing highly radio-active glycine. The smaller loss in activity from the soluble protein fraction is consonant with the presence of some RNA remaining in this fraction. Littlefield et al (1955) draw attention to the incompleteness of separation by deoxycholate, and this is supported by the data of Palade and Siekevitz (1956). The presence of a somewhat labile protein fraction associated with RNA may be the reason why in Experiment 1 (Table 34), the radio-activity data for the microsome fraction contrast with those in subsequent experiments.

The significance of these findings may be considered in relation to the structure believed to be present in each cell fraction. The microsome vesicle fraction contains proteins of the endoplasmic reticulum together with RNA-protein particles. The ultramicrosomes are considered to represent the RNA containing particles free from the endoplasmic reticulum. The ultramicrosome fraction has a lower radio-activity, and this agrees with the finding (Table 34) that, from the microsome vesicle fraction we can obtain in general a lower radio-activity in the RNA-protein precipitated by deoxycholate. This RNA-rich fraction should correspond to the particles forming the ultramicrosomes. In theory we might therefore expect to obtain identical radio activities in the RNA-protein fractions isolated from both the microsome and ultramicrosome fractions. In fact, Table 34 shows that this is not so. In experiment 3, the activities in the microsomal RNA fraction are higher, and in experiment 1 the difference is very considerable. We have already drawn attention to the lack of reproducibility of the separation procedure and, until this is improved it is not possible to say whether such discrepancies are real.

GENERAL DISCUSSION.

The Synthesis of Enzymes by the Pancreas.

A



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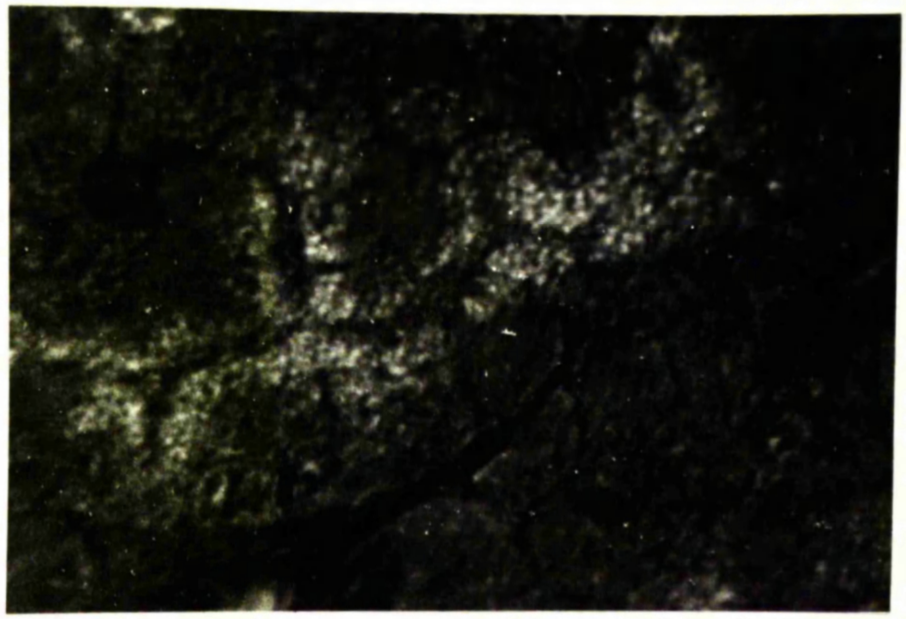


Plate 9. INTERFERENCE MICROGRAPH OF PIGEON PANCREAS.

Frozen-dried preparation, thickness 3μ, dry-mounted-wax removed in Xylol - section examined in xylol. Photographed at infinite fringe separation. Magnification x 2,000.

In (a) dark areas = high mass

(b) light area = high mass (depends on position in fringe in which photograph is taken). The picture obtained is consistent with a high degree of density, comparable to that of the spermatozoon head, and is thus suggestive of a solid protein particle.

GENERAL DISCUSSION.

The Nature of the Secretory Granules.

It is commonly believed that the secretory enzymes of the pancreas are located in the granules. This belief had its foundation in histological observation. Thus, a pancreatic cell was seen to contain a great number of granules and these disappeared on the receipt of a suitable stimulus. Since the secretion from the cell under these conditions contained enzymes, the assumption was made that the enzymes were located in the granules.

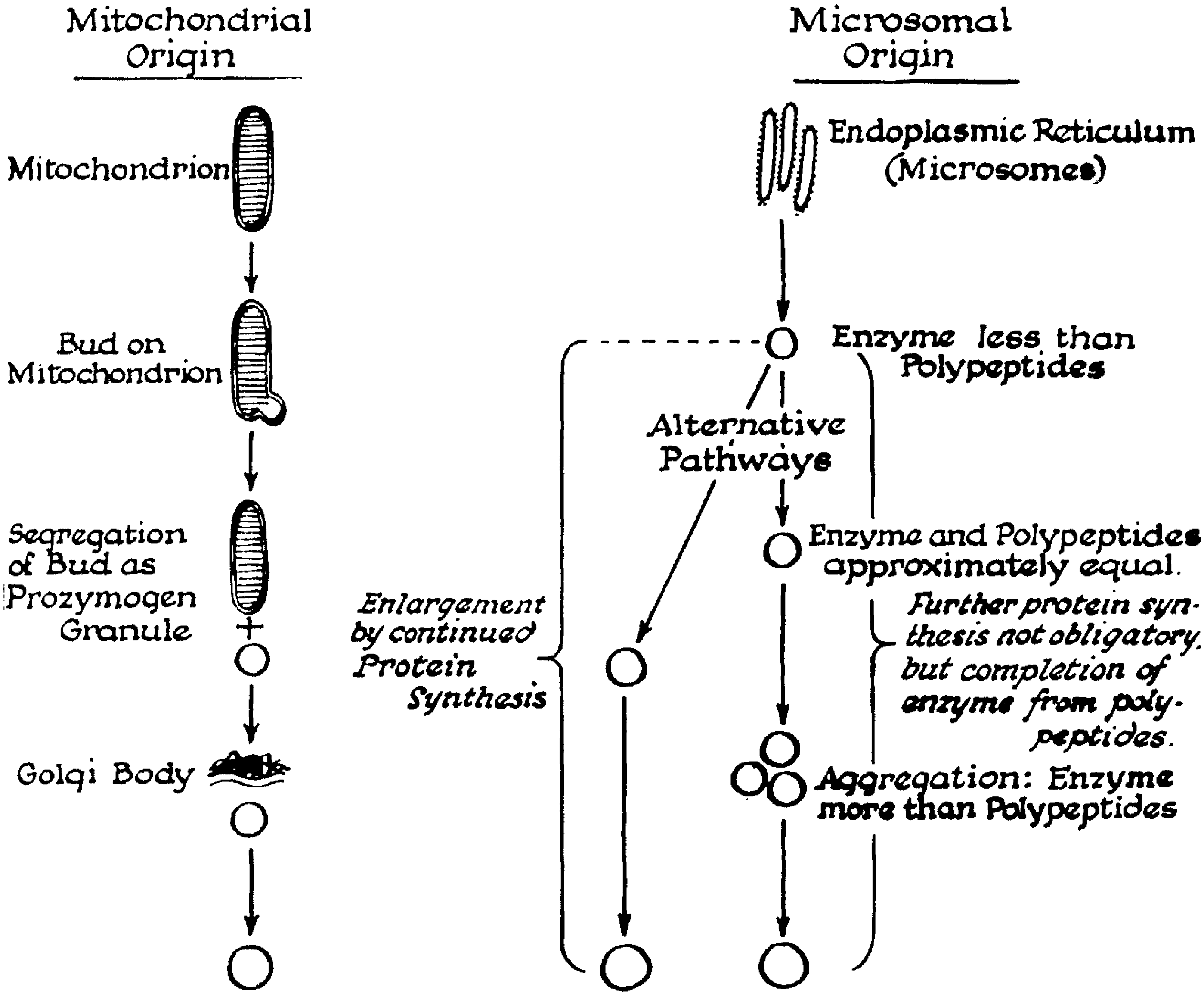
Recent studies on the composition of isolated pancreatic granules show that they do in fact contain secretory enzymes. Thus Hokin (1955) has demonstrated that the granules have a high concentration of amylase, protease and lipase, and that the concentration of both phospholipid and RNA is extremely small. The solids in the granules are therefore likely to be proteins. We have been able to show by microscopy that the granules are, in fact, of high density consistent with a high content of protein. Plate 9 shows the pictures obtained by interference microscopy and it will be seen that the density of the granules is much higher than that of the surrounding cytoplasm. The granules must therefore be solid masses of protein, presumably enzymic, and the question of the formation of granules is thus germane to the problem of protein synthesis.

The next question which arises is whether one granule contains a battery of enzymes or whether separate granules contain individual enzymes. Hokin (1955) has observed that, on solution of the secretory granules in water, 95% of the amylase is liberated free into the supernatant fluid, whereas the lipase and protease remain bound to the insoluble material. It is thus possible that there are different granules for different enzymes.

This question of the relationship of individual enzymes to the secretory granules should be considered in relation to the presence of enzymes in other parts of the cell. Despite the high enzymic content of the granules, the digestive enzymes of the pancreas are not wholly confined to the cell particulates. Our data (Table 5) reveal that the cell sap contains more amylase, both in total amount and per mg of N, than any other part of the cell. The distribution of other pancreatic enzymes has been investigated by Siebert (1955) who finds that 65% of the trypsin is contained in the nuclear fraction. The distribution of lipase has also been studied Siebert (1955) and the mitochondrial fraction contained the highest concentration. Previous to this report by Siebert, we had found that the lipase distribution was very different from that of amylase in that the mitochondrial fraction was the richest and that the cell sap contained little. The difference in distribution in enzymes and in particular of amylase suggests that, either the finding of the highest concentration of amylase in the cell sap represents the real distribution in the

Alternative Theories of Pancreatic Granule Formation

Fig. 8.



Mature Granules with very high enzyme content.

living cell, or the fragility of granules varies according to their enzyme content. Thus the amylase-containing granules might be more fragile than those bearing lipase etc. and consequently amylase may become liberated into the cell sap during homogenisation. A difference in the behaviour of enzymes after rupture of the granules noted by Hokin has already been mentioned, and from this it would appear that amylase is fairly easily liberated from granules, whereas lipase and protease are not. An alternative hypothesis is that each granule bears all the enzymes but that amylase is more readily lost on rupture.

Histological Observations on Granule Formation.

The origin of the granules has been of considerable interest to many workers, mainly histologists and cytochemists, and the theories which have been put forward tend to depend on interpretation of structure rather than on absolute proof. The current theory (shown diagrammatically in Fig. 8) on the formation of the secretory granules is that prozymogen granules are produced from the mitochondria and that these bodies gradually enlarge and migrate to the Golgi region of the cell where they become arranged along the long axis of the Golgi canal (Plate 1 page 26). The Golgi apparatus apparently makes a contribution to the prozymogen granule which allows it to mature to a secretory granule. During this maturation, the prozymogen granule becomes vacuolated and enlarged. The mature secretory granules aggregate to form larger secretory bodies in which form the enzymes are discharged.

The evidence in support of this theory has come from a variety of sources. The mitochondrial origin of the prozymogen granules was suggested first by Hirsch and Duthie (1953) who noted that these bodies would colour with Janus Green, a typical mitochondrial reaction. Challice (1954), with the aid of the electron microscope, has demonstrated the presence, attached to the mitochondria, of small structures which he claims are prozymogen granules. The movement and development of the granules has depended on visual observations and therefore involves subjective interpretation. The association of granules with the Golgi body has also been noted by the electron microscope (Sjöstrand and Hanzon, 1954) The contribution of the Golgi body to the maturation process is not known but it has been suggested that small particles approximately 400 Å in diameter are added to the prozymogen granules (Dalton and Felix, 1954). Lacy (1956) has noted that, before the prozymogen granules receive their contribution from the Golgi body, they will stain with neutral red. Once they have been in association with the Golgi body, however, the neutral red staining property is no longer so evident. The exact reactions involved here are not known, though neutral red is considered to be a selective fat stain.

In view of the importance placed on the Golgi apparatus in the process of granule maturation, modern opinion about this cell particulate may be presented. The Golgi apparatus has long been a bone of contention among the histologists (see Symposium on the Golgi Body - Microscopical Journal 1954). It would seem to be the consensus of opinion that the Golgi apparatus does exist in the living cell but its function within the cell is completely unknown. Electron micrograph studies have demonstrated its presence (Sjöstrand and Hanzon, 1954). Schneider and Kuff (1954) have prepared Golgi substance from the epididymides of rats by cell fractionation techniques using density gradients. Analysis of this Golgi fraction demonstrated that RNA, phospholipid and alkaline phosphatase were present. That the Golgi substance represented a particular cell constituent was shown by the finding that no ascorbic acid, DNA, cytochrome oxidase or deoxyribonuclease were present. These materials might have been expected to be present if this Golgi substance were just a random selection of the homogenised particles. The significance of the actual chemical constituents in the Golgi apparatus is not known, but the presence of alkaline phosphatase is worthy of note since this enzyme has been demonstrated to occur in other tissues where intense protein synthesis is taking place. (Davidson, 1949). Thus the contribution of the Golgi apparatus to the granules during their formation is not understood, but in view of the alleged presence of alkaline phosphatase and of RNA, it is possible that it could contribute to some part of the process of enzyme synthesis.

We may conclude that the evidence provided by the histological and cytochemical approach tends to give a highly speculative picture as to the origin and significance of the processes involved in granule formation and one not easily susceptible of proof.

The case for Granule Formation by Microsomes.

Since the formation of the granules involves accumulation of proteins, their origin becomes of interest in connection with protein synthesis. The modern theory of protein synthesis favours the view that the free amino acids are activated in the cell sap and that they are arranged in particular sequences by the RNA molecules acting as a template. There is, as we have stated in the introduction, no compelling proof of this template hypothesis. Since the microsomes contain the greatest quantity of RNA, these structures have been accorded great significance in the mechanism of protein synthesis. It would therefore be in keeping with the evidence obtained in other tissues, if pancreatic enzyme formation could be linked with microsomal activity. We shall therefore consider how far this link can be forged on the basis of the available evidence.

(a) Changes in Microsome Composition during the Secretory Cycle:

In our studies on the distribution of nitrogen in the different cell fractions of the pancreas in various stages of the secretory cycle, we obtained evidence that the microsome fraction is important in the formation of enzymes. Thus, during depletion the microsome fraction suffered a loss of 30% in protein content whereas the other cell fractions were virtually unaffected. During depletion the loss in amylase from this fraction was at the same rate as the loss in protein (Table 6). This parallelism of protein and enzyme discharge might lead to the supposition that the products secreted from the microsomes are pure enzymes, which are, of course, protein in nature. However, the amylase per mg. of nitrogen in the microsomes is very low, and, since the material lost has the same amylase content per mg. nitrogen as the whole microsome it must also be of low activity. On the other hand, the mature granules have a high amylase activity per mg. of nitrogen. This would suggest that the microsome fraction is losing particles of fixed composition in regard to amylase and protein, the non-enzymic proteins predominating at this stage. Then, at some later stage in the formation of large granules, enzyme enrichment must take place.

(b) Presence and Nature of Bound Amylase in Microsomes.

We have demonstrated that, associated with the microsome fraction, there is amylase which is not capable of being assayed without further treatment. This amylase appeared to be located in a structure which required vigorous disintegration to effect rupture and so render the amylase free for estimation. Moreover, if these structures were allowed to age in water then the amylase became available for estimation but did not diffuse freely into the supernatant fluid, apparently remaining attached to the particles. From these data we assume that the particle becomes more permeable to the substrate but that the enzyme is still attached to the particle.

It has already been demonstrated (Table 29) that the binding of amylase is associated essentially with the microsome fraction and that the ultramicrosome fraction contains very much less of this form of amylase. Since it is considered that the ultramicrosome fraction is composed of RNA-rich particles detached from the endoplasmic reticulum, this finding would imply that the bound amylase is not associated with the RNA-rich part of the endoplasmic reticulum but rather with larger structures of the reticulum.

This dissociation of the RNA-rich particles from the microsomal property of binding amylase is supported by the data presented in Section 6. In this section, we deduced that the process of unmasking amylase by ageing in water did not parallel the loss of RNA from these particles.

Electron micrograph studies have also demonstrated that the property of binding of amylase is associated with fairly large particles. When freshly isolated (Plate 6) the visible particles tend to be large (over 50 mμ), suggesting that the endoplasmic reticulum has been disintegrated rather than that the RNA-rich particles attached to this reticulum (10 - 15 mμ in diameter) have been sheered off. On ageing the microsome fraction, discrete swollen structures were produced (Plate 8) and in these the amylase is free for estimation but remains attached to the particle. There would thus appear to be structural changes in the microsome fraction during ageing which accompany the unmasking of the enzymic activity.

(c) ^{14}C-glycine Uptake by the Microsomes.

In Section 2, we demonstrated that the pancreatic microsome fraction isolated at 18,000 g exhibited the highest ^{14}C-glycine uptake into protein at short time intervals after injection, the ultramicrosomes being appreciably less active (Fig. 2). This accords with the view propounded on the basis of studies with other tissues, namely that the microsomes represent the site of initial amino acid incorporation.

In a later section of this thesis, we attempted to subdivide the microsomal pellet into RNA-rich and RNA-poor protein fractions. This led to the result that less radio-activity was generally found in the RNA-rich fraction (Table 34). This result is in keeping with the observation that less ^{14}C-glycine is taken up by the ultramicrosomes which, according to Palade and Siekevitz (1956) are made up of RNA-rich particles detached from the endoplasmic reticulum.

It would thus appear that the initial uptake of radio-activity, like bound amylase, is not very closely connected with the presence of RNA in the pancreatic microsomes. This contrasts with the findings of other workers using different tissues, generally liver (Hultin, 1955, Littlefield et al, 1955, Simkin and Work, 1957). However, the formation of secretory granules by the pancreas, places this organ in another class from these tissues and may explain why our data differ from those of others working on other organs. In view of this divergence between the pancreatic data and that of other tissues we feel that our case would be greatly strengthened by alternative approaches to the segregation of the RNA-rich fraction of the microsomes, such as the procedure described by Dallam (1955) and recently used with success by Simkin and Work (1957).

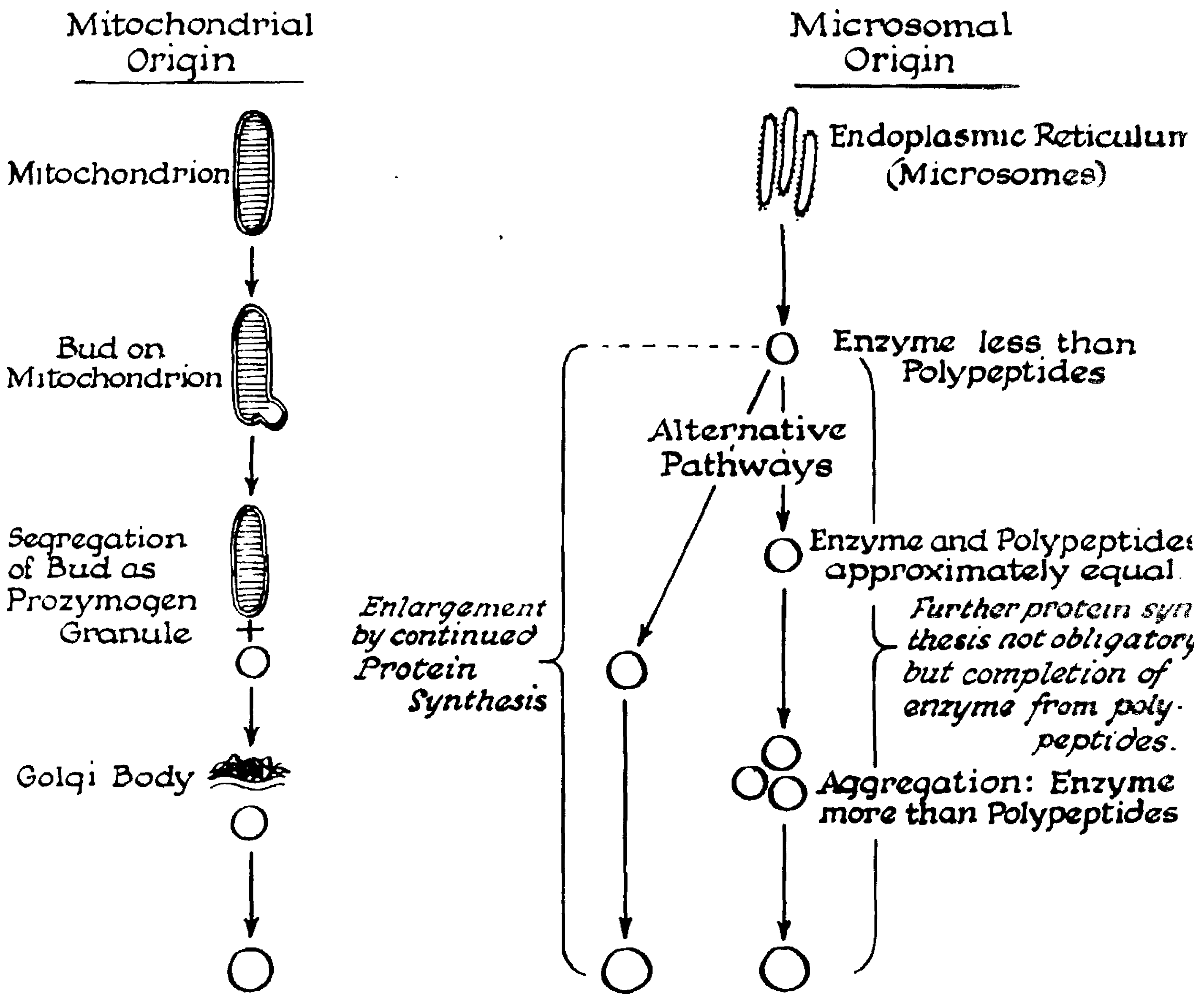
The case for the site of enzyme synthesis in the pancreatic microsomes may be briefly summarised in table form :-

Fraction	Protein Content during Depletion /Repletion	Bound Amylase.	^{14}C-Glycine Uptake.
Mitochondria	No change	None	+
Microsomes	Large change	+ + + +	+ + +
Ultramicrosomes	?	+	+

From the data presented we consider that there is a very strong case for the initial stages of granule formation occurring in the microsome fraction of the cell. We would suggest that the formation of the granules occurs initially in a microsome fraction

Alternative Theories of Pancreatic Granule Formation

Fig. 8.



Mature Granules with very high enzyme content.

as shown in Fig. 8. It is suggested that the microsomes liberate small resistant particles which apparently contain amylase and non-enzymic material, perhaps polypeptide in nature. Our protein distribution figures suggest that these particles contain a large proportion of non-enzymic proteins. The enzymic content of these particles must increase because of the difference which exists between the enzyme content of the microsome fraction and the granular fraction. Two alternative pathways are suggested in the diagram in Fig. 8, one being a process of aggregation of small particles without necessarily involving much new protein formation, the other being enlargement of individual particles by continued enzyme synthesis. There is some evidence for the maturation, i.e. development of enzymes in these particles, being an active metabolic process. Lacy (1956) has shown that immature granules, visible under the light microscope, will segregate neutral red which prevents the further development of these granules. Hence under these conditions neutral red granules accumulate in the cell. This suggests that neutral red interferes with some maturation process, possibly enzymic in nature. This, however, does not inevitably imply *de novo* protein synthesis. Thus Straub (1957b) has claimed that "mitochondrial" fraction of the pancreas will produce amylase given only two amino acids, arginine and threonine. Our experiments with this type of system suggest caution in accepting such results. Straub postulates that precursors formed in the microsomes are present in his mitochondrial fraction and that the two amino acids are required for the completion of the amylase molecules. Straub's method of fractionation brings down a broad spectrum of particles, which we would prefer to call mitochondria + small granules + large microsomal elements. His claim of amylase synthesis, even if accepted, is thus not indicative of amylase production in the mitochondria or even in the granules of mitochondrial size. In other words, the production of amylase molecules in microsomal elements is not incompatible with his data. The possibility that the granules continue to synthesise enzymes de novo from the free amino acids present in the cell sap does not seem likely on other grounds, namely the higher radio-activity found in the microsomes as compared to the granules. If de novo synthesis were continuing, the radio-activity in the granular fraction and mitochondrial fraction would be comparable to that found in the microsome fraction. From our own data, it is seen (Fig. 2) that the microsome fraction is about six times as active as the granule fraction at early stages of the experiment.

The maturation of the granules does not seem to involve the participation of RNA, since no loss in RNA or evidence of metabolic replacement is obtainable during the secretory cycle (Section 3). Thus the process of maturation is not similar to the maturation of the reticulocyte, where RNA is apparently involved in the synthesis of haemoglobin from free amino acids even after the reticulocyte has left the bone marrow (Holloway and Ripley, 1952). The RNA in the case of the granules may have already played its part in sequentialising the amino acids in the polypeptide intermediates and the final stages may well be independent of a template.

The essential difference between the histological findings and the biochemical data therefore lies in the origin of the prozymogen granule. Histologists, as indicated earlier (Fig. 8), favour a mitochondrial origin, whereas, we have presented evidence favouring a microsomal origin. From the biochemical viewpoint the role of the Golgi body is completely unknown as is the participation of the mitochondria in the maturation process. Further work is required to consolidate the data we have presented in this thesis, particularly in regard to the distribution of radio-active amino acids in the microsome fraction during early stages of the incorporation process. We are hoping, in future experiments, to investigate the uptake of radio-active amino acids into the fraction particularly associated with bound amylase.

SUMMARY.

Summary.

A study has been made of the biochemical changes associated with the formation of secretory granules in pigeon pancreas. This subject was treated as one aspect of the biosynthesis of proteins.

Section 1. The composition of the pancreas at rest and during secretion.

(1) The effect of different homogenising media on the distribution of cell constituents in the cell fraction obtained by differential centrifugation was investigated. The two media which were used were 0.25 M sucrose and 0.25 M sucrose buffered at pH 7.2 with 0.2 M phosphate buffer. The use of the latter medium, which had a higher molarity, led to less efficient sedimentation of particles, with the result that the cell sap fraction isolated in this medium was enriched both with total nitrogen and with amylase derived from other fractions.

(2) The distribution of amylase and nitrogen was determined in five sub-cellular fractions using 0.25 M sucrose as homogenising medium. The order of occurrence of amylase was as follows : -

Cell sap > Microsomes > Mitochondria > Granules > Ultramicrosomes.

The distribution of nitrogen was found to be thus : -

Cell sap > Microsomes > Ultramicrosomes > Mitochondria > Granules.

When the concentration of amylase per mg N was calculated it was found that the amylase was more concentrated in the cell sap than in the granules. These results indicated that the amylase of the cell sap could be derived from the rupturing of granules during the homogenising process. This interpretation would be valid only if the ruptured

granules had a higher amylase content than the granules which were recovered intact in the form of the granular fraction.

(3) The examination of the pancreas during the secretory cycle showed that neither the weight nor the total nitrogen content underwent any significant change. The amylase content underwent a reduction of about 50% during depletion. Two hours later, however, the amylase content had risen to a level above that found in the resting pancreas.

The cellular sub-fraction which underwent the greatest change in amylase content during depletion was the cell sap (- 54%); the microsomes and mitochondria changed by - 30% under the same conditions. During the recovery phase of the secretory cycle, the most marked gain in amylase content was to be found in the cell sap.

Compared with the other cell fractions, the microsomes lost the largest percentage of their initial protein content during depletion. On the other hand, the concentration of amylase per mg N in the microsome fraction was found to be constant during the secretory cycle. This suggested that the microsome fraction was losing particles of a fixed amylase-to-protein ratio.

Section 2. The uptake of ^{14}C-2-glycine by the proteins of different cell fractions of the pancreas.

(1) Measurement was made of the uptake of ^{14}C-2-glycine into the proteins of pancreatic cell sub-fractions. In the first instance, sucrose-PO_4 was the homogenising medium. The results obtained led to the conclusion that the cellular sub-fractions obtained with this medium were not homogeneous.

(2) On repetition of the ^{14}C uptake experiments using 0.25 M sucrose as homogenising medium we found that, at short time intervals, the

microsome fraction had the greatest uptake. At all time intervals the uptake into the ultramicrosomes was less than that into the microsomes.

(3) The pattern of incorporation of ^{14}C-2-glycine in pancreases of birds injected with carbamylcholine was very erratic and no definite conclusion could be arrived at as to any change in incorporation as a result of depletion.

Section 3. The metabolism of RNA during the secretory cycle in the pancreas.

(1) The quantitative change in RNA during the secretory cycle was investigated. It was found that there was no appreciable change in RNA content per gm. of pancreas as compared to controls.

(2) Using ^{32}P as a precursor, no definitely increased incorporation into nucleotides of RNA of the pancreas was obtained during the secretory cycle. The labelling of nucleotides required the use of very large doses of isotope (500 μc ^{32}P per bird).

(3) Using ^{14}C-2-glycine as a precursor of the purines of pancreatic RNA no significant increase in incorporation could be detected after stimulation with carbamylcholine. This confirmed the quantitative and the ^{32}P data.

Section 4. In vitro synthesis of amylase.

(1) Pancreatic slices were used to demonstrate *in vitro* synthesis of amylase. The use of complete or incomplete amino acid mixtures did not appear to influence the production of amylase.

(2) The uptake of labelled amino acids did, however, appear to be sensitive to amino acid supplementation. Omission of tryptophan decreased the incorporation of ^{14}C-2-glycine or ^{35}S-methionine. This would indicate that the synthesis of amylase and amino acid incorporation do not go hand in hand.

(3) The use of cell-free sucrose homogenates of pigeon pancreas resulted in an apparent increase in amylase content on incubation, but no corresponding labelled amino acid incorporation.

(4) Treatment of the homogenate with various agents which would break up any particulate material resulted in the apparent synthesis disappearing. The use of butanol was found to be unsatisfactory as a means of releasing combined amylase, since it apparently inactivates the enzyme.

(5) Various energy-producing substrates were added to the homogenised mixture but led to no increased synthesis of amylase or amino acid incorporation. 3-Phosphoglyceric acid was not apparently stimulant to amino acid incorporation by the proteins of pigeon tissues as it was to rat tissues.

(6) Acetone-extracted pancreas (Straub) was used in an attempt to get cell-free production of amylase, but without success.

Section 5. The location of bound amylase in the pancreatic cell.

(1) The bound amylase detected in homogenates was found to be confined to the microsome fraction.

(2) Freezing and thawing and freeze drying were found to be

effective in liberating the amylase when the microsomes were suspended in a saline medium but were of no value in a sucrose containing medium.

(3) More vigorous treatment was required for the disruption of microsome particles suspended in sucrose and the most effective was found to be homogenisation with Ballotini beads.

Section 6. The role of the microsomes in enzyme formation.

(a) The nature of bound amylase.

(1) Electron microscope studies were made of the pigeon pancreas to establish characteristic sub-fractions of the cell and to detect changes in the microsomal fraction under various conditions. It was confirmed that the microsome fraction consists of a vesicular and a particulate part, the latter being the part rich in RNA.

(2) The possibility that bound amylase was trapped mechanically was considered to be eliminated by the results obtained on vigorous washing and by ageing in water. Washing resulted in the removal of about 50% of the nitrogen and the RNA content. In some cases about 60% of the amylase was removed. Electron micrographs indicated that this was due to the removal of mitochondrial and granular elements. The bound amylase content of the particles was, however, unchanged. Ageing in water for seventy two hours was found to free all the amylase for estimation but did not liberate it into the supernatant fluid.

(3) The enzyme changes produced by ageing in water were correlated

with the changes in structure of the particles by electron micrographs. The particles appeared to undergo swelling and vacuolation.

(4) During the process of ageing in water, while all the amylase was made available for estimation about one third of the RNA had disappeared. Thus the binding of amylase did not appear to be directly associated with an RNA-template-amylase association.

(b) <u>^{14}C-2-glycine uptake into microsomal sub-fractions.</u>

(1) Sub-fractions of the microsomes were examined with a view to determining which fraction was responsible for the high uptake of labelled amino acids.

(2) In confirmation of data in an earlier section, the ultramicrosomes were found to have a lower uptake than the heavier microsomes.

(3) Treatment with deoxycholate to separate the vesicular and RNA-rich particulate proteins resulted in the highest uptake being found in most cases in the vesicular sub-fraction. It appeared that the pancreas may differ from the liver in this respect.

GENERAL DISCUSSION.

The data obtained are used in an attempt to formulate a theory for the production of zymogen granules by the pancreas, in which the microsomes are associated with the initial step in the formation of enzymes.

A P P E N D I X

of

EXPERIMENTAL METHODS.

Appendix of Experimental Methods.

Estimation of Amylase. Amylase was assayed by the method of Smith and Roe (1949) with the modifications recommended by Hokin (1951). This method is based on the starch-iodine colour reaction. The blue colour formed by the reaction of starch with iodine is measured photometrically before and after incubation of soluble starch with material containing the enzyme. The decrease in blue colour obtained after the incubation is a measure of the amylase concentration.

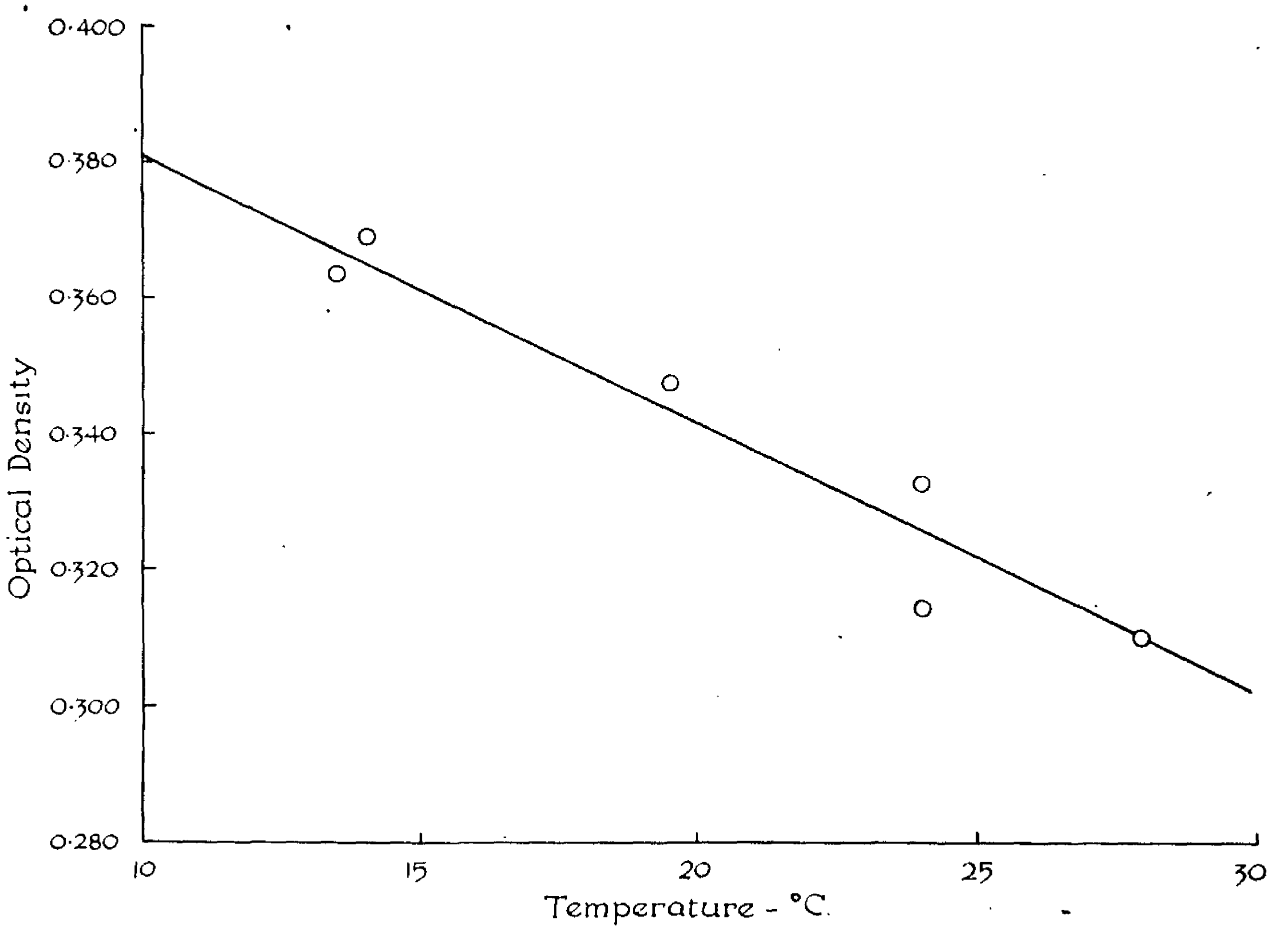
Reagents. (1) Substrate. 5 gms. of soluble starch were dissolved in 100 mls boiling distilled water. To this was added 60 mls Phosphate buffer pH 7.2 and 20 mls of 0.5 M Sodium Chloride. The mixture was allowed to cool and 9 mls of the buffered starch solution was pipetted into each assay tube.

(2) Iodine Reagent. 50 gms. Potassium Iodide and 3 gms. Iodine were dissolved in 1000 mls distilled water.

Assay. 1 ml of the enzyme solution was added to 9 mls buffered starch solution and incubated at 37° for 30 minutes exactly when 2 mls N HCl were added to stop the enzyme reaction. 2 mls of this solution were added to a 500 ml Volumetric Flask containing 400 mls water, 5 mls N HCl and 4 mls Iodine Reagent. The flask was made up to volume and well shaken. Samples were taken and placed in a water-bath at 25° and the colour density measured in the Unicam SP 600 at a wave length of 620 mμ. Control samples were prepared in the same way except

Fig. 9.

Relationship of Starch-Iodine Colour to Temperature



that water was used in place of the enzyme solution.

The use of the water-bath to maintain constant temperature in the final solution to be estimated was found to be necessary for accurate work since the temperature variation in the starch-iodine colour reaction is very great indeed (Fig. 9).

The <u>Smith and Roe unit</u> of amylase activity is defined as the amount of enzyme which, under the conditions of this procedure, will hydrolyse 10 mgs. of starch in 30 minutes to a stage where no colour is given with iodine at 620 mμ.

<u>Ballotini Bead Treatment.</u> 2 mls of the solution to be examined and 1 ml, by volume, of dry Ballotini beads are placed in the smallest homogeniser of the Nelco series which uses a 5 ml Universal container as the vessel. The homogeniser is then run at full speed for five minutes, after which the liquid is washed out of the vessel quantitatively and made up to a known volume with water. To check recoveries, a total nitrogen estimation is done before and after treatment.

<u>Butanol Treatment.</u> 5 mls of the solution to be treated together with 2 mls n-butanol are placed in a homogeniser tube and homogenised at 0° for two minutes with a glass pestle. The emulsion may be broken by centrifugation in the cold. Enzyme assays are then carried out on the aqueous phase.

<u>Estimation of Lipase.</u> Lipase was assayed by the method of Seligman and Nachlas (1950) in which β-naphthyl laurate is hydrolysed by the enzyme. The β-naphthol, produced by the enzymic hydrolysis, is converted to a purple azo dye by the coupling of two molecules with tetrazotized diorthoanisidine. This pigment is then extracted with ethyl acetate and the optical density measured colorimetrically.

The substrate is prepared by adding 5 mls of stock solution (200 mgs / 100 mls acetone) of β-naphthyl laurate in acetone through a submerged pipette into an agitated aqueous mixture of 10 mls Veronal buffer (0.1 M pH 7.4) and 35 mls. water. 5 ml portions of this suspension are pipetted into stoppered tubes followed by 1 ml of the enzyme solution. Incubation at 37° for five hours proceeds after which 1 ml of cooled tetrazotized diorthoanisidine (40 mgs./10 mls water) is added followed in two minutes by 1 ml of 40% TCA. The purple pigment is then extracted with 10 mls ethyl acetate. The tubes are centrifuged for five minutes and the optical density of the ethyl acetate is estimated at 540 m in the SP 600.

<u>Preparation of Protein for Counting.</u> The protein is precipitated with ice-cold 30% TCA to a final concentration of 10% and washed twice with ice-cold 10% TCA. Lipid solvents are used in the following order :- absolute ethanol, ethanol:chloroform (3:1),

ethanol:ether (3:1) and finally ether. The lipid-free material is allowed to dry in the air, ground finely in an agate mortar and counted at infinite thickness in a polythene planchet.

Nitrogen Determinations. Nitrogen was estimated by a modification of the micro-Kjeldahl method described by Ma and Zuazaga (1942). Digestion was carried out with 1.5 mls nitrogen-free sulphuric acid in the presence of metallic mercury as catalyst. The digestion mixture was transferred to the Markham apparatus (Markham, 1942) and 1 ml of a saturated solution of sodium thiosulphate was added to decompose the mercury-ammonium complex formed during the digestion. The ammonia, liberated in the distillation with 10 mls 40% NaOH, was trapped in 6 mls of 2% boric acid containing the mixed indicator and subsequently titrated with 0.01 N sulphuric acid.

Estimation of RNA by the Orcinol Method. The protein precipitated by TCA was lipid extracted as described above. The dry residue was digested with N NaOH (1 ml) for eighteen hours at 37° and the digest made up to a suitable volume with water. Aliquots were taken for ribose estimation by the orcinol method. (Kerr and Seraidarian, 1945). Orcinol reagent is prepared fresh daily by dissolving orcinol in a solution of 0.02% (w/v) $FeCl_3.6H_2O$ in concentrated HCl (6mg. orcinol per ml. of $FeCl_3$ solution). A known volume of digest was added to a clean tube and the volume adjusted to 3 mls with water. 3 mls. of the orcinol reagent were added and the tubes heated in a boiling-water-bath for thirty minutes exactly. The tubes were removed from the bath and placed in cold water and the optical densities measured in the SP 600 at 665 mμ. Reagent blanks and standard ribose solutions were estimated with each series of tubes.

<u>Determination of Radio-Activity of Phosphorus Compounds.</u> At suitable intervals after the injection of ^{32}P the pancreases were removed and were homogenised in a Nelco blender (ice-jacketed) with 20 mls ice-cold 10% TCA. The homogenate was centrifuged at 0° and the supernatant fluid filtered and set aside for the determination of the specific activity of the inorganic phosphorus of the tissue.

The protein precipitate was then subjected to a modified Schmidt and Thannhauser (1945) procedure as described by Davidson and Smellie (195[illegible]). The technique was as follows: - Washing twice with ice-cold 10% TCA and then lipid extracted as described above.

(1) <u>Specific Activity of Inorganic Phosphorus of Tissue.</u> The specific activity of the inorganic P in the TCA-soluble fraction was estimated by the method of Davidson, Frazer and Hutchison (1951). Mathison's reagent (1909) was added (1 ml to 10 mls. extract) to the acid solution and the mixture made alkaline to phenolphthalein with NH_4OH.

This was allowed to stand overnight at 0^{o} and the precipitate of $Mg(NH_4)PO_4$ separated by centrifugation and filtration, washed twice with 10% NH_4OH and then dissolved in N HCl. An aliquot of this was taken for the estimation of P by the method of Allen (1940) and the radio-activity measured in a liquid counter.

(2) Specific Activity of RNAP. The method described by Davidson and Smellie (1952) was used for the isolation of the nucleotides of RNA. By this method the four nucleotides can be obtained in a relatively pure condition.

The procedure is as follows : - The dry lipid-extracted residue was incubated with N KOH for eighteen hours at 37^{o}. The digest was then cooled to 0^{o} and the pH adjusted to 1 by the addition of ice-cold 60% Perchloric acid. The precipitate of DNA and $KClO_4$ was centrifuged down at 0^{o} and the supernatant containing the nucleotides of RNA was removed. The pH of the supernatant was then adjusted to 3 by the addition of KOH. A suitable aliquot of this fraction (100 to 120 μ g. P) was applied to a spot 6 cm. from one end of a strip of Whatman 3 MM filter paper 7 cms. broad and 72 cms. long and paper ionophoresis carried out in buffer solution (0.02 M citric acid-trisodium citrate, pH 3.5) for eighteen hours at a potential gradient of approximately 11 V/cm. length. The separated nucleotides were then eluted from paper with water and P estimations and radio-activity determinations carried out.

Determination of Radio-Activity of Carbon Compounds. Glycine was isolated from the proteins of sub-cellular fractions and from the free amino acid pool of the pancreas by the method described by Campbell and Work (1952). In this method the material is reacted with an excess of 1-fluoro-2:4-dinitrobenzene (FDNB), dissolved in a mixed organic solvent and the reaction mixture fractionated on a buffered celite column. The isolated DNP-glycine is subsequently purified on celite columns developed with ether. The radio-activity of this pure sample is then determined and the amount present estimated colorimetrically.

An amount of the sample containing about 200 μg. glycine was dissolved in 1.5 mls water and the solution made alkaline by the addition of a small quantity of $NaHCO_3$ and shaken with a 20-fold excess of a 10% solution of FDNB in methanol for four hours. At the end of this period the reaction mixture was diluted with 5 mls water, shaken with 20 mls ether to remove excess FDNB. This ether solution was then shaken with 5 mls water three times, the washings being added to the original aqueous layer. The latter was then acidified with 3 mls of 2.5 N HCl and extracted three times with 20 mls portions of ether. The ether extracts containing the DNP-glycine were combined and evaporated to dryness with a current of cold air. Any moisture in the residue was removed by desiccation over P_2O_5.

The dry residue was dissolved in a mixture of chloroform:n-butanol prepared by the method of Krol (1952). This solution was applied to a celite column, 1 cm. internal diameter, 15 cms. long, buffered at pH 5.2, packed in ether, and then washed with chloroform:n-butanol as described by Krol (1952). The column was developed with chloroform:n-butanol, the DNP-glycine band collected and the organic solvents removed by evaporation in a current of air. The residue was dissolved in ether (0.5 ml) and applied to a celite column prepared as above but using ether saturated with water as the developing solvent. The DNP-glycine was collected and the ether removed. The dry residue was dissolved in the minimum of ether and transferred to a stainless steel planchet on which it dried as an even film in air. The sample was counted using an end-window counter. The DNP-glycine was then dissolved from the planchet with 20 mls chloroform:n-butanol and then extracted from the latter with 10 mls 1% $NaHCO_3$. The amount of DNP-glycine present in the $NaHCO_3$ was estimated at 360 mμ. using the SP 600. The specific activity of the glycine was expressed as counts per minute per 100 μ g. glycine.

(1) Specific Activity of Free Glycine in the Pancreas. The TCA extract of the pancreas prepared as described in Section 2 was extracted with ether until the pH of the aqueous phase was about 4-5. The latter was then evaporated to dryness and the residue dissolved in 1.5 mls water, reacted with FDNB and the DNP-glycine isolated.

(2) <u>Specific Activity of Glycine in Proteins of Sub-Cellular Fraction.</u> DNP-glycine was prepared from the acid hydrolysed proteins of the various cell fractions obtained by differential centrifugation as described in Table 1.

<u>Determination of the Specific Activity of the Free Bases of Pancreatic RNA.</u> After administration of ^{14}C-2-glycine the nucleotides of the pancreatic RNA were separated by ionophoresis as described above. The adenylic acid and guanylic acid were eluted with water and the solution evaporated to dryness. The free bases were prepared by hydrolysing the residue with 72% perchloric acid for one hour at 100° (Wyatt, 1952). The digest was cooled and the pH adjusted to 7 by the addition of 5 N KOH. The precipitate of $KClO_4$ and carbon was removed by centrifugation. The pH of the solution was adjusted to 2 with HCl and applied to Whatman No.1 filter paper and subjected to two dimentional chromatography, the descending solvent being iso-propanol/HCl (Wyatt, 1951) and the ascending solvent being n-butanol/NH_3 (MacNutt, 1952). The spots were detected in ultra violet light and the appropriate areas cut out. NH_4Cl was removed by adsorption chromatography. The bases were extracted with HCl for twelve hours at 37° (0.1 N HCl for adenine and 1.6 N HCl for guanine). Aliquots of the supernatant were taken for radio-activity determinations by plating out on stainless steel planchets and for quantitative estimation by the method of Crosbie, Smellie and Davidson, (1953).

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