SYNTHESIS AND SECRETION OF PANCREATIC ENZYMES AS SHOWN BY RADIOAUTOGRAPHY USING TRITIATED AMINO ACIDS

by

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To my Wife

this thesis is dedicated

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INTRODUCTION

> "The body of anything whatsoever is nourished, continually dies and is continually renewed, ... just as the flame of a candle - a light which is also continually restored with the speediest of assistance from below by as much as is consumed above in dying, and the brilliant light is converted on dying into murky smoke".
>
> Leonardo Da Vinci (1452-1519)

The philosophical question of constancy and change, as applied to the proteins of the animal body, has been a subject of controversy for over half a century. The experiments of Folin (1905 a) on the effect of the protein level of the diet on the composition of urine marked the beginning of modern concepts of protein metabolism. On the basis of his experiment, Folin (1905 b) assumed that protein catabolism is not all of one kind, but could proceed via two pathways which are essentially independant and different. One pathway of catabolism is variable in quantity of end products, while the other process is constant.

> "I would (therefore) call the protein metabolism which which tends to be constant, tissue metabolism or endogenous metabolism, and the other, the variable protein metabolism, I would call the exogenous or intermediate metabolism" (Folin, 1905 b).

The endogenous metabolism would then account for the nitrogen necessary for the body maintenance, while the exogenous metabolism is dependant upon dietary nitrogen intake and excretion. This constancy of the tissue protein metabolism, as shown by Folin, was

the basis on which the concept of protein stability was established.

The validity of Folin's conclusions on the constancy of endogenous protein catabolism has been strongly confirmed (Mukherjee and Mitchell, 1949; Mitchell and Beadles, 1950; Blaxter and Wood, 1951).

The work of Schoenheimer et al (1939) on intermediary protein metabolism, using N^{15} labelled amino acids, has dramatically affected the concepts of endogenous metabolism of Folin (1905 a) by demonstrating the extraordinary lability of tissue nitrogen. By showing that dietary leucine was directly introduced into the body proteins, the distinction between endogenous and exogenous protein metabolism as two separate catabolic processes, had to be replaced by the concept of a "general dynamic state" of the tissue proteins, interpreted by Moss and Schoenheimer (1940) as

> ".... automatic and non-interruptable biochemical processes, of synthesis as well as degradation, which are balanced by an unknown regulatory mechanism so that the total amount of the body material and its composition do not change".

Rickenberg, Yanofsky and Bonner (1953), on the basis of experiments with Escherichia coli reintroduced the concept of body protein stability. Their experiments stemmed from the work of Spiegelman and Dunn (1947), who suggested that enzyme deadaptation involves active metabolic breakdown of the enzyme in question, while the work of Wainwright and Pollack (1949) indicated that in certain instances deadaptation can best be accounted for on a basis

of "diluting out" of performing enzymes. The results of Rickenberg et al (1953) show that in the case of β -galactosidase deadaptation, diluting out, and not metabolic breakdown was the process involved. Further data suggested that the protein β -galactosidase may not be unique in its stability.

This point of view was confirmed by Hogness, Cohn and Monod (1955), again with the β -galactosidase of Escherichia coli. In addition, their results lead to the conclusion that the non β - galactosidase proteins of the cell are stable in that they are not degraded to amino acids by any mechanism. On first consideration this seems to be in direct opposition to Schoenheimer's results, however, as pointed out by Hogness et al, the discrepancies of their data with those of Schoenheimer may be due to the fact that their experiments were of too short a duration to detect degradation rates of the order of magnitude of those found in mammalian systems.

The technique of radioautography (p. 8) with radioactive labelled amino acids, when applied to the problem of stability versus turnover of body proteins, yielded unequivocable evidence favouring the concept of instability of the body proteins. Leblond, Everett and Simmons (1957) applied this technique to the study of methionine-S^{35} distribution in intact, non-growing adult rats, under conditions in which protein synthesis for purposes of growth were eliminated. They found that 4 hours after the injection of the labelled methionine, the cells of all the tissues studied showed at least some degree of labelling. On the basis of the reaction intensities over

various cells and tissues, three categories indicative of the rapidity of protein synthesis were created. Those tissues associated with the formation of new cells form the first category, the second contains the cells which produce various types of secretions, and the third category consists of tissues in which neither renewal nor secretory processes are known to occur. The reactions over the latter category of tissues were attributed to turnover of intracellular protein material. It was further concluded that the reactions observed radioautographically in all three categories represented a de novo synthesis of proteins.

This, and subsequent studies with radioautography and other techniques tend to confirm the dynamic rather than static state of most tissue proteins. However, it must be pointed out that some proteins, such as the collagen of dentin, may be permanently stable (Greulich and Leblond, 1954; Carneiro and Leblond, 1959).

The Basic Problem, and Purpose of the Experiments

The experiments of Leblond, Everett and Simmons (1957), in addition to adding confirmation to the dynamic state of tissue proteins, had as their objective, the elucidation of the sites of protein synthesis in various tissues. The earliest time point selected for study, that of 4 hours after injection, was thought to reveal the sites of synthesis under the assumption that relatively long periods of time were required for the synthesis of a protein molecule. Thus, at 4 hours after injection of methionine-S^{35} into

rats, the zymogen granule regions were the most heavily labelled areas of the pancreatic acini. However, in keeping with the theory that proteins are synthesized in relation to the ribonucleoprotein particles (see page 26) it would be expected that the basophilic ergastoplasm,shown to contain the majority of these particles, is the site of protein synthesis.

In addition, the radioautographic results of Hansson (1959) obtained with mice, rats and guinea pigs on frozen- dried and routinely prepared sections after injection of S^{35} methionine and cystine, showed no definite concentration of radioactivity in any part of the exocrine cells of the pancreas in the first 30 minutes after injection. However, even with the rather poor radioautographic resolution obtained with S^{35}, the activity was found to be somewhat greater in the basal part of the cells than in the centers of the acini. At 1 and 4 hours, in confirmation of the results of Leblond et al (1957), the activity was concentrated in the centers of the acini. To resolve this apparent discrepancy as to the site of synthesis as well as to investigate more fully the process of secretion of the pancreatic enzymes, it was decided to study the distribution of labelled amino acids in the pancreas at short time intervals, such as 5 to 30 minutes, after injection. With the introduction of tritium (H^3), the radioactive isotope of hydrogen, a tool was provided which allowed for the fine radioautographic resolution required to localize radioactivity to the small cytological structures under consideration. Three amino acids, varying as widely as possible in

metabolic roles, viz. leucine, glycine and methionine, labelled with tritium, were injected into rats and mice, and their distributions were followed at very short, as well as longer time intervals after injection.

Before presentation of the methods and results, a description will be given of the theoretical aspect of the radioautographic technique, the structure of the pancreatic acinus, followed by a brief description of the most commonly accepted mechanism of protein synthesis.

The Radioautographic Technique

General Tracer Requirements

The advantage of the radioautographic method lies in the fact that radioactivity administered as part of a normal molecule, can be detected at the cytological level in tissue sections. Thus, the fate of a molecule can be studied by adding to the body a "tracer" amount of the labelled molecule, and localizing the label at a series of time intervals after administration. A tracer substance is defined as one which a metabolic system is incapable of distinguishing from the normal substance; thus, the tagged and untagged molecules can enter the process with equal probability, the advantage being that the tagged molecule is in some way distinguishable to the observer (Siri, 1949). However, in order to succeed in creating a tracer condition, four basic requirements must be fulfilled.

First, the H^3 introduced on amino acid molecules, must not exchange with hydrogen atoms independant of metabolic mechanisms. The

presumptive evidence indicating that exchange, as defined above, does not occur is the fact that with leucine, the label is in the C_{4-5} positions, a region known to be the least reactive in the molecule. Furthermore, the mass-ratio difference between tritium and hydrogen are such that a greater amount of energy is required to activate the carbon-tritium bond than for the activation of the carbon-hydrogen bond; thus, transfer of hydrogen atoms are more likely than transfer of tritium.

The second consideration is that of "isotopic effect", in that the mass of tritium is three times that of hydrogen; however, this is only likely to be significant when tritium is used as a tracer of hydrogen, and should not apply when it is used as a tracer of amino acids. Therefore, it is assumed that there is no appreciable "isotopic effect".

The third requirement is that the amount of labelled substance injected should constitute a tracer concentration; that is, in order to satisfy the condition of indistinuishability the tracer must be present in minute quantities. It has been shown, in the case of the leucine-H^3 experiment to be described, that the quantity of amino acid injected was small enough to act as a tracer (Mitmaker, 1960). Furthermore, it is assumed that the quantity of the other amino acids used in this study, being of the same order of magnitude, also fulfilled tracer concentration requirements.

The fourth requirement is that a minimum radiation effect should be produced on the tissues. In most of the experiments, the animals were killed long before any radiation effect could have occurred. Those animals which were allowed to survive for several days con-

tinued to gain weight according to the normal pattern expected. This, as well as the lack of cytological evidence of radiation effect, eliminated the possibility of significant radiation damage.

It is therefore concluded that tracer requirements were fulfilled in all the above respects, and that the dosages of amino acids given to the animals served as adequante "tracers" of the fate of corresponding, unlabelled amino acid molecules.

Radioautographic Theory (Gross et al, 1951)

Fundamentally, radioautography is a technique in which a radioactive tissue section is placed in direct contact with a sensitive photographic emulsion. The energy emitted as a result of disintegration of the radioactive nucleus causes a reaction to occur within the silver bromide crystals in a manner analogous to the reaction with light, resulting in the formation of a latent image. Upon development of the emulsion in standard photographic developers, silver ions tend to be deposited on crystals, which make up what is known as the latent image. As a result, silver grains, produced by development, overlie the radioactive regions of the histological section. Localization of these grain accumulations implies an accurate association of these areas of grain density with tissue structures, and that a distinction can be made between areas of increased grain density closely adjacent to one another. The term resolution is applied to the latter implication. Both resolution and density of silver grains are dependant on three considerations. One, the geometrical relationship of the radioactive source and the overlying emulsion;

two; the energy and intensity of the radiation; and three, the characteristics of the photographic emulsion. For detailed consideration of these factors, reference is made to the article of Gross et al (1951).

Briefly, the geometrical relationship implies that for maximum resolution and density, the distance of the tissue from the emulsion, that is, the interspace, should be at a minimum. Using the coating technique (Messier and Leblond, 1957), with no intervening celloidin coat, as in the experiments to be described, the interspace can be considered as negligible. In addition, by focusing the microscope so that the grains and the tissue can be seen almost simultaneously, the advantage of accurate localization is obtained.

The low energy β -particle emitted by tritium has a maximum penetration path of 6 micra in an aqueous medium, (Slack and Way, 1959) and probably less through emulsion. Thus, the "spreading" effect of higher energy emittors is not present with tritium, and the silver grains resulting from the disintegrations of this isotope can be associated with accuracy to the underlying structures.

Concerning the characteristics of the emulsion, the resolution and sensitivity are determined by the grain size, concentration and uniformity of distribution. Kodak NTB 2 emulsion, employed in all the experiments to be described, is a "high contrast" emulsion, that is, it includes crystals of uniform size, making up about 85% of the emulsion weight. These conditions are compatible with maximum radioautographic resolution and sensitivity.

Morphology of the Pancreas

Development of the Pancreatic Ducts and Acini

The pancreas (Arey, 1941) arises as two outpouchings of the endodermal lining on opposite sides of the duodenum of 3-4 mm human embryos. One, the Dorsal Pancreatic bud, pushes out from the dorsal wall, cephalic to the hepatic diverticulum. The other, the Ventral Pancreatic bud, appears ventrally in the caudal angle between the gut and the hepatic diverticulum.

The dorsal pancreas grows more rapidly than the ventral and during the sixth week of development an axial duct is formed to drain it. The ventral bud remains smaller, and its duct is carried from the duodenum by the lengthening common bile duct, and then arises directly from the latter. The unequal growth of the duodenal wall shifts the bile duct dorsally bringing the ventral pancreas into the dorsal mesentary near the dorsal pancreatic stem. Both buds fuse indistinguishably, and the longer distal portion of the dorsal duct fuses with the distal end of the ventral duct to form the adult duct of Wirsung. The remaining proximal part of the dorsal duct forms the accessory duct of Santorini. The terminal portions of the branching duct systems develop into exocrine secretory units or acini.

Histology of the Acinus

In any histological section through the pancreas, the expanded

tubular acini, are cut in all possible planes. Statistically, the predominating plane passes obliquely through the acinus.

According to Ham (1957), the cells composing the acinus are more or less pyramidal in shape, with their apices toward the center of the acinus and the opposing sides in close contact, so that the cell membranes are indistinct. Because the nuclei lie towards the base of the cells, they appear to form a ring around the outer part of the circular acinus.

Within the center of some acini, nuclei of duct cells may be seen. In other acini, no such nuclei are evident. Thus, a duct may lead directly off an acinus, or it may be invaginated into it, in which case the nuclei of the duct cells are visible within the acinus. These cells are termed centro-acinar cells.

A separate basement membrane, 100-150 Å thick has been described as enclosing and surrounding each acinus, both in the guinea pig pancreas (Palade and Siekevitz, 1956) and in the mouse (Sjöstrand and Hanzon, 1954 a).

Acinar Cell

The acinar cell, as stated above, is pyramidal in shape, with its apex directed toward the center of the acinus, where it forms part of the boundary of a very small lumen. The base of the cell is in contact with the above-mentioned basement membrane.

Cell Membrane

The plasma membrane, as studied with the electron microscope in

the guinea-pig pancreas (Palade and Siekevitz, 1956), sends small finger-like projections or microvilli into the lumen of the acinus. The membrane shows thickenings or desmosomes along the upper sides of the cell body, and appears frequently infolded or invaginated along the lower sides and the basal pole of the cell. The invaginations are usually shallow and frequently associated with small vesicles which appear singularly or in clusters or rows. These are morphologically similar to the smooth surfaced elements of the endoplasmic reticulum.

Nucleus

The nuclei of the acinar cells are rounded and lie toward, but not in contact with the bases of the cells (Ham, 1957).

Opie (1932) described within the nucleus a chromatin network formed of fine threads and nodules. The chromatin is in contact with the inner surface of the nuclear membrane, which he said, is formed by a thin layer of chromatin. Within the chromatin meshes are found one or more spherical bodies which stain with acid dyes; these are the nucleoli. Chromatin granules are applied to the nucleolus and may form a complete shell around it.

Dolley (1925) found that as much as half of the acinar cells of the rat were binucleated.

Nuclear Membrane

In 1954 (a) Sjöstrand and Hanzon described the general morphology of the exocrine cells of the mouse pancreas with the electron microscope.

They described the cell cytoplasm as consisting of concentrically arranged membranes studded on one side with small dense particles. The membranes are arranged in pairs with the smooth sides facing each other. These membranes face the cell membrane, the zymogen granules, the Golgi apparatus and the mitochondria with the particle covered side, but face the nucleus with the smooth side. They therefore concluded that the membrane immediately adjacent to the nuclear membrane represents only one of the membrane pair, the other half of the pair being unaccounted for.

Watson (1955) has shown that in the pancreas of the rat, the nuclear "membrane" is actually composed of two membranes, the inner nuclear membrane which borders the nucleoplasm and the outer nuclear membrane which resembles the granule studded membranes described by Sjöstrand and Hanzon. The space between them being the perinuclear space. In addition, Watson described two types of communications or pathways of exchange between the nucleus and the cytoplasm. One via a system of pores which lead from the nucleus into the cytoplasm and two, by the perinuclear space which he showed being in open communication with the cavities enclosed between the membrane pairs described by Sjöstrand and Hanzon. (These membranes and cavities are believed to be components of the endoplasmic reticulum described below).

Basal Region (Ergastoplasm)

The cytoplasm located between the nuclei and the bases of the cells, as well as that extending up along either side of the nucleus is

usually basophilic due to the accumulation of ribonucleic acid (Ham, 1957).

Electron microscopic studies of this basophilic region in the mouse pancreas (Sjöstrand and Hanzon, 1954 a) showed that the cytoplasm contains concentrically arranged, densely packed, 40 Å thick membranes. The membranes are arranged in pairs and bear on one side small dense particles, 140 Å in diameter. The particles are spaced 150 to 450 Å apart.

Similar structures have been observed in the guinea pig pancreas by Palade (1956 a) and Palade and Siekevitz (1956).

The following description of the ergastoplasm or endoplasmic reticulum applies to the guinea pig pancreatic acinar cells (Palade and Siekevitz, 1956).

In the guinea pig, as in the mouse, the entire cytoplasmic space in the basal half of the cell is occupied by numerous, tightly packed profiles of the endoplasmic reticulum which are frequently disposed with remarkable regularity. Practically all these profiles are of the "rough-surfaced" variety, that is, bearing the small dense 100 - 150 Å in diameter ribonucleoprotein (RNP) particles attached to the outer surface of their limiting membrane (Palade, 1955). The profiles vary in shape from circular to elongate, the elongate forms predominating. They are usually disposed in rows which may extend as long as 5 to 10 μ, generally parallel to one another, and at more or less regular intervals. Occasionally the regularity is disrupted by branching profiles and anastomoses that connect adjacent rows. The arrays are usually oriented parallel to the various surfaces provided by the nuclear surface or the cell membrane.

Within or among these variously disposed arrays of rough surfaced elements, smooth surfaced profiles are exceedingly rare. Where present, they are circular (40 to 100 Å in diameter) and occur in small clusters. These smooth surfaced profiles in short rows and clusters, are also found in the vicinity of the cell membranes.

Numerous investigations have shown occasional continuity of the membranes and contents of a smooth and rough surfaced profile. This is interpreted as indicating that the two varieties of profiles do not represent two unrelated structures, but are differentiated portions of a common system, the endoplasmic reticulum (Palade and Porter, 1954; Palay and Palade, 1955; Palade and Siekevitz, 1956). The electron micrographs offered in evidence of this do not, however, constitute unequivicable proof that this interconnection is a universal occurrence in the cells.

The rough surfaced elements of the endoplasmic reticulum usually have an amorphous content which varies in density. Frequently, it is less dense than the cytoplasmic matrix, but this situation may be reversed.

In the guinea pig, the endoplasmic reticulum profiles may contain round, homogeneous bodies of high density and relatively large size, the intracisternal granules (Palade, 1956 b). In three dimensions these correspond to spherical granules embedded in the light substance which fills the cavities of the endoplasmic reticulum. In such cells the rough surfaced profiles are randomly arranged, are of relatively large diameter and predominantly circular in shape.

In density and texture, the granules are similar to zymogen granules, but their size is smaller and their location different.

These granules have never been observed in the mouse (Munger, 1958).

The cytoplasmic matrix is disposed in narrow, more or less regular bands around the profiles of the endoplasmic reticulum. Some free ribonucleoprotein particles are found in this matrix.

The profiles of the endoplasmic reticulum in three dimensions can be interpreted as fenestrated cisternae, when there are few and small interruptions, or as reticular sheets when the interruptions are numerous and large. The continuity of the system is maintained by the branching and anastomoses described.

Mitochondria

The lightly stained striations, usually seen in the basophilic region of the cell may be due to the disposition of mitochondria in this region (Ham, 1957). In classical light microscopy the mitochondria, as visualized by special techniques, are rods or filaments, which neither branch nor anastomose (Opie, 1932).

Palade (1953) described the structure of the mitochondria of various tissues from the rat, mouse and rabbit. They were seen to consist of an outer limiting membrane, 70 to 80 Å thick, and a second or internal mitochondrial membrane, which he could observe under favourable conditions. Projecting internally from the internal membrane were folds which Palade called the Cristae Mitochondriales. The cristae are usually perpendicular to the long axis of the organ-

elle and occur in a series which lie parallel to one another, spaced at more or less regular intervals.

Between the projecting cristae an almost structureless matrix fills the body of the mitochondria.

Apical Region: Zymogen Granules

The round refractive zymogen granules in the apex of the cell were first described by Claude Bernard in 1856 (Opie, 1932).

Bensley (1911) described the acidophilic zymogen granules as occupying the spaces in a continuous cytoplasmic partition which surrounds them.

Tsukaguchi and Takagi (1921) also described a clear area (apparently cytoplasm) which surrounds the individual zymogen granules.

With the electron microscope, Palade and Siekevitz (1956) showed that most of the apical region of the guinea pig acinar cell is occupied by predominately circular, large (0.5 to 1.2 μ) homogeneously dense zymogen granules. The granules are usually surrounded by a complete membrane of the smooth surfaced variety.

Between the zymogen granules, in the rim of cytoplasm are numerous profiles of endoplasmic reticulum of the rough surfaced variety. Most profiles are circular or oval in shape, and show no preferred orientation. In three dimensions the random network of endoplasmic reticulum in the apical part of the cell merges around the nucleus with the basal part.

The Centrosphere Region: Golgi Apparatus

In 1898, Camillo Golgi described an "internal reticular apparatus"

in the cytoplasm of nerve cells after treatment with silver salts. Since then, with the use of silver and osmium impregnation methods, as well as recently with the electron microscope, this apparatus has been the subject of numerous investigations and much controversy. Although the function of the apparatus remains unravelled, its ultrastructure has been well established.

Classical impregnation methods showed that the Golgi apparatus lies in a fairly constant supranuclear position between the nucleus and the excretory pole, in the pancreatic acinar cell. In the mouse pancreas, Sjöstrand and Hanzon (1954 b) localized the Golgi apparatus as restricted to the distal part of the exocrine cell and showing intimate topographical relations to the zymogen granules. In general the position of the apparatus is described as supranuclear, that is, between the nucleus and the cell apex and in close contact with the zymogen granules (Dalton and Felix, 1956; Munger, 1958; Lacy and Challice, 1956).

It was felt to be unnecessary to present a comprehensive survey of the literature and controversy pertaining to the results of classical methods of studies on the Golgi apparatus. The only endeavour in this thesis will be to outline very briefly the concept of the Golgi internum, externum and Golgi substance of Hirsch (1939, cited in Bourne, 1951), because this concept approaches closest to interpreting the pictures obtained with Aoyama's technique (Fig. 25, p.124a) and the results obtained with radioautography (Fig. 26, p.124a).

The Golgi apparatus consists basically, of two parts, an outer, chromophilic portion (which absorbs osmium and silver), and a chromo-

phobic inner portion. Hirsch has found that the Golgi bodies of the cell do not always have this double structure. Hirsch states that the solid granules of Golgi substance, which show no differentiation into external and internal portions, really constitute the "pre-substance" of the Golgi apparatus. These pre-substances are able to build up nets which are the only sort of nets which Hirsch recognizes. The other nets described, he claims are due to over-impregnation of separate bodies by excessive amounts of osmium or silver, which causes them to link up and simulate a net. The pre-substance, which may be aggregated near the nucleus (e.g. pancreas) or distributed throughout the cell (e.g. neurone) gradually develops a double structure, with a chromophilic cortex. At this stage it is referred to as a Golgi system. The outer part or cortex of the Golgi system is known as the "externum", the inner part as the "internum". Hirsch believes that the product (zymogen granules) of the secretory cell is formed in the Golgi internum.

The electron microscopic study by Sjöstrand and Hanzon (1954 b) of the mouse pancreas has revealed that the Golgi complex is composed of three elements; the Golgi membranes, the Golgi ground substance and the Golgi granules.

The Golgi membranes occur in several groups, each consisting of 2 to 5 tightly packed membrane pairs. These membranes are free of opaque particles (described by Palade (1955) as ribonucleoprotein granules). Large vacuoles are frequently seen between the split membrane pairs.

The Golgi ground substance has embedded in it the Golgi membranes.

The ground may appear either homogeneous or granulated but the granules do not resemble the RNP particles.

The Golgi granules have a dimension range from 40 Å units in diameter to the size of zymogen granules. These granules show an electron dense limiting membrane, similar in appearance to the membrane enclosing the zymogen granules, however, the membrane may at times be incomplete.

Dalton and Felix (1954, 1956) have also described three components of the Golgi apparatus, these are the large vacuoles, membranes forming the boundaries of flattened sacs which correspond to the Golgi membranes of Sjöstrand and Hanzon (1954 b), and small vesicles or granules, presumably part of the Golgi "ground substance".

In the exocrine cells of the pancreas, the system of large vacuoles is arranged in the form of a ring (Dalton and Felix, 1956). The authors are uncertain as to whether these vacuoles are connected, or are separate entities. The vacuoles are bounded by an 80 Å thick membrane, which is at least twice as thick as the membranes of the ergastoplasm of the same cell. The profiles of flattened sacs are occasionally seen between two adjacent vacuoles. Peripheral to the vacuoles, between them and the surrounding ergastoplasm is a layer of small granules with electron dense membranes surrounding less dense centers. The ground substance in which the vacuoles, membranes and granules lie is continuous with the cytoplasm in which all the other cell organelles are embedded. In the area where the Golgi granules or vesicles and the ergastoplasm are in juxtaposition, it can be seen that the Palade or RNP granules appear only on the mem-

branes of the ergastoplasm. Dalton and Felix's original description of Golgi granules (1954) is contradicted by Weiss (1955) who claims these are actually the smallest of the Golgi vesicles. These vesicles are occasionally seen to be continuous with the paired lamellae.

Lacy and Challice (1956) studied the pancreatic acinar-Golgi apparatus of the mouse with a classical impregnation method (Aoyama's) 1930) in combination with electron microscopy.

The silver deposits which were observed in the cell were confined entirely to two main regions: within the dense cytoplasmic material, in the form of many fine submicroscopic grains, and along one or both sides of a series of closely apposed vacuoles lying within the Golgi zone. The latter corresponds to the light microscopist's chromophilic region (externum), while the vacuoles correspond to the chromophobic part (internum).

On the whole they (Lacy and Challice, 1956) confirm the findings of Sjöstrand and Hanzon (1954 b), but they were unable to see a distinct ground substance. In addition, they observed that some of the pairs of Golgi membranes gradually diverge from one another as they approach the vacuolar substance while the dense material they enclose decreases in density. It was further noted that the Golgi vacuoles correspond morphologically to terminal dilations of the paired Golgi membranes.

Dilated terminal portions of the smooth surfaced Golgi membrane array have been noted by numerous investigators (Dalton and Felix, 1954; Sjöstrand and Hanzon, 1954 b; Weiss, 1955; Dalton and Felix,

1956; Lacy and Challice, 1956). This has been interpreted as a budding off of the vesicles which are so numerous in this region.

Summary of the Ultrastructure of the Acinar Cell

An attractive theory, based on the evidence of observed continuity between the various membraneous structures described above, as proposed by Palade (1956 a) suggests that the endoplasmic reticulum is a continuous network of membrane-bound cavities which permeate the entire cytoplasm from the cell membrane to the nucleus. Within this network there are a number of local differentiations which include the nuclear envelope and the Golgi apparatus.

Palay and Karlin (1959) expanded this theory by stating that the endoplasmic reticulum is a continuous system, with the ergastoplasm, Golgi apparatus and nuclear envelope as differentiations of an otherwise generalized cytoplasmic vacuolar system dynamically interrelated and interconnected. Although the electron micrographs do indicate connections between rough and smooth surfaced profiles, and regardless of the attractiveness of this theory, the published micrographs alone do not constitute a strong enough basis for this hypothesis.

Biochemistry of Protein Synthesis

The unique importance of proteins in biology lies in their capacity to act as "building stones", or structural units of the animal cell. That this is not their primary function, however, is evidenced by the fact that at the botanical level, this role is almost entirely assumed

by polysaccharides. The functions through which proteins gain their unique position are as biological catalysts or enzymes, and indeed, it has been shown that all known enzymes are proteins. The vast majority of biochemical reactions proceed, in vitro, at a rate which is too slow for the high metabolic activity of most organisms. Enzymes act to increase the velocity of these reactions to an adequate rate, accounting for the fact that almost all biochemical reactions are enzymatically controlled.

Although proteins are so diverse, functionally, and their involvement in a wide range of metabolic reactions is recognized, whatever little is known of their own biosynthetic mechanism suggests that it is uniform for all proteins.

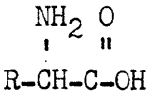
General Consideration of Proteins

Upon hydrolysis, simple, non-conjugated proteins, yield a mixture of amino acids. The number and kind of amino acids, as well as the molecular weight of the entire molecule varys greatly from one protein to another, although all three are constant for each protein species. Typically, a protein with a molecular weight of 25,000 may contain about 230 amino acid residues joined end to end. It is interesting to note that the peptide bond (formed with the elimination of a water molecule) which links the amino acids together is the same regardless of the amino acids involved. Topographically, then, the protein is a linear molecule, but as has been shown for some proteins, it may be folded upon itself in a complicated manner. Although there is theo-

retically no limit to the number of residues which may occur in a protein molecule, there are only 20 different species of amino acids found in all proteins, with certain noteworthy exceptions, as for example tryptophan, which does not occur in the protein insulin. Also, the amino acid hydroxyproline forms a unique residue, found only in collagen.

Amino Acids

The basic units of proteins, the amino acids, are nitrogen containing compounds of the general form,



the amino and carboxyl group being α to one another.

Normally, mammals cannot make use of nitrate or nitrite, nor can they utilize atmospheric nitrogen. They must therefore receive their nitrogen in the form of amino acids from ingestion and subsequent digestion of proteins. The amino acids thus received are the so-called essential amino acids.

That amino acids are precursors of protein is shown by supplying an essential amino acid to a cell and observing its incorporation into the cellular proteins. Studies of this nature have shown that if one or more essential amino acids are lacking in the diet, the animal fails to grow, indicating that the body does not synthesize

proteins deficient in an essential amino acid, but rather produces less of that protein. Moreover, the cell does not synthesize that portion of the protein molecule which does not contain the deficient amino acid.

It becomes apparent that protein synthesis required the presence of the complete complement of amino acids, yet they are not randomly arranged in the protein chain, but have an ordered and precise sequence. A given protein is highly homogeneous in that all the molecules contain the same amino acids and in the same proportion. There is also evidence that the sequence of arrangement of the amino acids is constant; thus every molecule of, for example, hemaglobin, insulin or ribonuclease is exactly similar to every other molecule of the same protein species in the same individual.

It has been shown that a Mendelian gene can alter the structure of hemoglobulin, causing sickle-cell anemia (Neal, 1949). This disease was shown to be due to the gene altering the amino acid sequence in one small part of the polypeptide chain (Ingram, 1956), the difference being due to a valine residue occurring in place of a glutamic acid one in a molecule which contains about 300 amino acid residues in all (Ingram, 1957). If the gene is homozygous, the disease is usually lethal before adult life, illustrating the tremendous significance of the sequentialization of amino acids.

Essential Points in the Mechanism of Protein Synthesis

It is apparent from the foregoing that the synthesis of protein

is unlike the synthesis of other molecules, although as previously pointed out, the mechanism is probably uniform throughout nature.

It is known that protein synthesis and turnover of preexistant proteins occurs even in the tissues of animals in nitrogen balance, that is, even in the absence of growth, renewal or secretion (Leblond et al, 1957). This universality is support for the contention that protein synthesis is a general characteristic of life (Leblond, 1960).

Three basic factors must be considered in postulating a theory of the mechanism of protein synthesis. First, it must be taken into account that the formation of peptide bonds is a reaction which requires considerable energy; second, the high degree of protein specificity must be considered; and third, the question of utilization of multiple units of smaller peptides in the formation of larger ones versus the direct assembly of single amino acid units simultaneously or by successive addition to the ends of a rapidly growing peptide chain, must be resolved.

Mechanism of Protein Synthesis

Basically, the mechanism of protein synthesis may be arbitrarily divided into two reaction sequences. The first, for which a good deal of evidence is available, consists of a series of reactions which prepare the amino acid molecule for subsequent incorporation into the protein molecule. The second part, which is mainly in the realm of hypothesis, consists of actually bringing these amino acids together in the proper proportions and sequences which are characteristic of

the particular protein being synthesized.

Activation of Amino Acids

The initial reaction to which amino acids are subjected is an activation of the carboxyl group of the amino acid by adenosine triphosphate (ATP). The activated amino acyl groups would then be bound through adenosine monophosphate (AMP) to an enzyme which acts in transfering the amino acid to a postulated acceptor. The reaction may be summarized as follows:

Amino acid + ATP + Enzyme $\longrightarrow$ Enzyme - AMP $\sim$ Amino acid + PP_i

Thus, the R group of the amino acid and the adenine moiety of ATP are first bound to a specific activating enzyme, pyrophosphate being cleaved off the ATP leaving the bond energy in a carboxyl-phosphate linkage (Fig. 1). This, presumably is the source of the high energy required for peptide bond formation. In addition, it was found by Lipmann (1954, cited in Lipmann et al, 1959) that the liberation of a pyrophosphate group indicated the formation of an acyl adenylate.

Hoagland (1955) and Hoagland, Keller and Zamecnik (1956), working with a system devised by Zamecnik and Keller (1954), containing rat liver microsomes, pH 5 precipitable proteins from the supernatant of microsomes (obtained by centrifugation of the microsome at 105,000 x g), ATP, and ATP generating system, plus guanosine di- or triphosphate, showed that the pH 5 precipitable proteins contained the amino acid activating enzymes. They showed that several amino acids could be activated in that system. Lipmann et al (1959) theorized that this indicates a general activation process for all amino acids. In-

PLATE 1

Fig. 1 Mechanism of protein synthesis (modified from Hoagland, Keller and Zamecnik, 1956; and Lipmann et al, 1959).

The three primary reactions involved in the mechanism are depicted.

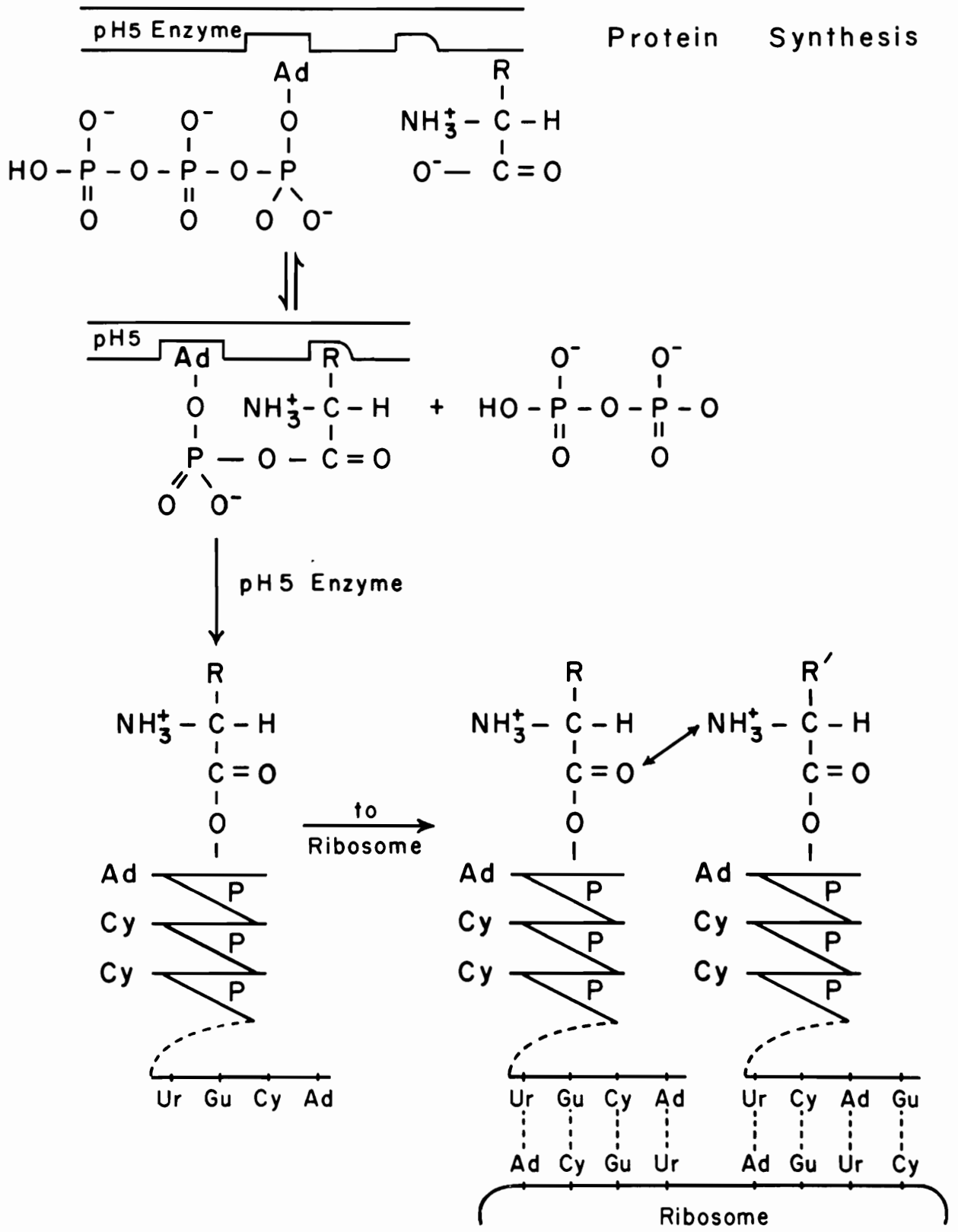
The first reaction (represented at the top) involves the activation of the amino acid with ATP in the presence of the pH 5 enzyme, with the splitting off of pyrophosphate.

The second reaction (lower left) depicts the transfer, presumably again mediated by the pH 5 enzyme, of the amino acid residue to the soluble RNA.

The third reaction (lower right) involved the transfer of the amino-acyl-s-RNA complex to the RNA of the microsomes, so that peptide bonds can be formed between neighbouring amino acid residues.

Fig. I

Mechanism of Protein Synthesis



direct evidence such as the work of Hoagland (1955) and Hoagland et al (1956) above, strengthened the belief that activating enzymes were truly amino acid specific. Further indirect evidence was added by Davie et al (1956) when they isolated a tryptophan activating enzyme from beef pancreas (where it is very abundant) and produced a homogeneous tryptophan specific enzyme preparation.

The Role of Soluble RNA (s-RNA)

Hoagland, Zamecnik and Stephenson (1957) demonstrated that the activated amino acid was transferred to a soluble RNA molecule, being joined to it by relatively weak bonds. Soluble RNA differs from microsomal and nucleolar RNA by virtue of it's smaller molecular weight, which renders it soluble in the ordinary extraction procedures for proteins and nucleic acids. It is apparent that the "postulated acceptor", mentioned above, is the s-RNA molecule.

Further work, by Zachau et al (1958) led to the conclusion that the amino acid was transferred from the initial activation product, amino acyl adenylate, to the terminal adenosine moiety in s-RNA. The complex formed is shown as part of Fig. 1. The characteristic cytosine- cytosine-adenosine ending, appears to be common to all species of s-RNA. Furthermore, the activating enzyme may also be responsible for the transfer of activated amino acids to soluble RNA. The evidence presented by Zachau et al, showed a definite intracellular occurrence of these amino acid esters of s-RNA.

The idea of a specific s-RNA for each amino acid is suggested by

the work of Porath (1956) and Smith et al (1959). Holley and Merrill (1959) differentiated more clearly between RNA's that are specific for their respective amino acid. Thus,

> "..... we now feel pretty well assured that we are dealing with a "fleet" of different s-RNA's each specific for only one of the whole set of amino acids".

Lipmann et al (1959) further concluded that essentially all amino acid esters of s-RNA must be freely diffusible in the cell, since they are the active amino acids ready to enter the peptide-linking process in protein synthesis.

The Role of Microsomal RNA

The guanosine triphosphate (GTP) present in the system devised by Zamecnik and Keller (1954), was shown to be unnecessary in the transfer of labelled activated amino acids to s-RNA. Hoagland et al (1957) extracted and purified this labelled amino acid s-RNA complex and added it to the microsome fraction. They found that a high percentage of the labelled amino acid is transferred from the s-RNA to the microsomal protein only in the presence of GTP. Thus, they concluded that GTP mediates the transfer of the activated amino acid to peptide linkage via the microsomes.

Littlefield and Keller (1957) have shown that only the ribonucleoprotein particles (RNP) of the endoplasmic reticulum are required for the transfer of activated amino acids. These RNP particles, termed "ribosomes" are defined as the small particles isolated from the microsomes after solubilization of the membranous part by bile salts such as

desoxycholate. They were found to contain a higher concentration of RNA than the corresponding microsomes (Brachet, 1960).

Incorporation of single Amino Acids into In Vivo Systems

Regarding the question of the acceptance of a single amino acid by microsomes for protein synthesis, versus the presence of a total complement of amino acids, Zamecnik and Keller (1954) found no requirement for addition of a full complement of amino acids to a system containing the microsomes and their supernatant. However, they postulated that there may be present in the microsome complex sufficient amounts of free or bound amino acids so that addition of more amino acids does not increase the initial incorporation.

Lipmannet al (1959), using threonine activating enzymes, found a small incorporation of labelled threonine into microsomal protein using a system containing threonine-activating enzyme, labelled threonine and the microsomes only. However, the addition to the above medium of other amino acids and their activating enzymes, which are normally found in the crude microsomal supernatant, caused a four fold increase in labelling of the microsomal protein. They concluded that in the isolated liver system, a complete complement of amino acids, such a s is present in the crude microsomal supernatant, is required for incorporation into protein.

Thus, it appears that the synthesis of a protein proceeds only in the presence of all the amino acid residues which constitute that protein.

The Utilization of Peptides as Intermediates in Protein Synthesis

(Summarized from a review by Simkin and Work, 1958)

The reasonable assumption that long chain protein molecules may be formed by linking together of smaller peptide fragments, has initiated considerable work in trying to isolate peptide intermediates from cell extracts.

Turba and Esser (1955), using Torula utilis (yeast cells) and Connell and Watson (1957), with Pseudomona hydrophila, have extracted small quantities of peptides. However, there is uncertainty as to the involvement of these peptides in protein synthesis. Turba and Esser have shown that peptides are rapidly synthesized from amino acids, but in attempting to correlate peptide intermediates, with protein synthesis, it is not sufficient to demonstrate the existence, or even the synthesis of the peptides; it must be shown that they are incorporated into proteins without being first degraded to amino acids. Meinhart and Simmonds (1955) presented evidence which strongly suggests that when peptides are utilized by bacteria as a nitrogen source, they are first degraded to amino acids.

Godin and Work, (1956) have attempted to demonstrate the utilization of peptides for casein synthesis in a goat, however, the casein analyzed immediately following the administration, revealed no direct incorporation of the peptides.

Several conflicting reports may be mentioned in connection with the assumption that if protein synthesis did occur as a step wise

addition of peptides, it would be expected that proteins synthesized from labelled amino acids would show uneven distribution of radioactivity.

Askonas et al (1955) found a uniform distribution of radioactivity between different portions of the proteins β -lactoglobin and casein, after administration of labelled amino acids. Similar results were obtained in the synthesis of several enzymes, after labelled amino acid administration (Heimberg and Velick, 1954; Simpson and Velick, 1954; Simpson, 1955).

Non-uniform distribution of labelling over different parts of the same protein molecule has been reported in tissue minces (Steinberg and Anfinsen, 1952; Vaughan and Anfinsen, 1954). Although labelled ovalbumin, ribonuclease and insulin were obtained, no net synthesis of protein was demonstrated under these conditions.

The conflicting results on tissue minces and on intact animals led Steinberg et al (1956) to conclude that none of the evidence is decisive, and that their own results could be explained without assuming the existence of peptide intermediates in protein synthesis.

Thus, on the whole, there is no evidence indicating that proteins may be synthesized by a step wise addition of peptide intermediates.

Assemblage of the Amino Acids into Protein

Although the part of the mechanism of protein synthesis concerned with the assimilation of amino acids into the final product is highly speculative, (Crick (1958) proposed three hypotheses which together may lead to a reasonable interpretation of how the activated amino

acids are assembled into a complete and specific protein molecule in which the amino acids are arranged in the correct sequence.

The first hypothesis assumes that the specificity of nucleic acids is expressed by the sequence of its purine and pyrimidine bases, and that this sequence is a code for the amino acid sequence of a particular protein. The second hypothesis states that information, defined as the precise determination of sequence, either of nucleic acid bases, or of amino acid residues in proteins, can only be transferred from nucleic acid to nucleic acid or to proteins.

From the above assumptions, there must exist in the cytoplasm a nucleic acid code which can transmit information to the proteins synthesized here. This involves the postulation of a nucleic acid "template" which would act as a "die" on which the protein molecule could be cast. The idea of a template was first generalized by Haurowitz (1950) to include not only the synthesis of any one protein, but to include the synthesis of all proteins throughout nature. Where then is the location of this template? There being no DNA in the cytoplasm, the most logical place to look for a template would be the RNA present in the ribosomes as a ribonucleoprotein complex. However, the original genetic information is known to be present in the DNA molecule; thus, the DNA must impart this information again presumably via a template mechanism, to the RNA which eventually finds its way into the cytoplasm.

That a transfer of genetic information from DNA to RNA must occur is evident from indirect argumentation. For example, it is known that

the amino acid sequence in hemoglobin (assumedly determined by the RNA template, according to the proposed mechanism of protein synthesis) is gentically controlled (Neel, 1949). Spermatozoa, the conveyors of male genetic patterns contain DNA but no RNA, indicating that the DNA must in some way impart this information to RNA, which will then determine the production of specific proteins in the new individual. In addition, direct evidence is available which shows that RNA synthesized within the nucleus migrates into, and probably forms the only source of cytoplasmic RNA (Amano and Leblond, 1960).

Thus, in the ribonucleoprotein particle (ribosome), the main function of the protein component is as a structural framework for the RNA molecule. Each particle is composed of a similar protein moiety with the same arrangement of RNA within it, but one particle differs from another in that the purine and pyrimidine bases of the RNA have different sequential arrangement, hence producing different proteins. Therefore, according to Crick (1958), the RNA forms the template, the protein merely supports and protects the RNA.

The third hypothesis of Crick concerns the adapter molecule, previously discussed and shown to be the soluble RNA. The s-RNA, carrying a specific activated amino acid, would attach to a specific place on the ribosomal RNA template by hydrogen bound base pairing (Lipmann et al, 1959; Fig. 1). It is apparent that at this stage GTP is involved in the transfer of the activated amino acid - s-RNA - complex to the ribosomal RNA (Hoagland et al, 1957).

The steps following this are obscure, but Crick suggests that the s-RNA, bound by hydrogen bonds to the template, polymerizes to form a high molecular weight RNA molecule which is released from the template bearing the attached, sequentialized amino acids. The RNA then folds up to a new configuration, probably inducing the amino acids to polymerize and form the polypeptide chain, which then folds up as it is made to produce the finished protein. The RNA thus formed, is now free of its amino acids and breaks down to replenish the supply of s-RNA.

The speculative nature of the latter part of the mechanism of protein synthesis is evident; however, adequate proof exists to support the first part of this proposed mechanism. In studying the synthesis of proteins from labelled amino acids in vivo with radioautography, where most small molecules are washed out of the histological section during processing, all the steps up to those concerning the binding of amino acids to the ribosomes cannot be detected. Thus, the earliest stage in the synthesis of protein which is of concern in a radioautographic study of this nature is the actual binding of the amino acids to the ribosomes.

Biochemical Investigations of the Process of Synthesis and Secretion of Pancreatic Enzymes

By way of integrating the description given of the pancreatic acinar cell with the mechanism of protein biosynthesis, two purely biochemical studies pertaining to the events in the synthesis and secretion of pancreatic enzymes will be discussed in detail. It is the purpose of this section to integrate, wherever possible, the structures described

in the acinar cells with specific stages in the synthetic mechanism of proteins. These investigations also form part of the rationale which adds confirmation, at the biochemical level, to the radioautographic experiments to be described.

The process of secretion of pancreatic enzymes has been studied by various techniques. However, investigations such as the two to be described, using radioactive tracers to study the formation and migration of specific enzymes are of particular importance.

The Formation of α -chymotrypsinogen

Siekevitz and Palade (1960) have investigated the synthesis of α -chymotrypsinogen, the inactive zymogen of α -chymotrypsin.

Guinea pigs which had been fasted for 48 hours and refed one hour prior to injection received an intravenous injection of DL-leucine-C^{14}. An interval of one hour was chosen because it was found that at this time the rate of protein synthesis apparently increases simultaneous with the appearance of intracisternal granules in the cavities of the endoplasmic reticulum (Siekevitz and Palade, 1958 a, 1958 b).

The pancreas of every animal from each time interval was homogenized and the individual homogenates were pooled. The tissue was fractionated by centrifugation into several components. The nuclear fraction was obtained by centrifuging the homogenate for 15 minutes at 760 x g. The supernatant was spun for 20 minutes at 20,000 x g to sediment the zymogen granules and mitochondria. This supernatant was centrifuged for 1 hour at 105,000 x g yielding the main micro-

somal fraction shown to consist of disrupted elements of the endoplasmic reticulum. A differential density gradient was employed to separate the mitochondria from the zymogen granules.

The microsomes were subfractionated after treatment with sodium desoxycholate. The cleared suspension (cleared presumably by dissolution of the membranes) was spun at 105,000 x g for 30 minutes to isolate a heavy microsomal subfraction which consisted of intracisternal granules plus various microsomal debris. The supernatent was further centrifuged for 120 minutes at 105,000 x g obtaining an intermediate fraction consisting almost entirely of RNP particles and a light supernatant containing the fluid phase of the microsomal content.

α -chymotrypsinogen was chemically isolated from each fraction, purified by ion-exchange column and the eluates were collected. They were counted employing a carrier-method with bovine crystallin chymotrypsinogen.

The results (Table 1) show that at the early time points, 1 to 3 minutes after injection, the attached RNP particles yield α -chymotrypsinogen with a specific activity higher than in any other fraction; the same protein isolated at 3 minutes from the attached RNP particles, the intracisternal and zymogen granule fractions were less active than the RNP particles by 3.5 and 7 times respectively. The specific activity showed little variation among the various fractions at 15 minutes, while at 45 minutes the chymotrypsinogen in the zymogen granules was twice as radioactive as that in the microsomal fraction.

They concluded that the findings are compatable with the hypothesis

TABLE 1

MODIFIED FROM SIEKEVITZ AND PALADE (1960)

SPECIFIC RADIOACTIVITY OF α-CHYMOTRYPSINOGEN FROM VARIOUS PANCREATIC CELL FRACTIONS AFTER IN VIVO LABELLING WITH LEUCINE-1-C^{14}

(Counts/min/mg enzyme)

Cell Fraction	Minutes after injection				
	1	2.5	3	15	1.5
Total microsomes	-	-	-	-	27,700
Attached RNP	22,100	13,780	10,000	15,480	
Endoplasmic reticulum content	-	8,160	5,740	14,950	-
Intracisternal granules	7,970	2,920	2,770	18,300	-
Zymogen granules	-	-	1,770	10,300	58,500

that the α -chymotrypsinogen is synthesized in or on the attached RNP particles; is subsequently transferred to the cavities of the endoplasmic reticulum and finally concentrated and stored in the zymogen granules.

It is pointed out that an alternative interpretation is that synthesis is carried out at different rates in all the sites mentioned, however, morphological and cytochemical findings favour the first alternative.

The Formation of Ribonuclease

The second investigation is that of Morris and Dickman (1960), in which the formation of ribonuclease in the pancreas of adult male and female mice was investigated.

A group of animals each received an injection of pilocarpine, which is know to act as a parasympathomimetic agent causing depletion of zymogen granules from the acinar cells. Injection of pilocarpine causes maximal depletion in 1 to 3 hours in mice and rats. Eighteen hours after the pilocarpine injection each mouse received an intraperitoneal injection of valine-C^{14}, and was sacrificed at specific time intervals thereafter.

The pancreatic tissue was pooled, homogenized and the homogenate fractionated by differential centrifugation. The nuclei fraction was separated by centrifuging the homogenate for 10 minutes at 600 x g. The supernatant plus the nuclear washings were centrifuged for 16 minutes at 11,125 x g to yield the combined zymogen granule and mito-

chondria fractions. This was not separated because previous work had indicated only minimal amounts of ribonuclease in the mitochondria. A microsomal fraction was prepared by centrifuging the washes and supernatant from the zymogen granule fraction for 30 minutes at 105,000 x g. The remaining supernatant and microsomal washings are referred to as Supernatant I. (In some cases this was subfractionated into 2 post-microsomal fractions and a Supernatant II.)

A modification of Hirs' et al (1953) procedure was used to prepare ribonuclease of the cell fractions for chromatography. The extracted ribonuclease was counted for C^{14} and the specific activity was expressed as the ratio of radioactivity to enzymatic activity.

The results show a strikingly rapid labelling of the microsomal ribonuclease. The radioactivity of this fraction at 5 minutes is higher than that in any of the other cell fractions, reaching a maximum at 15 minutes and decreasing thereafter.

The supernatant I showed the second most rapid rise in radioactivity, followed at 30 minutes by a rise in the zymogen granules which then exceed the supernatant.

The conclusion from this data is that the microsomal portion of the acinar cell is the site of synthesis of ribonuclease. The high radioactivity in the supernatant I may be due, in part, to the possibility of a significant portion of labelled ribonuclease remaining in the supernatant which presumably has a different function from that which enters the zymogen granules.

The results, therefore, clearly implicate the endoplasmic retic-

ulum (microsomes) as the site of synthesis of mouse pancreatic ribonuclease.

It is interesting to note that from this data it was found that the synthesis of a biologically active ribonuclease molecule requires 3 minutes.

From the above experiments the microsomes or more specifically the ribosomes, contained mainly in the ergastoplasm are implicated as the site of protein synthesis of two enzymes in the acinar cells of the pancreas. Presumably, in accordance with the proposed mechanism of protein synthesis, this applies to all the other proteins synthesized by the cell. In addition, a short time after maximal labelling of the ribosomes, the labelled proteins appear in the contents of the cavities of the endoplasmic reticulum, and then in the zymogen granule fraction.

However, the steps between the synthesis of the enzymes and their occurrence in the zymogen granules remain unclear.

With radioautography, a technique is presented whereby not only the site of synthesis of the enzymes and their subsequent fate can be followed at any time interval after the injection of labelled amino acids, but the intermediate stages between synthesis and the final product (zymogen granules) can be determined.

MATERIAL AND METHODS

Male Sprague-Dawley rats and male Royal Victoria Hospital mice were used in the investigations to be described. The choice of these two species was obvious on the basis of availability and facility of handling. The relatively small weight of these animals permitted smaller doses of precursors to be used, thereby reducing the cost of the radioisotope. Furthermore, due to the differing metabolic rates, it was possible to compare the two species insofar as the rate of protein production in the pancreas was concerned.

The incorporation and distribution of three labelled amino acids were examined in detail. Amino acids with widely differing metabolic pathways were selected to determine if a different cytological localization was detectable. Leucine, glycine and methionine labelled with tritium were investigated. Glycine differs from other amino acids in that it is not subject to transamination reactions. Furthermore, it is both a ketogenic and glycogenic amino acid. Methionine, a sulphur containing compound, also is believed to act as a one carbon fragment donor. Leucine is a branched chain, ketogenic amino acid.

In addition, leucine labelled with carbon 14 was used to analyse the radioactive material found in histological sections of mouse tissues. For this analysis, routine biochemical extraction techniques were employed. A Geiger-Müller counter was used to assay the radioactivity content of each fraction.

Animal Techniques

The rats were obtained from the Holtzman Co., Madison, Wisconsin. They were received at least 10 days prior to the experiment and housed under constant temperature conditions at 84° ± 5° F. The animals were maintained under a system automatically controlled to alternate 12 hours of darkness (between 8.00 P.M. and 8.00 A.M.) with 12 hours of light.

The animals were housed in groups of 6 in standard 15" x 12" x 9" cages, the walls of which were of galvanized tin, the floor and front wall, of wire mesh. The size of the mesh permitted the excreta to fall through the floor into a pan covered with sawdust. The sawdust was periodically changed, and the cages replaced at least once during the experimental period.

Food, in the form of Canadian Purina Fox Chow pellets and tap water, was provided ad libitum.

Daily weight records were kept to ensure that the animals gained weight steadily. A regular increase in body weight was taken as an indication of the health of the animal. During the daily weighings, each animal was petted and handled in a manner similar to that used during injection. This procedure was intended to accustom the animals to being handled and to reduce their nervousness.

The mice were obtained from the Royal Victoria Hospital's breeding stock. They were received at least one week prior to injection and maintained in cages similar to the ones described

for the rats, but the dimensions were 10" x 5 1/4" x 6".

Canadian Purina Fox Chow pellets and tap water were available ad libitum. Daily weight charts showed a slow increase in weight which is compatible with the normal pattern.

Although the mice were frequently handled, this seemed to have little effect on reducing their nervousness. They were, consequently, very difficult to hold at the time of injection.

Biochemical Extraction Experiment

In order to analyse the nature of the radioactive material present in histological sections, the following procedure was followed.

Four adult male mice were treated as described above under Animal Techniques. Mice, weighing 22 to 24 grams were injected with 1 uc per gram of body weight of DL-leucine-1-C^{14} which was obtained from the Radiochemical Centre, Amersham, England (specific activity 5.45 mc/mM). The powder was dissolved in normal saline and the dilution calculated so that the dosage did not exceed 0.5 ml. The animals were killed in 2 groups of 2 animals each, at 30 minutes and 24 hours after the injection. The pancreas, as well as the liver, kidney and brain were removed and fixed in Bouin's fluid for 48 hours. The tissues were treated (up to Paraffin Embedding) as described in Histological Techniques (page 53). After embedding, the entire piece of tissue was cut into 5 μ sections and all ribbons collected.

Deparaffinization

The sections thus obtained were deparaffinized in 12 ml. of toluene. After centrifugation the supernatant was discarded and the

packed sections were washed 5 times with 12 ml. of toluene. They were then washed in a mixture of absolute alcohol and toluene and finally with absolute alcohol.

Homogenization

The sections were suspended in 5 ml. of distilled water and homogenized using a Teflon homogenizer, after which the proteins were precipitated with 5 ml. of 30% tricholoracetic acid (TCA). The final concentration of TCA was about 15%. Homogenization was continued for a short time, the solution centrifuged and the supernatant discarded.

Nucleic Acid Fractions

The precipitate was resuspended in 5 ml. of 5% TCA and incubated for 15 minutes in a water bath at 90° C. The suspension was centrifuged and the supernatant, containing nucleic acids, retained for subsequent analysis for radioactivity.

The precipitated proteins were washed twice with cold 5% TCA to remove all traces of nucleic acids and the supernatants pooled with the original nucleic acid fraction.

Lipid Extraction

The precipitate was washed once in 5 ml. of 95% ethanol and then treated with 5 ml. of a mixture of 3 parts ethanol to 1 part ether. The suspension was incubated for 10 minutes in a water bath at 60° C,

centrifuged and the supernatant retained.

Alkaline Extraction

The precipitate was divided approximately into 2 equal aliquots; one was left untreated as a control, the other was extracted with alkali.

The aliquot for alkali extraction was dissolved in 2 ml. of 1N NaOH and allowed to stand for 1 hour at room temperature. The proteins were reprecipitated from this solution with 3 ml. of a solution consisting of 2 ml. of 15% TCA and 1 ml. of 2N HCl. This gave a final concentration of 10% TCA in 0.7 N HCl (the H Cl served to neutralize the NaOH).

The suspension was centrifuged and the supernatant retained.

Preparation of Protein Precipitate for Plating

The alkali extracted precipitate was dehydrated by washing with 95% alcohol.

Both alkali extracted and non-extracted precipitates were washed in 5 ml. of pure ether and suspended in 0.5 ml. of a mixture of 4 parts chloroform to 1 part ether.

Plating of the Protein Precipitate

Aluminum foil planchets were prepared according to the specifications used in the McGill-Montreal General Hospital Research Institute. The inner aspect of the planchets were covered with a thin film of vaseline and weighed in a Gram-o-matic balance to one tenth of a milligram.

The entire 0.5 ml of the suspension in the chloroform-ether mixture was pipetted into a planchet and the volatile solution allowed to evaporate at room temperature. The planchets were placed in a horizontal position, as nearly level as possible, to ensure an even plating. After initial evaporation the planchets were completely dried in the heat of a lamp.

The planchets were again weighed and the weight of the vaseline coated plate was subtracted to give the total amount of protein (in mgms).

The NaOH extraction, lipid and nucleic acid fractions were first reduced in volume by evaporation under reduced pressure, then plated in a manner similar to the protein precipitates.

Radioactive Assay

The plated samples were counted with a Geiger-Müller tube in a Berkeley sealer. With a low energy β emittor, such as C^{14}, it is known (Kamen, 1947) that varying the relationship of the sample to be counted with respect to the Geiger-Müller tube by as little as 1 mm. can introduce an error of 5%. Therefore, each planchet was counted 4 times, each time in a position rotated 90° from the previous location and the four counts for each sample were averaged. The duration of counting and/or the total number of counts were sufficiently large to be statistically reliable.

The results of the effect of NaOH extraction on the radioactivity of the protein residue, were expressed as the number of counts per minute per mgm of protein after correction for background

radiation. The results pertaining to the amount of radioactivity in each of the extracted fractions, were expressed as a percentage of the total radioactivity. The latter value was obtained by adding the number of counts per minute of the alkali extracted protein residue, non-alkali extracted protein residue, the nucleic acid fraction and the NaOH extractable fraction.

The radioactivity of the nucleic acid fraction, as a percentage of the total radioactivity, was calculated from the number of counts per minute in this fraction. However, for the protein and NaOH soluble fractions, further modifications had to be made. Since the non-alkali extracted proteins contain a small amount of NaOH removable radioactivity, this amount had to be subtracted from the protein fraction and added to the NaOH soluble fraction. To calculate this, an amount of radioactivity proportional to the amount of protein was deduced from the known amounts removed from the alkali treated aliquot. The percentages of the total radioactivity were then calculated.

No self absorption was found in the protein residue if the amount of precipitate did not exceed 3 mgms per square cm. In the experiment, this amount was never exceeded. The nucleic acid residues showed no self absorption, but the NaOH extractable fraction, consisting of large flaky precipitates of NaOH, showed a large degree of absorption. Correction curves were calculated and applied to these results.

To verify and extend the results obtained, the experiment was repeated with 4 mice weighing 15 to 18 grams. The specific activity

of the L-leucine-C^{14} (The Radiochemical Centre, Amersham) used in this experiment was 7.14 mc/mM. In all other respects, except that the entire protein residue was extracted with NaOH, the two experiments were identical.

Radioautographic Experiments

Experiment I - Leucine-H^3

Sixty-nine young adult male rats, weighing 122 ± 6 grams were selected, using the random number method, from a stock of 106 animals. The animals were maintained for ten days under the conditions of the departmental animal room. Thirty-three animals were injected with one dose of 2.5 µc per gram of body weight of leucine-H^3. The leucine-H^3 was obtained from the New England Nuclear Corporation, Boston, Mass., (specific activity 29.1 mc/mM) and diluted with normal saline. The dilution was adjusted so that a minimum volume, not exceeding 0.32 ml. of solution, could be injected. Three animals were killed at 10 minutes, and the remaining 5 groups of 6 animals each were killed at 30 minutes, 4 and 36 hours, 7 and 30 days after injection.

Experiment II - Glycine-2-H^3

Five adult male RVH mice, weighing 25 to 30 grams, each received one single intraperitoneal injection of 3.5 or 5 µc per gram of body weight of glycine-2-H^3. The animals were killed in pairs at 5 and 15 minutes, and a single animal at 30 minutes. One animal of each group received 3.5 µc, the other 5 µc per gram. The 30 minute animal

received 3.5 μc per gram of body weight.

Experiment III - DL-Methionine-H^3 (generally labelled)

Six mature male RVH mice, weighing 23.5 to 27.5 grams, were each injected intraperitoneally with 5 μc per gram of body weight of methionine-H^3, and killed in pairs at 5, 15 and 30 minutes.

The glycine-H^3 (specific activity 15.4 mc/mM; New England Nuclear Corporation, Boston, Mass.) and the methionine-H^3 (specific activity 28.0 mc/mM; Radiochemical Centre, Amersham, England) were received as powders and dissolved in normal saline, the dilution being such that the volume of the injection, in the case of methionine, did not exceed 0.2 ml. The glycine solution was prepared for a previous experiment and the radioactivity was in a greater dilution, thus a larger volume, about 0.8 ml. was injected. However, no detectable effect was produced on the animal.

Injection Techniques

In all experiments, the animals were injected during the morning hours. Partly digested food was found in the stomach of each animal at autopsy, indicating that digestive processes were under way.

Injections were given with a 1 ml. tuberculin syringe and number 25, one-half inch needle. The injection was intraperitoneal, via the anterior abdominal wall, usually lateral to the midline. Care was taken to insert the needle very obliquely so that no material

could leak out after the needle was withdrawn.

Following injection, each animal, suitably marked by ear clipping, was replaced in its cage.

Sacrifice

Rats

Because the radioautographic experiment with leucine-H^3 was performed with Dr. B. Mitmaker (M.Sc. Thesis) whose particular interest was the thyroid gland, the method of sacrificing the rats was as follows. Under ether anesthesia, the thyroid gland was exposed by a mid-line incision in the neck. The abdominal wall was then opened and the animal exsanguinated by aortic puncture using a heparinized syringe. The thyroid gland, together with the trachea, was immediately removed. The pancreas was removed immediately after the thyroid extirpation, followed by other organs and tissues. All were fixed in Bouin's fluid.

Mice

Under chloroform anesthesia, the pancreas, as well as other organs of the mice used in the radioautographic experiments, was removed and fixed in Bouin's fluid. The mice died under the anesthetic, usually before complete removal of the organs.

For the biochemical extraction experiment, the mice were anesthetized with chloroform and killed by exsanguination via aortic section.

Histological Techniques

Paraffin Embedding

The tissues were fixed for at least 24 hours in Bouin's fixative, then transferred to 70% alcohol. Each piece of tissue was trimmed with a razor blade so that a flat plane, with a large surface area, was presented for sectioning.

The various tissues, enclosed in a cheesecloth bag, were dehydrated over a period of about 12 hours in several changes of dioxane.

After dehydration, the tissues were placed in a 1:1 solution of dioxane and liquid paraffin, then into several changes of clean paraffin, until infiltration was judged to be complete.

All the soft tissues from each animal were embedded together in a single block. Sections were cut at 6 μ and mounted on a glass slide using egg albumin as an adhesive.

Staining: Hematoxylin and Eosin

The sections were deparaffinized in xylol and hydrated in a graded series of alcohols, and finally into water.

Hematoxylin:

Harris' hematoxylin was used because it was shown to have little effect upon the photographic emulsion. After staining for 5 minutes, the slides were washed in tap water and differentiated with a solution of 0.5% HCl in 70% alcohol. The slides were again washed in tap water and blued with lithium carbonate, followed by washing in

tap water, then in distilled water.

Eosin

From distilled water the slides were brought through 50 and 70% alcohol to 95% alcohol and stained for 5 minutes with 1% eosin in 95% alcohol. After differentiation in 50% alcohol, the slides were washed in water and allowed to dry in air.

Radioautographic techniques

The dry histological sections were processed for radioautography by the coating technique (Messier and Leblond, 1957; Markus-Kopriwa and Leblond, in press).

Coating

Coating of the histological sections was carried out at a distance of 3 feet from a Wratten Safelight filter in a completely light-proof dark room maintained at 17 to 18° C. Bulk Kodak NTB2 emulsion was melted in a 40° C water bath. The slides, bearing the histological sections, were dipped by hand vertically into the fluid emulsion, retained for a few seconds and withdrawn. Excess emulsion was drained off the lower portion of the slide and the backs cleaned of emulsion, with a soft tissue. The slides were allowed to dry vertically in plastic racks.

Exposure

The coated slides were stored in plastic slide boxes, the humidity being minimized by 15 to 25 grams of Indicating Drierite contained in

a tissue bag. The boxes, sealed with black adhesive tape, were stored in a refrigerator (4-5° C) in such a way that the slides were exposed in the horizontal position, with the emulsion facing upwards.

The leucine-H^3 radioautographs were exposed for 15 days; the glycine and methionine-H^3 radioautographs for 21 days.

Processing

For processing, the exposed slides were placed in a plastic box, similar to the one in which exposure took place, but with holes bored in the sides and most of the top and bottom cut out to allow for circulation of fluids.

All solutions were kept in the darkroom and were, consequently, at 17-18° C. The radioautographs were developed for 2 minutes in Eastman Kodak Dektol (D-72) developer, carried through a distilled water stop bath and fixed for 3 minutes in Eastman Kodak Acid Fixer. After fixation the slides were washed for 15 minutes in running tap water at 17-18° C, dehydrated in 95% alcohol and two changes of absolute alcohol.

The radioautographs were taken from the dark room, removed from the slide carrier and placed in a 1:1 mixutre of cedar oil and absolute alcohol for 1 hour, then in a 1:1 mixture of Canada balsam and xylol for 1 hour, and permanently mounted in balsam under a coverslip.

The preparations were dried at 37° C for a few days and cleaned with xylol.

Quantitative Radioautographic Methods

Quantitating radioautographic intensity (expressed as number of silver grains in the emulsion overlying cytological structures) necessitated the selection of a convenient unit for analysis. The heterogeneity and small size of the cellular structures in the pancreas made counting silver grains per unit area of an ocular grid superimposed on these structures virtually impossible. Indistinct cellular limits in hematoxylin-eosin stained sections precluded the use of individual cells as a basic unit. Therefore, the choice was restricted to the entire acinus as the unit for study.

Method for Analysis of Radioautographs

The heterogeneity of the tissue prevented the use of ordinary grain counting methods. However, a more accurate analysis was made possible using drawings made to scale of acini and the silver grains over them.

Thus a more precise localization of all silver grains was made possible. In addition, acini could be further subdivided to gain more detailed information on grain distribution.

Criteria for Selection of Acini for Drawing

Drawings were made of acini selected on the basis of the following:

1. Clear and distinct separation from adjacent acini.
2. Basophilic ergastoplasm being clearly delimited from the eosinophilic zymogen granules.

3. Entire acinus ringed by a more or less complete ergastoplasm enclosing a central region of zymogen granules.
4. Acinar size not exceeding an area of 2500 sq. μ (as determined by a known ocular grid) with a minimum not smaller than 100 μ.
5. Intensity of radioautographic reactions.

In practice, acini which were well stained, clearly defined and with a readily countable radioautographic reaction were selected.

Preparation of Drawings for Glycine and Methionine Analysis

The drawings were made in relation to a Whipple micrometer ocular disc (1 square = 10 x 10 μ) at an original magnification of 800 times under oil immersion. Squared paper was used, one square inch representing one squre of the disc grid. First, the acinar limit (basement membrane), ergastoplasm-zymogen granule junction, nuclei and nucleoli were drawn. Then the silver grains were drawn in, being superimposed on the acinar outline drawing. Proceeding one square at a time the acinar details in the square of the disc were drawn freehand onto the paper.

Thus, three morphological subdivisions of the acinus were drawn (1) ergastoplasm, (2) zymogen granule area, (3) nuclei (only those judged to be at the surface of the section were included in the drawing). Of these three morphological divisions, the ergastoplasm and zymogen granule region were further subdivided.

The ergastoplasm was divided by a line drawn approximately parallel to the basement membrane and to the ergastoplasm-zymogen granule junction and about one half the perpendicular distance between them. This line separated two regions which were referred

to as proximal and distal ergastoplasm respectively. The distal ergastoplasm does not, therefore, include the small amount of ergastoplasm scattered in the apex of the cell.

The zymogen granule area was divided by a line drawn parallel to the ergastoplasm-zymogen granule junction and at an arbitrarily set distance of 2.8 μ from it.

Therefore, five acinar components in all were drawn (Fig. 2): viz.

1) Proximal ergastoplasm
2) Distal ergastoplasm
3) Proximal zymogen
4) Distal zymogen
5) Nuclei.

Preparation of Drawings for Leucine Analysis

The lines of a stage micrometer, ruled 10 μ apart, were photographed at a magnification of approximately 550 times. At the same magnification, the surface of the acinus immedately beneath the level of the silver grains was photographed. The negative image of the micrometer lines was projected using a photographic enlarger onto graph paper ruled in 1 inch squares. The magnification was so adjusted that the micrometer lines coincided with those of the graph paper. Thus a final magnification was achieved such that 10 μ corresponded to 1 inch or x 2540.

The negative image of the acinus was projected at this magnification and traced on the square paper in such a way that the drawn

PLATE 2

Preparation of the drawings for the radioautographic analyses.

Fig. 2 a Masson's trichrome stained section of the pancreatic acinus of the rat.

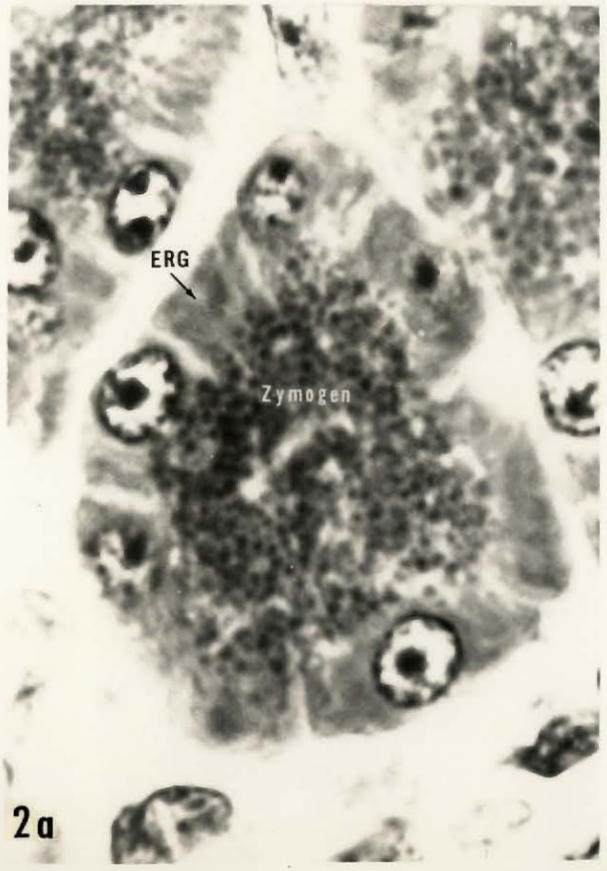
The acinus is completely enclosed by the ergastoplasm (Erg), which in turn encloses the central zymogen granule region. The nuclei appear entirely within the ergastoplasmic band. A more complete description of this photograph is given for Plate 3.

Fig. 2 b A line drawing representation of the acinus in Fig. 2 a

This drawing is similar to the ones made for analysis of the radioautographic grain distributions. The ergastoplasm (Erg) as well as the nuclei contained therein were drawn, then divided by the interrupted line into proximal (P) and distal (D) portions.

The zymogen granule region was divided by the interrupted line, drawn 2.8 μ from the limit of the ergastoplasm. Thus, a proximal and distal portion was also created in the zymogen granule mass.

The positions of the silver grains in the radioautographs were then plotted on such drawings.



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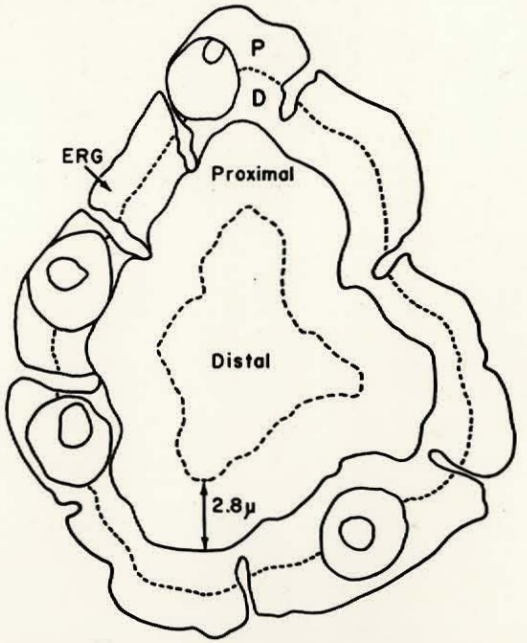


2b

acinus had the same relations to the squares of the paper as the microscopic image of the acinus had to the squares of an ocular grid superimposed on it.

By using the lines of the ocular grid and the lines of the graph paper as coordinates, the radioautographic silver grains overlying various acinar structures were mapped on the drawing.

The drawings were subdivided exactly as described for glycine and methionine (above).

Counting of Silver Grains

The number of silver grains drawn over each of the five components were counted in all drawings. Those grains which occurred at the border of two components were considered as originating half from each component.

Histometric Technique

The results of the grain counts alone were not comparable from one acinus to another due to the variation in size. They were, therefore, expressed in terms of concentration, or grains per unit area of the section.

Since the acinar components are irregular in outline it was impossible to calculate the surface area by conventional mathematical means. However, the drawings represent the relative proportions of the various acinar components. The paper used for the drawings was shown to be relatively homogeneous, as far as weight per unit area

is concerned, and it may be assumed that the weight of paper representing each acinar component is proportional to the area of that component.

Therefore, the prepared drawings were cut out and the paper comprising each of the 5 components was separately weighed on an analytical balance.

Relative weights of the paper-cut-outs were obtained and concentration of radioactivity, expressed as number of grains per weight of acinar component (paper) was calculated.

RESULTS

The results are presented in three parts; an introductory description of the structure of the pancreatic acinus; the results of the biochemical analysis justifying the use of radioautography to detect newly formed proteins, followed by the results of the radioautographic analysis, which forms the main subject of this thesis.

PART I

Structure of the Acinus

Structurally, the pancreatic acini from both rats and mice were found to be almost identical. The following description, therefore, applies to both species (Fig. 3).

The Acinus

In sections stained with hematoxylin and eosin (H & E) the acini appear as more or less distinct, irregularly circular or oval units. The amount of separation of one acinus from another varies from one region to another, probably due to the degree of shrinkage produced by uneven penetration of the fixative. Small amounts of loosely and irregularly arranged connective tissue elements are found between adjacent acini.

In section, the cells forming the acini are irregularly triangular in shape (Fig. 3). The apex of the cell is flattened,

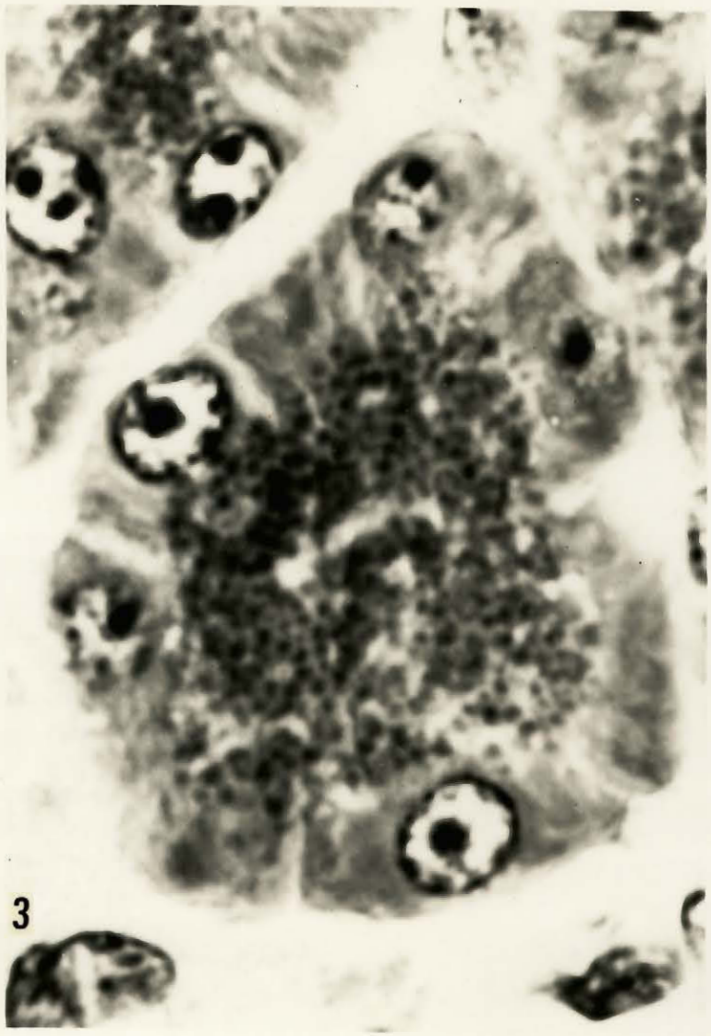
PLATE 3

Fig. 3 Masson's trichrome stained section of the pancreatic acinus of the rat. (x 2600)

The acinus is composed of triangular cells, the outline of which can be distinguished in several places. Surrounding almost the entire acinus is the basophilic though lighter staining ergastoplasm in which the circular nuclei, containing prominent nucleoli, are located. The central region is occupied by the acidophilic darkly stained, zymogen granules.

Within the center of the zymogen granules, an elongated clear space indicates the apical lumen. A clear area, can usually be distinguished between the zymogen granules and the ergastoplasm, this presumably indicates the position of the Golgi apparatus.

Between the bases of adjacent cells, indentations can be seen. The basal indentation at the right of the acinus contains fine connective tissue elements, and is lined by the basement membrane.



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forming a very small border which abuts on the acinar lumen (see description below). The base of the cell is in contact with a periodic acid-Schiff (PA-Schiff) positive basement membrane. The sides, or lateral borders of adjacent cells are in close contact at their apical ends, where they are indistinguishable in H & E stained sections, but may diverge from each other as they approach the basement membrane leaving narrow gaps (referred to hereafter as basal indentations) between the basal portions of the cells. At this level the separation gives an indication of the cellular limits.

Observations on sections of rat pancreas stained with the TPA technique (see Acinar Lumen, below), PA-Schiff technique and reticulin silver impregnation technique, reveals two types of basal indentations. One type, which is narrow and extends for only a short distance apically, is always devoid of connective tissue elements. These indentations are more numerous in poorly fixed regions of the sections, giving the impression that they may be due to shrinkage caused by fixation. A second type of indentation is usually much larger, and extends further apically between adjacent cells. This type of indentation is associated with connective tissue, and is usually in close proximity to a capillary.

The basement membrane, not seen in H & E stained preparations, and only faintly visible in sections stained with the PA-Schiff technique, encloses the entire acinus, but does not extend into the narrow indentations at the basal junction of adjacent cells. The basement membrane does, however, extend into the deeper in-

dentations.

Acinar Lumen

The lumen of the acinus is extremely small, more often than not, indistinguishable. With the technique devised by Puchtler and Leblond (1958), which consists of mordanting with tannic acid, followed by phosphomolybdic acid and stained with amido black (TPA), a ductular lumen, not exceeding 1.5μ in diameter, as indicated by the apical cell web (Leblond et al, 1960), may be seen between the cell apices (Fig. 4). The shape of the apical lumen differs according to the number of cells abutting on it. Thus, in an acinus composed of only three cells, the lumen is triangular in cross-section with a terminal bar at each angle (Fig. 4), in a four celled acinus, the lumen is square or rectangular, again with terminal bars at each angle.

Frequently, the ductular lumen is elongated between two adjacent cells, forming an intercellular canaliculus, characterized in cross-section by a circular appearance with two terminal bars at opposite points on the circumference (Fig. 4). Cross-sections of intercellular canaliculi may be seen anywhere along, but enclosed between, the lateral borders of adjacent cells. When the canaliculi appear close to the basal end of the cell, the ergastoplasm which would normally be seen at this point, is separated from the canaliculus by zymogen granules. Judging from their size and number in comparison to the apical lumen, these caniculi probably represent the main lumen of some acini.

PLATE 4

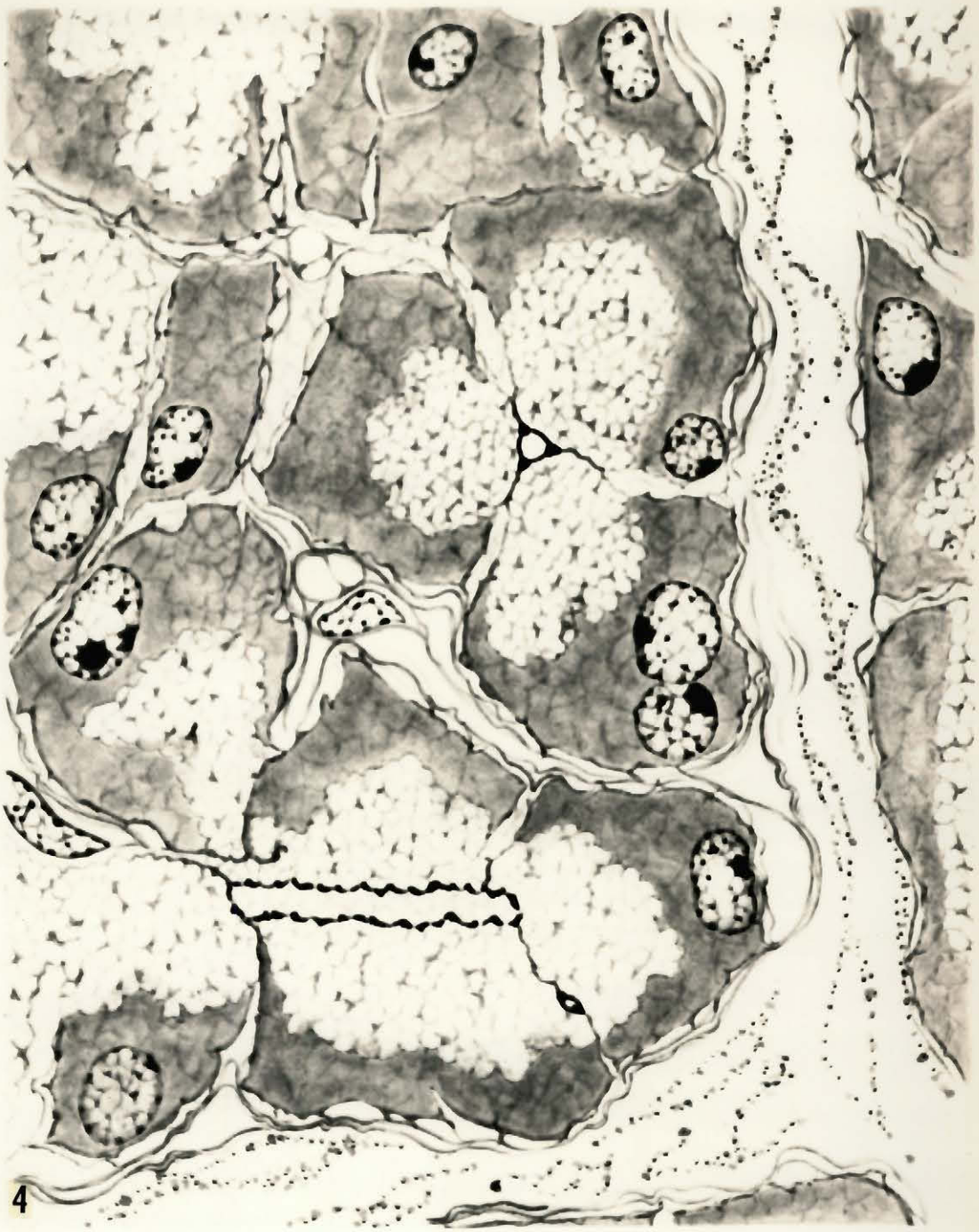
Fig. 4 Drawing of a TPA stained section of the pancreatic acini of the rat.

Several acini have been drawn. At the periphery of each acinus, there is an almost complete band of ergastoplasm, here shown in grey. Several nuclei can be seen within the ergastoplasm.

The lacy pattern enclosed by the ergastoplasm is due to the presence of zymogen granules. In the direct center of the acinus at right center, desmosomes can be seen at the apical pole of the three cells which form the acinus. Connecting the desmosomes are three lines, which represent the delicate apical cell web. From the desmosomes, cell membranes may also be seen extending outwards. These are first seen as a single line and then diverge to form the basal indentations.

The lower acinus shows a pattern essentially similar, except that a longituidnally cut lumen can be seen transversing the zymogen granule accumulation. The walls of the lumen are seen to be composed of a row of granules; the entire row being the terminal bar, while the darkly stained granules are the desmosomes (whose alignment makes up the terminal bar). The duct originates blindly at the right, but its termination to the left, presumably into an excretory duct, cannot be seen in this section.

Branches of the main lumen may extend as outpouchings between two adjacent cells. These consist of 2 terminal bars, joined by a layer of cell web at the surface of each one of the two cells. These form the intercellular canaliculi, one of which is seen in the lower right portion of the acinus.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Nucleus

The nucleus is large, round and basally placed, where, except at it's distal pole, it is surrounded by the basophilic ergastoplasm. A large number of binucleated cells were observed in both species, however, on a qualitative basis, the proportion of binucleated acinar cells appears to be somewhat greater in rats than in mice. The nuclear details observed correspond to those described in the introductory section on morphology of the pancreas.

Basal Region

With H & E the entire basal portion of the acinar cell stains deeply basophilic. The basophilia extends around either side of the nucleus, usually completely enclosing it. As the band of basophilia approaches the border between two cells, it usually extends for a short distance apically along the lateral cell membranes, appearing as a peak of basophilia projecting into the mass of zymogen granules in the center of the acinus. This basophilic region ends abruptly in contact with the acidophilic zymogen granules. The term ergastoplasm coined by Garnier in 1900, will be used to denote this region.

Two varieties of striations are evident in the ergastoplasm. Intensely stained striations, irregularly alternating with and parallel to lightly stained, longitudinally disposed striations, are usually seen in H & E stained sections. The dark striations

are probably due to the preferentially oriented ergastoplasmic sacs, while the light areas are due to the mitochondria located in the ergastoplasm. Both types of striations are more prominent in tissues fixed in Carnoy's fluid.

With Masson's trichrome, the ergastoplasm stains deep purple, leaving the fine striations, due to mitochondria, visible as lightly stained areas (Fig. 3).

Apical Region

The apical portion of the cell, in H & E preparations is occupied by numerous refractile, eosinophilic zymogen granules. These organelles are embedded in the substance of the apical cytoplasm; however, in the 6 μ sections used for this study, the granules overlapped to such an extent that almost no trace of the cytoplasm could be seen.

The zymogen granules stain a brilliant red with Masson's trichrome stain; the central portion of each granule appears paler while the periphery is more intensely stained.

A clear area, particularly noticeable in Masson's trichrome preparations, is invariably seen separating the ergastoplasm from the zymogen granule accumulation. This region occupies the morphological site of the Golgi apparatus.

The Golgi Apparatus

Classical silver impregnation methods, in particular Aoyama's (1930) technique, were applied to the rat pancreas. In sections prepared

according to this technique (Baker, 1956), the internal reticular apparatus of Golgi was always seen in a position either directly between the nucleus and the cell apex, or between the nucleus and the lateral cell membrane (Fig. 25). Measurements of the extent of the Golgi apparatus, as well as of various distances from the apparatus to other cellular structures are shown in Figure 23 . From these measurements, it is interesting to note that a distance of at least 2 μ separates the Golgi apparatus from the apical pole of the nucleus.

The silver deposition blackens a series of interconnected linear structures, which subdivide a non-pigmented area, presumably corresponding to the chromophilic and chromophobic portions of the Golgi apparatus (Lacy and Challice, 1956).

Summary

In summary, the general picture of the acinus which is considered for quantitative purposes is that of a rounded structure divided into three distinct morphological units. These units are (1) the ergastoplasm which forms an almost complete basophilic band of rather uniform width around the entire acinus; (2) the nuclei, large round structures almost entirely within the ergastoplasm, and (3) the esinophilic zymogen granule accumulation, which occupies the entire central portion of the acinus within the ergastoplasmic band.

For purposes of orientation, the basement membrane is considered as the proximal end of the cell and the apex bordering on the lumen

as the distal end. Thus, all subdivisions of the acinus may be referred to as being proximal or distal with respect to the basement membrane.

PART II

Biochemical Extraction Experiment

Introduction

The biochemical extraction experiment was designed to determine precisely what compounds, of those remaining after histological processing contain radioactive material.

The crude TCA insoluble fraction (see Methods), obtained after homogenizing the sections, contains all the simple unconjugated proteins, the nucleoproteins, lipoproteins (though practically none of the lipid prosthetic groups remain after histological procedures) and the labelled compounds which may adhere to these simple and conjugated proteins, by bonds other than peptidic linkage.

Extracting the crude TCA insoluble fraction with hot TCA hydrolyses the nucleic acids and frees them from the protein. Thus, the hot TCA soluble fraction contains the ribonucleic and the deoxyribonucleic acids (RNA and DNA), of the tissue.

Lipids were extracted from the crude TCA insoluble fraction (After nucleic acid extraction) merely for the sake of completeness, however, no radioactivity was found in this fraction.

After alkaline extraction, the TCA insoluble fraction consists

of the purified proteins. The alkali soluble fraction contains labelled compounds liberated from the proteins; these are presumably not held by peptide bonds.

Results

To assess the effect of NaOH extraction on the radioactivity content of the tissue proteins, the two aliquots, viz. non-alkali extracted, acting as a control, and alkali extracted fractions, were compared. The results are expressed as the number of counts per minute per mgm of protein, after correction for background radiation (Table 2). In some cases, the count following alkali extraction is lower than the corresponding control, but in others, the opposite is found. However, in all cases, radioactivity is found in the alkali soluble fraction; this is taken to indicate that the experimental conditions are not sensitive enough to detect slight losses due to NaOH extraction.

A more informative picture is obtained when the results are expressed as a percentage of the total radioactivity contained in the histological sections of the entire piece of tissue (Table 3). To obtain the percentage of the total radioactivity in the tissue the calculations described on page 49 were made.

The results show that 90 to 97% of the total radioactivity in tissue sections is found in the protein residue; a slight amount occurs in the nucleic acid fraction and a smaller quantity is found in the NaOH soluble fraction.

Conclusion

The principle conclusions drawn from these experiments, relevant

TABLE 2

EFFECT OF NaOH EXTRACTION ON THE RADIOACTIVITY IN THE PROTEIN FRACTION FROM HISTOLOGICAL SECTIONS OF VARIOUS TISSUES FROM LEUCINE-C^{14} INJECTED MICE

(COUNTS/MINUTE/MGM OF PROTEIN)

Tissue	Time after injection	Non-NaOH extracted	NaOH extracted
Pancreas	30 min	418.9	499.7
		501.4	499.7
	24 hrs	78.6	78.1
		52.9	54.6
Liver	30 min	64.9	68.9
		69.7	106.3
	24 hrs	65.5	75.6
		43.7	37.9
Kidney	30 min	53.4	68.1
		127.2	101.4
	24 hrs	112.4	94.5
		115.8	131.1
Brain	30 min	24.3	21.8
		25.2	22.9
	24 hrs	38.9	43.7
		24.3	23.9

TABLE 3

FRACTIONATION OF THE RADIOACTIVITY RETAINED AFTER HISTOLOGICAL PROCESSING IN VARIOUS TISSUES OF LEUCINE-C^{14} INJECTED MICE

Tissue	Time after injection	% Total radioactivity		
		Insoluble in cold TCA	Soluble in hot TCA	Soluble in NaOH
Pancreas	30 min	91.0	4.0	5.0
	24 hrs	94.0	3.0	3.0
Liver	30 min	96.7	2.3	1.0
	24 hrs	96.0	2.2	1.8
Kidney	30 min	96.4	1.9	1.7
	24 hrs	97.2	1.1	1.7
Brain	30 min	90.8	3.5	5.7
	24 hrs	96.5	1.2	2.3

to the radioautographic results, is that over 90% of the radioactivity visualized by radioautography originates from labelled material firmly fixed to the proteins of the histological section. Thus, it is confirmed, that the injection of labelled amino acids does in fact give rise to labelled proteins.

PART III

Radioautographic Results

Preliminary observations of the radioautographs at the earliest time intervals, 5 and 10 minutes, show photographic silver grains in the emulsion overlying every acinus throughout the entire section of pancreas. Thus, as early as 5 minutes in mice, and 10 minutes in the rat, the labelled amino acids must have diffused throughout the pancreas; have passed through the various reactions involved in protein synthesis and have been incorporated into the newly elaborated proteins. The radioautographic reactions, though relatively light at the early time intervals, increase to a maximum at 4 hours and drop to a minimum at 36 hours.

Leucine-H^3

Cytoplasm

Qualitative analysis of the leucine-H^3 radioautographs show that most silver grains at 10 minutes are located over the ergastoplasm of the acini (Fig. 5). The grains, predominantly random in distribution over the basophilic region, may however, be concentrated over one area of the ergastoplasm, and only scattered over another

ergastoplasmic area of the same cell. The concentration of grains, often confined to either the basal portion, close to the basement membrane, or to the apical part of the ergastoplasm, could not be correlated with any specific substructure within this region.

The proximal zymogen granules are also heavily labelled, but only rare and scattered reactions are seen over the distal zymogen granules.

Thirty minutes after the injection, the most intense reaction is seen over the proximal zymogen granules (Fig. 6). The grains appear as more or less distinct clusters somewhat removed from the distal pole of the nucleus. The ergastoplasm remains strongly labelled, and an increased, though still slight reaction is seen over the distal zymogen granules.

By four hours after the injection the maximum reaction intensity is located diffusely over the proximal and distal zymogen granules (Fig. 7).

Concomitant with the reactions seen over the cells at 30 minutes and particularly at 4 hours, reactions are also seen over the material in the lumen of the excretory ducts (Figs. 8 and 9).

Quantitative Results

Four acini, selected according to the criteria stated in the section on Material and Methods, from each animal in the 10 and 30 minutes, 4 and 36 hour groups, were drawn as described on page 58 . For the 10 minute, 4 and 36 hour groups, 3 animals were studied; for the 30 minute group, all six animals were analyzed. Thus, the results represent the average of 60 different drawings.

PLATE 5

Radioautographic localization of leucine-H^3 in the pancreatic acini of the rat. (x 1300, 15 day exposure, H & E staining).

Fig. 5 Acinus 10 minutes after injection.

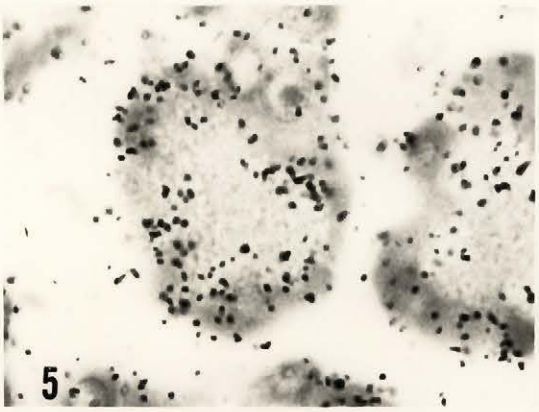
The silver grains are localized mainly over the ergastoplasm (darker staining zone), with a few grains over the proximal zymogen granule region. The distal zymogen granules show no reaction.

Fig. 6 Acinus 30 minutes after injection.

The ergastoplasm shows some reaction, but most of the grains are now located over the proximal zymogen granule region. Again the distal zymogen granules shown no reaction.

Fig. 7 Acinus 4 hours after injection.

While some reaction persists over the ergastoplasm, most reactions are distributed over both proximal and distal portions of the zymogen granules.



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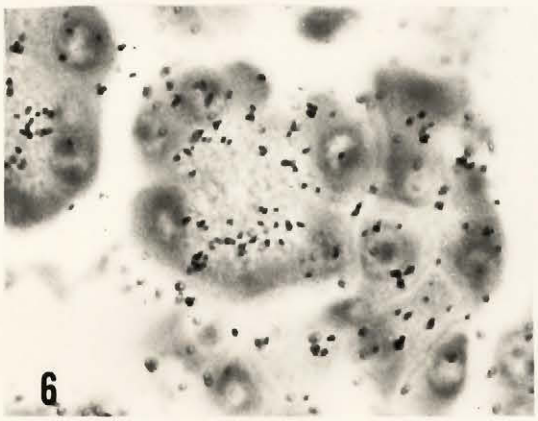

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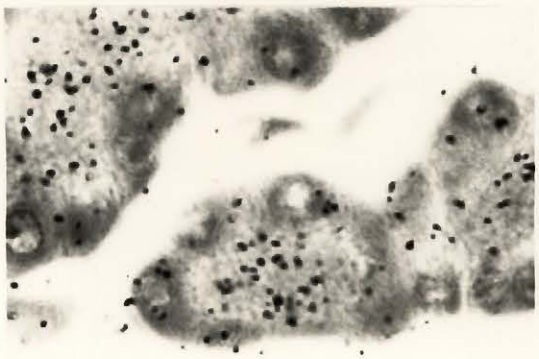

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PLATE 6

Radioautographic localization of leucine-H^3 in the excretory ducts of the rat pancreas (x 500, 67 day exposure, H & E staining).

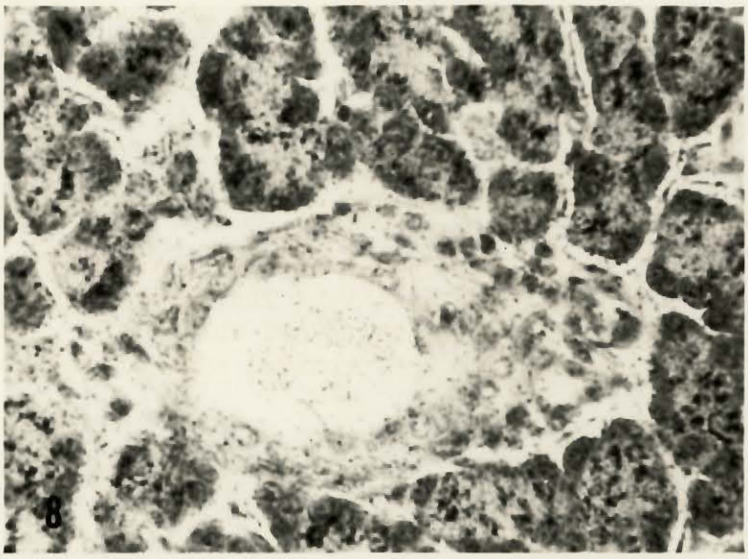
Fig. 8 Pancreatic duct, 30 minutes after injection.

The material in the lumen of the duct is seen to be slightly radioactive. The surrounding acini show dark clumps of silver grains, which under high power can be localized to the proximal zymogen granule region.

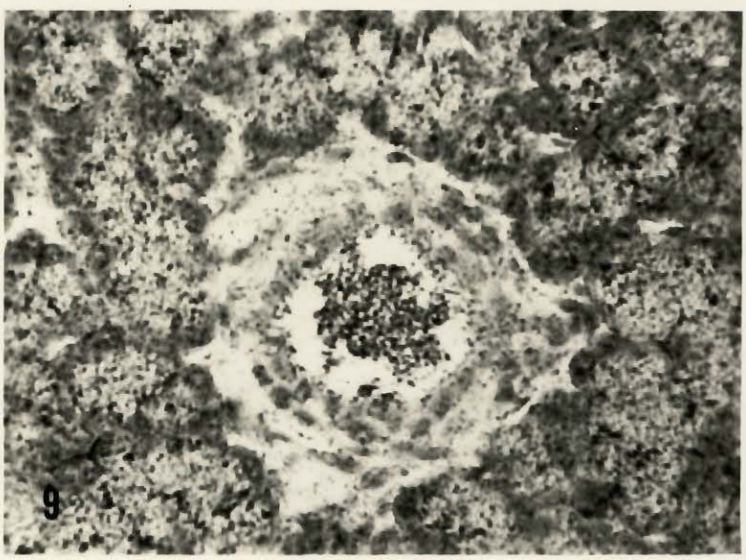
Fig. 9 Pancreatic duct, 4 hours after injection.

The material within the lumen is now intensely radioactive. The radioautographic reactions over the surrounding acini are distributed over the entire zymogen granule region, however, localized clumps of grains can still be seen over the proximal zymogen granule region.

At 30 minutes after the injection, the duct cells show a slight reaction, which increases somewhat at 4 hours.



8



9

Grain Counts

The number of silver grains was counted over the acinar components from every drawing at each time interval and averaged (Table 4). Expressed as the average number of grains per component, the reactions over each component are comparable only to the same components at the other time intervals; even then, a comparison can be made only if the areas under consideration are similar. However, from this table it can be seen that the proximal and distal portions of the ergastoplasm are almost equally labelled at all time intervals. The number of grains over both regions remains about the same from 10 to 30 minutes, decreases somewhat at 4 hours and drops even lower at 36 hours.

The number of grains over the proximal zymogen granules increases about two fold from 10 to 30 minutes, drops slightly at 4 hours and falls to a very low level at 36 hours.

The number of grains over the distal zymogen granules increases steadily up to 4 hours, then decreases to a very small number at 36 hours.

Grain Concentration

Because of the area differences between the components, the counts were expressed in terms of concentration. Multiplying the grain concentrations by the weight of an area of paper corresponding to 100 μ^2 (using the scale described on page 57), gave the con-

TABLE 4

AVERAGE NUMBER OF SILVER GRAINS OVER EACH CYTOPLASMIC ACINAR COMPONENT AFTER INTRAPERITONEAL INJECTION OF LEUCINE-H^3 IN THE RAT

Acinar component		Time after injection			
		10 min	30 min	4 hrs	36 hrs
Ergastoplasm:	Proximal	30.3	28.0	15.0	10.9
	Distal	26.0	27.4	15.7	7.3
Zymogen:	Proximal	30.5	59.1	50.4	9.9
		6.2	8.5	20.2	1.9

centration of grains per 100 μ^2 thus,

Concentration (Grains/Gram of paper) X Weight of unit area of Paper (Grams/100 μ^2)

= grains/100 μ^2

The results expressed in this way, with the standard deviations are shown in Table 5 . The large standard deviations indicate the high degree of variation from one acinus to another. From this table it becomes clear that there is no functional difference between the proximal and distal portions of the ergastoplasm and that the entire ergastoplasm may be considered as a homogeneous unit (combined ergastoplasm, Table 5).

The results, expressed per unit area, show that the ergastoplasm and the proximal zymogen granules have the highest grain concentration at 10 minutes. The ergastoplasmic grain concentration decreases during the period from 30 minutes to 4 hours, then continues to decrease slightly until 36 hours after injection. The grain concentration over the proximal zymogen granules increases sharply between the 10 and 30 minute intervals, remains almost constant until 4 hours, then decreases abruptly at 36 hours. The grain concentration over the distal zymogen granules increases gradually from 10 to 30 minutes, rises sharply to a peak at 4 hours, then decreases markedly at 36 hours.

The graphical representation, obtained by plotting the number of grains per 100 μ^2 area against the time in minutes after the injection gave the curves shown in Figure 10 . The proximal and distal zymogen granule regions are combined as a single entity.

TABLE 5

AVERAGE CONCENTRATION OF SILVER GRAINS PER 100 μ^2 OF EACH CYTOPLASMIC ACINAR COMPONENT AFTER INTRAPERITONEAL INJECTION OF LEUCINE-H^3 IN THE RAT

Acinar component		Time after injection			
		10 min	30 min	4 hrs	36 hrs
Ergastoplasm:	Proximal	23.7	20.8	12.2	8.5
	Distal	22.3	22.3	14.0	6.9
Combined ergastoplasm		21.2 ± 4.2	21.6 ± 7.5	13.2 ± 2.9	7.7 ± 3.5
Zymogen:	Proximal	22.5 ± 8.2	50.2 ± 16.1	51.2 ± 20.0	8.8 ± 4.1
	Distal	9.4 ± 8.4	21.0 ± 14.0	61.5 ± 13.1	6.5 ± 10.1

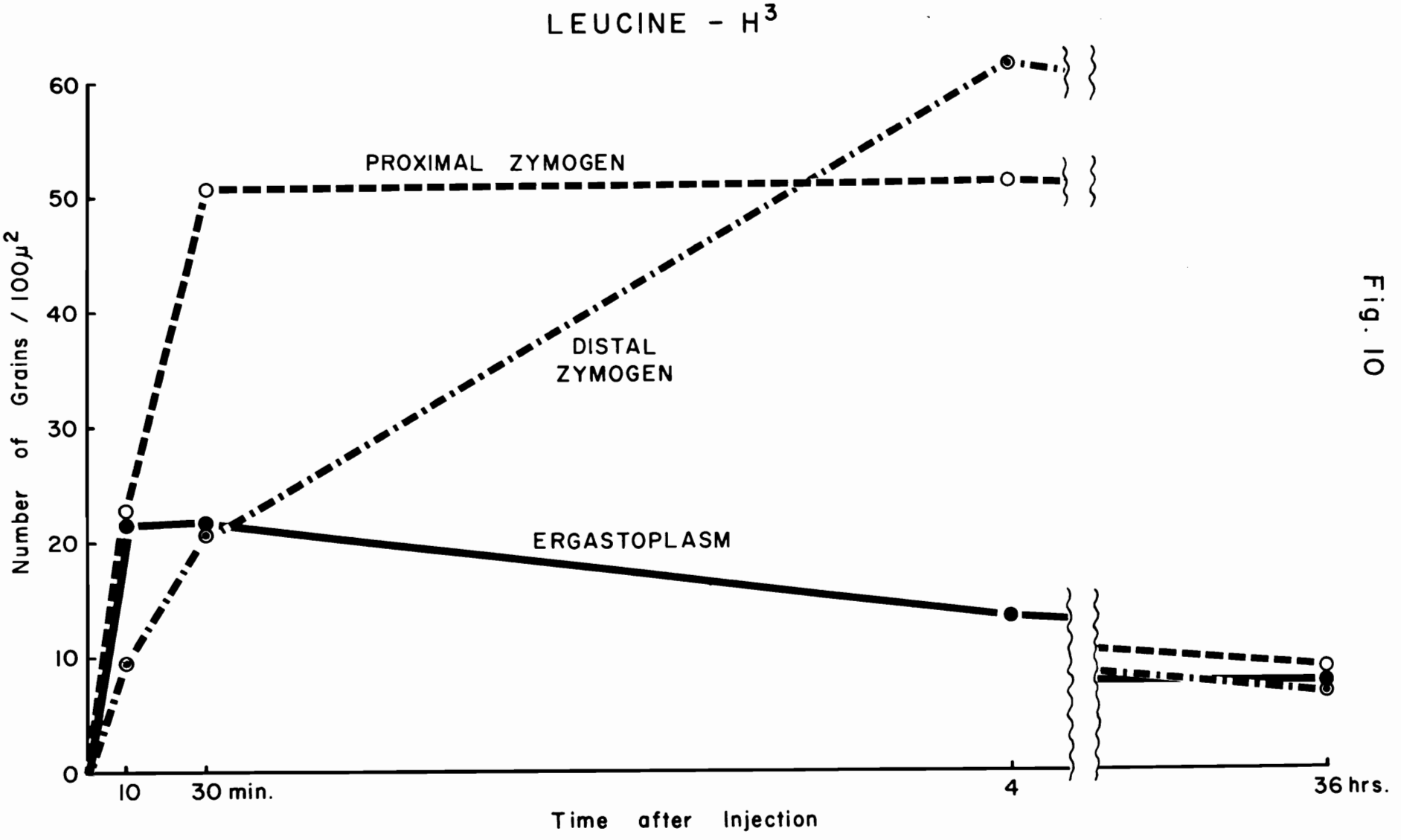

LEUCINE - H3
PROXIMAL ZYMOGEN
DISTAL ZYMOGEN
ERGASTOPLASM
Number of Grains / 100μ2
60
50
40
30
20
10
0
10
30 min.
4
36 hrs.
Time after Injection

Fig. 10

From the graph an indication of the sequence of peaks of radioactivity in the various components can be obtained. The peak of radioactivity concentration is first reached by the ergastoplasm at 10 and 30 minutes after the injection followed by the proximal zymogen granules at 30 minutes and 4 hours, followed by the distal zymogen granules at 4 hours. After 36 hours, the radioactive concentrations in all three components fall to almost equally low levels.

Total Radioactivity

Due to the misleadingly low concentration of radioactivity shown in the ergastoplasm, it was thought that a more representational way of expressing the results would be to obtain the total content of radioactivity in the entire volume of each of the three acinar cytoplasmic components. By assuming that an acinus is a spherical structure, the volume of each region was calculated from its area in the section, which was obtained from the weight of the paper cut-outs (see Methods). Thus, in a sphere, the outer portion, corresponding to the ergastoplasm, occupies a considerably larger volume than the inner sphere, which corresponds to the distal zymogen granules. From the value obtained for the ergastoplasm, a calculated average volume, accounting for the nuclei in this region, was subtracted. The ratio of ergastoplasm to distal zymogen granules by volume was found to be 21:1. The volume of the proximal zymogen granule region is about 3.2 times smaller than that of the ergasto-

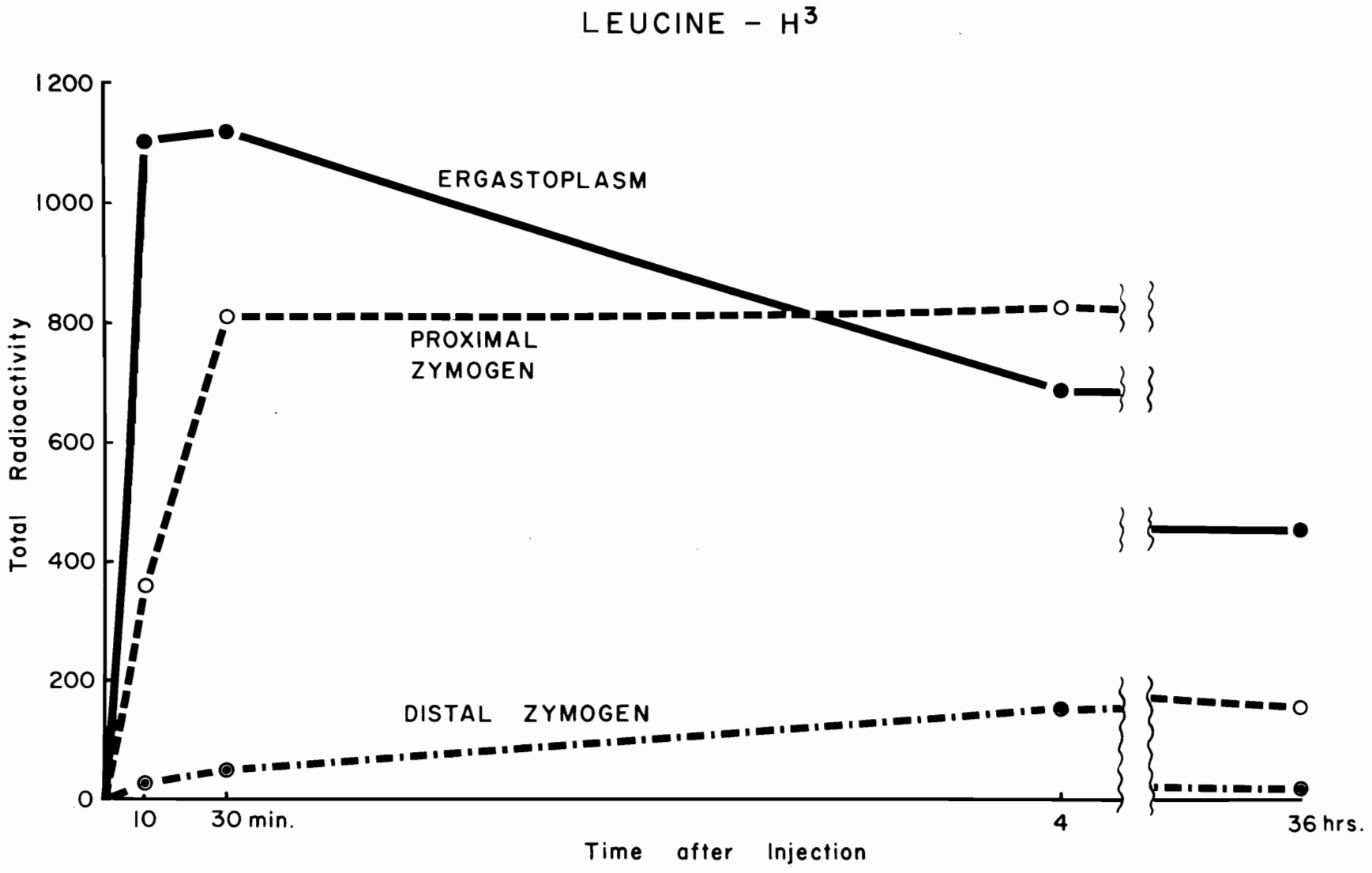

LEUCINE - H3
1200
1000
800
600
400
200
0
Total Radioactivity
ERGASTOPLASM
PROXIMAL
ZYMOGEN
DISTAL ZYMOGEN
10
30 min.
4
36 hrs.
Time after Injection

Fig. 11

plasm. From the grain counts per unit area, it was then possible to calculate the total radioactivity in each region. This was plotted against the time after injection and the results are shown in Fig. 11. The graphs clearly indicate that the maximum amount of radioactivity appears in the ergastoplasm at 10 and 30 minutes. This is followed by a peak, somewhat lower than the ergastoplasm in the proximal zymogen granule region. The distal zymogen granule region increases gradually with time to a peak at 4 hours, which is well below that of the ergastoplasm or proximal zymogen granule regions. The reason for this low value may be the fact that labelled proteins, in the form of zymogen granules, are continuously lost from this region. In evidence for this may be cited the occurance of moderately labelled material in the lumen of the excretory ducts as early as 30 minutes after injection, and of very strongly labelled material at 4 hours (Figs.8 and 9).

Nuclei

Most nuclei show some degree of labelling at 10 minutes after the injection. The grains are mainly localized over chromatin substance, with very few reactions over the nucleoli.

The grain concentration per nucleus, expressed per 100 μ^2 represents the averages of 320 nuclei.

The results, expressed as the concentration of silver grains per 100 μ^2 of nuclei, are as follows:

	Time after injection			
	10 min	30 min	4 hrs	36 hrs
Number of grains per 100 μ^2	9.4	15.3	10.3	8.3

The degree of labelling increases over all nuclei up to 30 minutes, then decreases with time. However, no clear-cut pattern of distribution or migration could be distinguished.

Glycine and Methionine-H^3

The radioautographic experiments with glycine and methionine tend to support and extend, as far as the very early time intervals are concerned, the findings of the leucine experiment. No radical differences were noted between the rates of protein synthesis and secretion in the two species. Thus, the results with mice can be compared to those obtained with rats.

Qualitative Results

Cytoplasm

Qualitative observations of both glycine and methionine radioautographs show that most silver grains at 5 minutes are located over the ergastoplasm of the acini (Figs.12 and 13). The zymogen granules show only rare and scattered grains.

At 15 minutes, the intensity of reaction over the ergasto-

PLATE 7

Radioautographic localization of glycine and methionine-H^3 in the pancreatic acini of the mouse (x 1300, 21 day exposure, H & E staining).

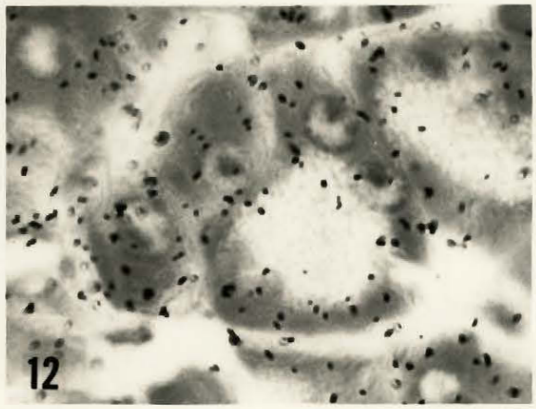
Fig. 12 Glycine
Fig. 13 Methionine. Acini 5 minutes after injection.

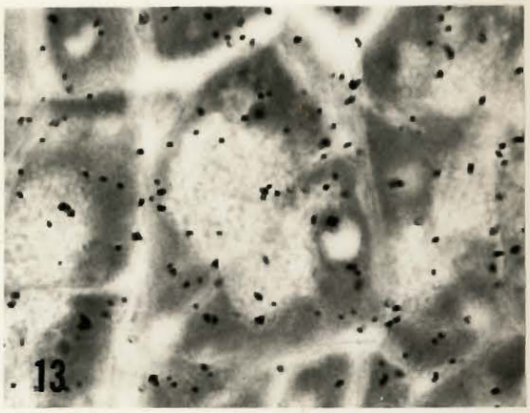
At this early time interval the reactions are localized mainly over the ergastoplasm, with rare and scattered reactions over the zymogen granules.

No difference in the intensity of the nuclear reaction can be seen with either amino acid. Two nuclei in the central acinus of Fig. 13 (methionine) show clear nucleolar reactions.

Fig. 14 Glycine
Fig. 15 Methionine Acini 30 minutes after injection.

The most intense reaction is localized over the proximal zymogen granule region. The grains form more or less discrete clusters which are particularly distinct in Fig. 14, where they are clearly seen to be supranuclear in position. The grain accumulations in the two cells to the right lie between the nucleus and the lateral cell membranes, presumably in relation to an intercellular canaliculus.


12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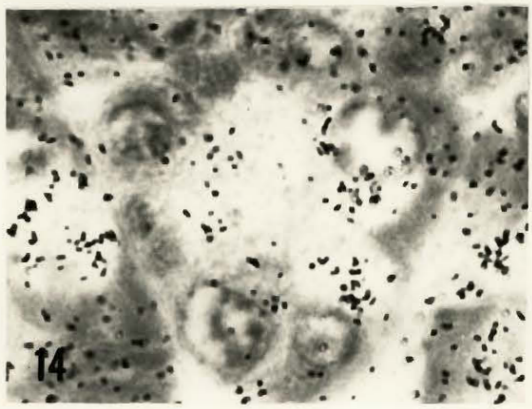
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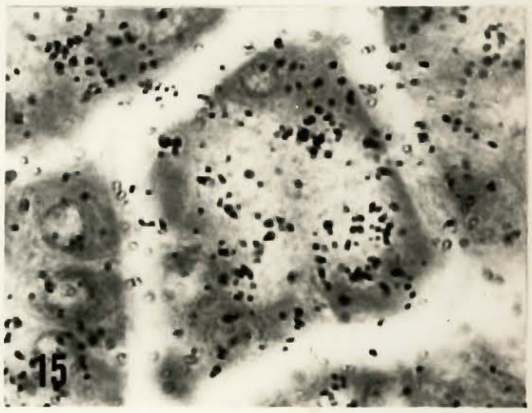

15

plasm increases and an accumulation of grains is observed in the proximal zymogen granules, very close to the ergastoplasm. Only scattered grains are seen over the distal zymogen granules.

Thirty minutes after the injection (Figs.14 and 15), the ergastoplasm remains strongly labelled, the proximal zymogen granules are very strongly labelled and more grains are seen over the distal zymogen granules. In confirmation of the picture seen with leucine-H^3 at this time interval, the grains over the proximal zymogen granule region give the appearance of clusters located in a superanuclear position. In addition, as with leucine, radioactive material is present within the lumen of the pancreatic ducts.

Thus, at the 30 minute time interval, both rats and mice show a similar distribution of radioactive labelled substance, regardless of the amino acid used.

Quantitative Results

Twelve acini were selected and drawn from each of the 5 animals in the glycine experiment. For the methionine experiment, 6 drawings were made from each of the 6 animals. The results of the two experiments are, therefore, based on the averages of 96 drawings.

Grain Counts

Since only two animals were used per group in these experiments the results of the grain counts for each animal are shown in Tables 6 and 7 . The grain counts show a similar behaviour for the two

TABLE 6

AVERAGE NUMBER OF SILVER GRAINS OVER EACH CYTOPLASMIC ACINAR COMPONENT AFTER INTRAPERITONEAL INJECTION OF GLYCINE-H^3

		Minutes after injection				
		5		15		30
Dose (per gm body wt)		3.5 µc	5 µc	3.5 µc	5 µc	3.5 µc
Ergastoplasm:	Proximal	33	28	39	38	51
	Distal	23	21	36	27	41
Zymogen:	Proximal	25	25	54	37	87
	Distal	3	5	7	5	27

TABLE 7

AVERAGE NUMBER OF SILVER GRAINS OVER EACH CYTOPLASMIC ACINAR COMPONENT AFTER INTRAPERITONEAL INJECTION OF METHIONINE-H^3

		Minutes after injection					
		5		15		30	
Dose (per gm body wt)		5 μc	5 μc	5 μc	5 μc	5 μc	5 μc
Ergastoplasm:	Proximal	21	16	47	32	42	63
	Distal	12	9	33	26	36	42
Zymogen:	Proximal	20	17	46	39	95	77
	Distal	4	2	14	6	34	16

amino acids.

Similar to the results with leucine, the proximal and distal portions of the ergastoplasm are about equally labelled at all time intervals. The intensity of the reaction increases over both regions up to 30 minutes.

The proximal zymogen granule region is somewhat less labelled than the ergastoplasm at 5 minutes after injection. The reaction increases with that of the ergastoplasm up to 15 minutes, at which time it is slightly higher, but becomesthe most strongly labelled area at 30 minutes.

The reaction over the distal zymogen granule region is very light at 5 minutes, increases slightly at 15 minutes and reaches a slightly lower degree of labelling then the corresponding ergastoplasm at 30 minutes.

Grain Concentrations

The grain concentrations are expressed as the number of grains per 100 μ^2 area of component. The graphs of these results were plotted using the number of grains per weight of paper; although the values are not as meaningful as when expressed per unit area, the shapes of the curves plotted with either figures are the same in that the one figure is obtained by multiplying the other by a constant factor.

The results for both amino acids, Tables 8 and 9, indicate that the proximal and distal portions of the ergastoplasm are equally labelled at all time intervals. (The results shown in Table 9 represent the average of the two animals at each time

TABLE 8

AVERAGE CONCENTRATION OF SILVER GRAINS PER 100 μ^2 OF EACH CYTOPLASMIC ACINAR COMPONENT AFTER INTRAPERITONEAL INJECTION OF GLYCINE-H^3 IN MICE

Acinar component		Minutes after injection				
		5		15		30
		3.5 μc	5 μc	3.5 μc	5 μc	3.5 μc
Ergastoplasm:	Proximal	21.6 ± 9.3	19.1 ± 8.9	26.3 ± 9.3	24.7 ± 8.6	28.4 ± 11.6
	Distal	18.3 ± 9.4	20.0 ± 7.6	28.6 ± 9.7	25.3 ± 12.1	31.8 ± 15.5
Zymogen:	Proximal	14.5 ± 6.8	16.6 ± 6.3	31.9 ± 9.7	26.1 ± 10.1	54.8 ± 24.0
	Distal	8.1 ± 10.1	7.1 ± 7.4	11.6 ± 4.8	10.0 ± 8.2	44.2 ± 30.6

TABLE 9

AVERAGE CONCENTRATION OF SILVER GRAINS PER 100 μ^2 OF EACH CYTOPLASMIC ACINAR COMPONENT AFTER INTRAPERITONEAL INJECTION OF METHIONINE-H^3 IN MICE

Acinar component		Minutes after injection		
		5	15	30
Ergastoplasm:	Proximal	14.7 ± 3.7	28.6 ± 12.7	44.3 ± 13.6
	Distal	10.8 ± 5.6	30.7 ± 11.7	47.9 ± 7.7
Zymogen:	Proximal	12.4 ± 3.2	34.9 ± 8.2	61.9 ± 14.9
	Distal	3.9 ± 2.9	17.0 ± 16.1	46.3 ± 18.0

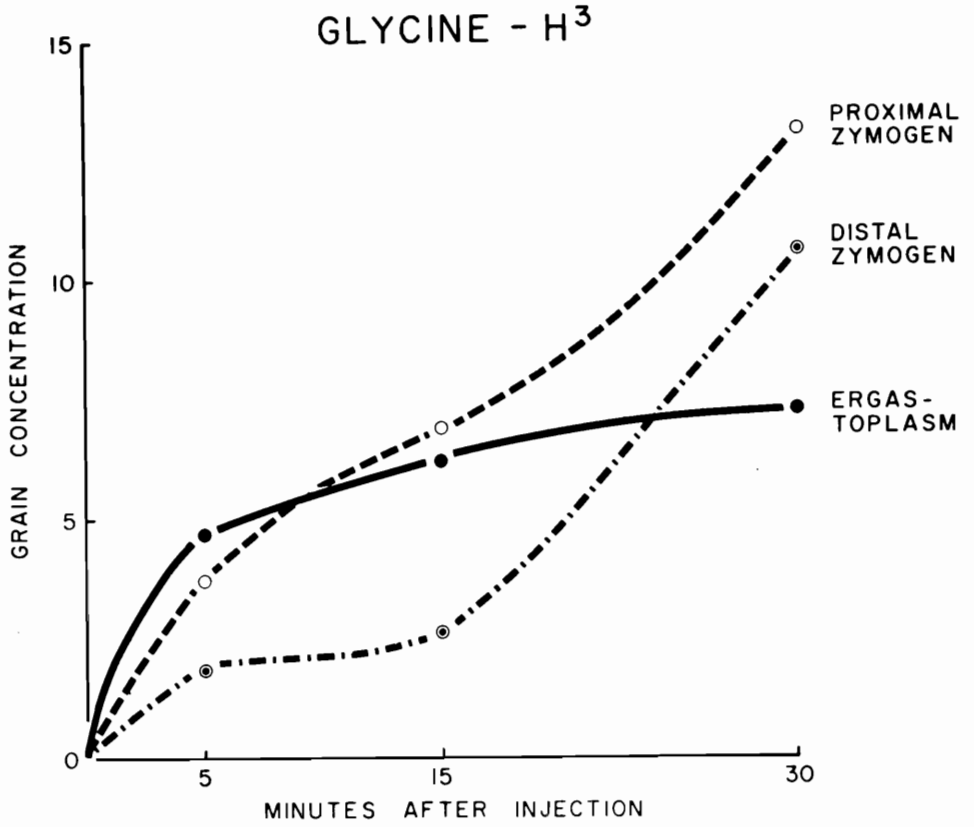
interval, all animals having received similar doses of methionine-H^3.) The whole ergastoplasm is, therefore, considered as a single entity. Furthermore, the number of grains over all components tends to increase with time, but the rate of increase is greatest for the distal zymogen granules.

Graphical representations of the grain concentrations, Figures 16 and 17 , made from the combined values for the entire ergastoplasm the proximal and distal zymogen granule regions show a definite trend, particularly for glycine; at 5 minutes, the highest concentration of radioactivity is in the ergastoplasm and at 15 minutes and later, in the zymogen granules.

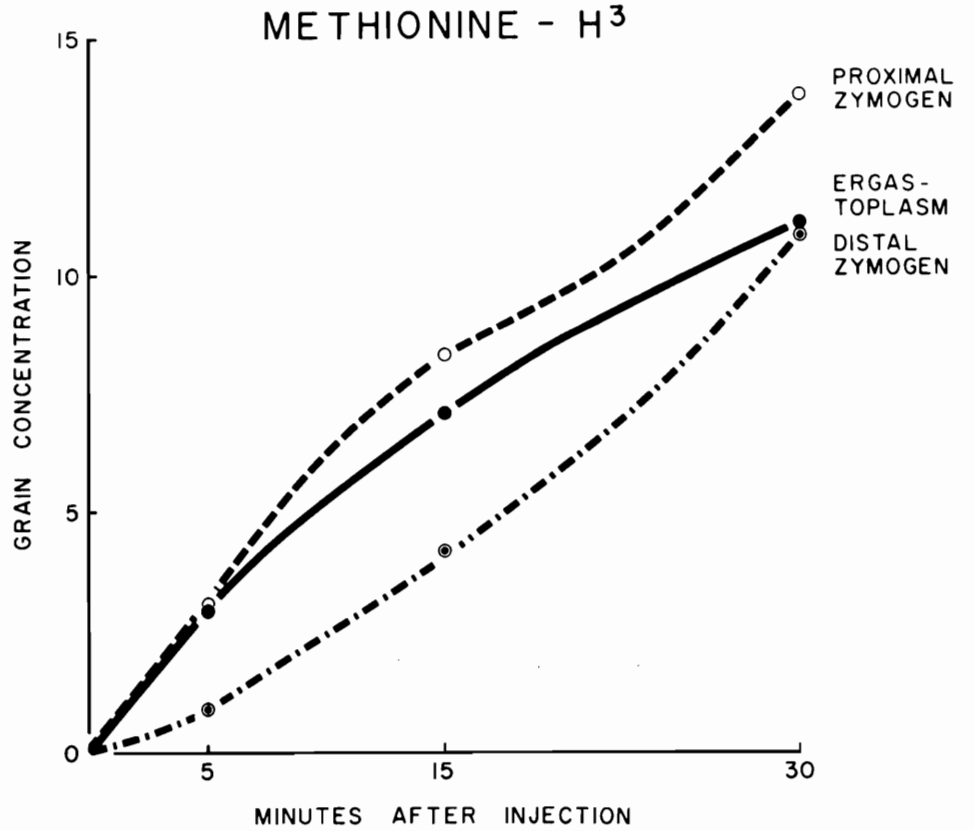
Nuclei

A pattern, essentially similar to that seen with leucine, is also found for the nuclei of the acinar cells of the mouse with both glycine and methionine.

The grain concentration per 100 μ^2 area of nucleus in both the glycine and methionine series, representing the averages of 260 nuclei, is low and very variable. Table 10 shows the average grain concentration per 100 μ^2 of nucleus for each animal with both amino acids.


GLYCINE - H3
15
10
5
0
GRAIN CONCENTRATION
PROXIMAL ZYMOGEN
DISTAL ZYMOGEN
ERGAS-TOPLASM
5
15
30
MINUTES AFTER INJECTION

16


METHIONINE - H3
15
10
5
0
GRAIN CONCENTRATION
PROXIMAL ZYMOGEN
ERGAS-TOPLASM
DISTAL ZYMOGEN
5
15
30
MINUTES AFTER INJECTION

17

TABLE 10

AVERAGE CONCENTRATION OF SILVER GRAINS PER 100 μ^2 OF NUCLEI AFTER INTRAPERITONEAL INJECTION OF H^3 AMINO ACIDS

(Each animal received 5 μc per gram of body weight, except those marked with an asterisk, which received 3.5 μc per gram of body weight)

	Minutes after injection					
	5		15		30	
Glycine	11.7 ± 4.2	11.5 ± 8.8	18.3 ± 9.7	12.3 ± 7.9	25.9 ± 11.9	
Methionine	14.7 ± 11.3	8.8 ± 6.7	32.8 ± 28.8	14.35 ± 10.1	34.4 ± 17.6	29.4 ± 13.4

DISCUSSION

Metabolic Fate of the Amino Acids

As pointed out previously, biochemical investigations have led to the idea that the three amino acids selected for this study each have a different pathway of metabolism. However, the experiments devised for this purpose with isotope-labelled amino acids were specifically designed to yield an amount of labelled product large enough for chemical analysis and identification of metabolites; thus in most instances, the amounts of precursor which were used were far in excess of tracer concentrations. Hence, even some of the most widely accepted concepts concerning the metabolic pathway of amino acids may not apply under physiological conditions. Therefore, in the following brief description of the metabolism of leucine, glycine and methionine, it must be borne in mind that most of the studies concerned did not conform to tracer requirements.

Leucine

Initially, transamination of leucine yields the corresponding α-keto acid, which is then decarboxylated with the formation of the acyl CoA derivative, isovaleryl CoA, containing one less carbon atom (Bloch, 1944; Zabin and Bloch, 1950; Coon, 1950).

Bloch's experiments (1944) were performed by feeding deuterium labelled DL-leucine to mice and rats. The mice received, as part of their diet, 40 mgm of labelled leucine per day for a period of

8 to 22 days, while the rats ingested 443 mgm of leucine per day for up to 16 days. After isolating substantial quantities of labelled isovaleric acid, he concluded that this substance is an intermediate in oxidative breakdown of leucine. In addition, Bloch found that labelled acetic acid was formed from the administered leucine.

Zabin and Bloch (1950) incubated 1.5 gms (wet weight) of liver with 10 mgm of sodium isovalerate containing C^{13} and C^{14}. After incubating for 3 hours at 37° C they were able to extract labelled ketone bodies from the liver tissue, indicating that isovaleric acid is an intermediate in the formation of ketone bodies.

Coon (1950) incubated 2.5 gms (wet weight) of rat liver slices with 2.0 mgm of C^{14} labelled sodium isovalerate. The incubation was carried out at 38° C for 4 hours. When carboxyl-labelled isovalerate was used, the recovery of radioactivity in the extracted acetoacetic acid was 32%. With β -labelled isovalerate a recovery of 44% was obtained in the extracted acetoacetic acid. Thus, it was confirmed that isovaleric acid is an intermediate in the breakdown of leucine, and that leucine eventually gives rise to ketone bodies.

The acyl CoA derivative formed from the isovaleric acid, is then degraded via pathways of fatty acid oxidation, allowing for the conversion of labelled leucine into labelled lipids.

Judging from the large quantity of the labelled precursors used, and the small amount of label which the products in question incorporated, it seems likely that these intermediate metabolites are

not formed to any significant extent under physiological conditions. However, assuming that some of the leucine did follow this pathway with the physiological doses used in the experiments described in this thesis (0.012 mgm of leucine to a rat of 125 grams), then these small molecular weight metabolites would in all probability be washed out of the sections, and therefore, not account for any labelling.

Glycine

The metabolism of glycine is much more complex than that of leucine. In the course of its breakdown, glycine enters many diverse reactions which may give rise in addition to proteins, to a number of compounds. Glycine enters the synthesis of the polypeptide glutathionine as one of its three amino acid residues. It is also involved in the synthesis of creatine and hippuric acid.

Shemin and Rittenberg (1945) first demonstrated that the glycine nitrogen is incorporated into the pyrrole rings of protoporphyrins. Their experiments were performed in a human subject and confirmed with rats. In the human experiment, one of the authors ate 66 gms of glycine-N^{15} over a period of 3 days, and protoporphyrins were isolated from blood samples withdrawn at various time intervals after the feeding period.

Altman et al (1948) showed that the methylene carbon atom is also incorporated into protoporphyrins. Altman et al fed rats 1 µc per 100 gms of body weight of glycine-C^{14} labelled in the methylene group. They found that after 1 day feeding, 0.49% of the dose was present

in the hemin part of hemoglobin, and 1.42% of the dose was in the globin part. The total amount of the fed dose which was incorporated into hemoglobin was 1.83%, and generally about 0.5% of the total dose of C^{14} administered was incorporated into hemin.

It is obvious that such a small fraction of radioactivity, if present in hemin, will not influence the radioautographic results.

The formation of α -amino β -ketoadipic acid, an intermediate in the synthesis of protoporphyrins forms one mechanism for the catabolism of glycine to CO_2. Glycine degradation to CO_2 may also occur via glycoxylic acid formation, and the formation of serine, which may be converted to pyruvate, providing a glycogenic pathway for glycine metabolism. Via this pathway, the methylene carbon atom of glycine may, in part, be used for acetyl CoA formation.

In addition, Heinrich et al (1949) have shown, by feeding carboxyl-labelled glycine to rats for a ten day period, that the carboxyl carbon atom of glycine is incorporated into the purines of nucleic acids.

Reichard (1949) working on the incorporation of glycine-N^{15} into the nucleic acids, used the intestine of normal and the regenerating liver of partially hepatectomized rats. Two subcutaneous injections of 50 mgm of glycine-N^{15} per 100 gms of body weight were given 6 hours apart; 6 hours after the last injection, the animals were killed and the organs homogenized. Sufficient quantities of N^{15} labelled purines were isolated from the cytoplasm of these tissues to demonstrate the uptake of glycine nitrogen into the purines of rat nucleic acid.

It is known that glycine enters the biosynthesis of purine bases via the formation of glycinamide ribotide.

The use of tracer doses of glycine-H^3 probably results in labelling only a minute quantity of the above compounds. However, the only compounds of concern in radioautographic studies with glycine are nucleic acids, and particularly proteins.

Methionine

Methionine metabolism, via demethylation and transmethylation, presumably gives rise to one carbon methyl fragment.

DuVigneaud et al (1941) hypothesized that the methyl group of methionine behaves differently from the rest of the methionine molecule. Thus, the loss of the methyl group from most of the methionine molecules would leave a sulfur-bearing moiety which may be incorporated into several compounds including cysteine. Hence, the distribution of S^{35}-methionine would then indicate the location of sulfur containing metabolites. However, Karpishka and Carneiro (1960) compared the radioautographic distribution of methionines labelled with S^{35}, C^{14} and H^3 and found no difference in the localization of the three isotopes.

They explained their discrepancies with DuVigneaud's work on the basis that the latter may have injected unphysiological doses of methionine. The doses used by Karpishka and Carneiro were thought to be too small for significant demethylation to occur. They, thus concluded that in tracer proportions the methionine molecule may

act as an entity rather than be degraded into two separate units.

The formation of cysteine from homocysteine forms another major metabolic pathway (Brinkley and DuVigneaud, 1942). The α -keto-butyrate formed in methionine metabolism (Carroll et al, 1949) is transformed to proprionate by enzyme systems in brain (Long and Peters, 1939) and liver. Proprionate is converted to glycogen (Kisliuk et al, 1956) by way of succinate, which gives pyruvic acid by operation of the tricarboxylic acid cycle.

Kisliuk et al (1956) found that the administration of methionine labelled with C^{14} at two positions on the molecule, resulted in the formation of labelled glycogen. It was found that a subcutaneous injection of methionine-2-C^{14}, with a specific activity of 1.09 x 10^7 cpm/mgm of carbon, yielded after 4 hours, labelled glucose with a specific activity of 2.9 x 10^3 cpm/mgm of carbon. Thus, they could account for only 0.026% of the injected dose in glucose.

There is evidence to suggest that some of the methionine which enters the proteins may have first been converted to cystine (Tarver and Schmidt, 1939; Forker et al, 1951).

Conclusion

It has been pointed out by Siri (1949) that in some biological systems, the addition of labelled substances in excess of the normal amounts present lead to processes that are not characteristic of the normal system. In most of the experiments described in the above section, tracer requirements as defined on page 6 were not fulfilled. Therefore, these experiments have proven the existance of the metabolic

pathways, but in most instances there has been no attempt to determine the proportion of the amino acids which would follow these pathways under physiological conditions. What is clear, however, is that following the injection of tracer concentrations of labelled leucine, glycine and methionine, the radioautographic distribution obtained from all three, particularly in the pancreas, is exactly the same. Since all three amino acids can be incorporated into proteins, it must be concluded that most of the amino acid molecules incorporated into the compounds which remain in the histological sections are present in the proteins, with the possibility of some incorporation into nucleic acids, especially in the case of glycine.

Moreover, as pointed out previously, even if these metabolites were formed, they would not be present in histological sections. However, in view of the fact that these alternate pathways do exist, it was essential to determine which biochemical fractions, of those present in the histological sections, contain the most radioactivity.

The Nature of the Material Studied

Biochemical Extraction Experiment

Introduction

Since it is known that proteins are composed of amino acids linked together by peptide bonds, it is reasonably to assume that labelled amino acids given to an animal will be incorporated into the proteins

being synthesized at that time. However, it is also known that amino acids may be metabolized in various ways which may eventually yield glycogenic or ketogenic compounds, as well as nucleic acids. A further possibility is that the labelled amino acid, or just the label itself, may be adsorbed onto the proteins by physicochemical bonds, such as ionic linkage (Godin, 1960). It is also known that the histological procedure renders the tissues free of most small molecular weight compounds, which include the free labelled amino acids. Therefore, the radioactivity visualized by radioautography must originate from the large molecular weight compounds which constitute the histological section. To test the assumption that a labelled amino acid, when administered to an animal, is incorporated into, and is therefore, capable of acting as a fairly specific tracer of the proteins being synthesized, the radioactivity of the proteins, nucleic acids and lipid fractions of various tissues from mice injected with leucine-C^{14} were compared.

Lipid Fraction

As stated in the results, the lipid fraction obtained from the histological sections contained no radioactivity. The results of an experiment designed to determine the loss of radioactivity incurred at each step in the histological procedure, contributed considerable data on the question of lipid extraction from histological sections.

In this experiment (forthcoming publication), 4 adult male mice received a single intraperitoneal injection of 1 µc per gram of

body weight of L-leucine-C^{14}. The animals were sacrificed in pairs at 30 minutes and 24 hours, and the pancreas, liver, kidney and brain were promptly removed and fresh aliquots were weighed and fixed in Bouin's fluid.

The radioactivity lost at the various steps of the histological procedure was determined with a Geiger-Müller tube as described on page 48.

The results (Table 11) show that in the pancreas of the animals killed 30 minutes after injection, fixation in Bouin's fluid for 48 hours, and storage in 70% ethanol for 7 days, conditions to which all the tissues in the radioautographic experiments were subject, removed about 10% of the total radioactivity originally present in the fresh tissue. Subsequent treatment of the pancreas, in preparation for paraffin embedding, with dioxane, paraffin and toluene, all of which are lipid solvents, removed only about 0.1% of the total radioactivity. It is, therefore, apparent that even before treatment with known lipid solvents, much lipid soluble material has already been removed, presumably in 70% ethanol (which is also a lipid solvent). Similar results were obtained from the pancreas of the animals killed 24 hours after injection.

In the light of the above, it is not surprising that no radioactivity was found in the lipid fraction extracted from processed histological sections. Thus, it is certain that the radioactivity seen in histological sections cannot be attributed to lipids.

TABLE 11

FRACTIONATION OF THE TOTAL RADIOACTIVITY FROM THE PANCREAS OF LEUCINE-C^{14} INJECTED MICE

Steps with Bouin's fixation	30 min. after injection	24 hrs after injection
% lost in each of the following:		
Bouin's fluid	8.8	2.1
Ethanol (70%)	1.6	12.2
Dioxane	0.5	0.9
Paraffin	0.0	0.0
Toluene	0.0	0.0
Ethanol (graded series)	0.1	0.2
Hot water (used to spread paraffin sections)	2.1	0.2
Extraction of tissue sections		
% retained		
Cold TCA soluble	0.5	0.5
Hot TCA soluble	3.9	3.8
Ether soluble	0.0	0.1
NaOH soluble	4.7	1.7
Protein residue	77.7	78.2

Nucleic Acid Fraction

Schneider's method (1945) for the quantitative removal of nucleic acids (DNA and RNA) was employed in the present study. Kaufmann (1950), working on onion root tips, and Davidson et al (1950), on bone marrow smears, have shown that extraction by this method leaves behind a protein component of the nucleus which can be demonstrated by its increased affinity for acid dyes.

The results obtained from the nucleic acid fraction of this study, show that less than 5% of the total radioactivity recovered from the histological sections is found in this fraction.

In addition, radioactive sections from the leucine-H^3 experiments were extracted by Schneider's method and radioautographed; quantitatively the results of counts over the Purkinje cell nucleus, divided into chromatin and nucleolus, showed no statistically significant difference between the extracted and control sedtions (Leblond and Amano, in press). Observations on the nucleus and ergastoplasm of the pancreatic acinar cells revealed no qualitative difference between the control and treated sections.

Thus, it is concluded that with leucine-C^{14} only a small fraction of the total radioactivity is recovered in the nucleic acid fraction. However, even the small amount of radioactivity which was removed, may not consist of nucleic acids, since the possibility that the nucleic acid fraction may be contaminated by a small amount of protein has to be considered. Lepage and Heidelberger (1951) using the method suggested by Schneider (1946) of combining

the original method of Schneider (1945) with that of Schmidt and Tannhauser (1945), found that the DNA solution thus obtained contains approximately 8% proteins.

In this connection, it is interesting to note that glycine-H^3, the only amino acid in this study known to be a precursor of nucleic acids, shows a lower radioautographic grain concentration over the nuclei than was obtained with methionine-H^3 (Table 10). The problem of glycine incorporation into proteins and nucleic acids, is discussed below under Protein Fraction.

Protein Fraction

With leucine-C^{14} in mice, from 90 to 97% of the total radioactivity in various tissues (Table 3) was recovered in the protein residue. It is, therefore, concluded that most of the radioautographic reactions originate from labelled proteins in the histological section.

Concerning the incorporation of glycine into proteins and nucleic acids, Lepage and Heidelberger (1951) administered 1.0 mgm of glycine-2-C^{14} by stomach tube, and found that 12 hours after the feeding, 56% of the radioactivity contained in the proteins and nucleic acids of normal rat liver was in the protein fraction (based on the average of two mature female rats). In all the animals studied, the incorporation of glycine into proteins was generally greater than into the nucleic acids.

Coleman and Ashworth (1959), studied the incorporation of glycine-

1-C^{14}, using a dosage of 1 µc per 10 grams of body weight, into proteins and nucleic acids of normal and distrophic rats. Extracting the nucleic acids by Schneider's method (1945) and the proteins with TCA, they found that from 1 to 6 hours after the injection of glycine-C^{14} the highest percentage of radioactivity obtained from adult rat liver was in the protein residue; the activity in the proteins as a percentage of the radioactivity from the proteins plus nucleic acid fractions changed from 69% at one hour to 50% at 6 hours.

To explain the apparent discrepency between the results of the above mentioned authors, and the results shown in Table 3, it must be borne in mind that the value of more than 90% in the protein residue, represents the percentage of the total radioactivity obtained from the histologically processed tissue sections. The results of Lepage and Heidelberger, and Coleman and Ashworth, represent the percentage of the total radioactivity of only the protein and nucleic acid fractions. Furthermore, the experiments were performed with fresh tissue aliquots, in contrast to the processed histological sections used in this study; therefore, the results obtained by the above authors, and those presented in Table 3 cannot be compared.

The results of the radioautographic study shows no difference between glycine and methionine concerning the pattern of incorporation into nuclei and ergastoplasm, the only areas known to contain nucleic acids in significant proportions. Hence, it appears that glycine was not appreciably incorporated into the nucleic acids of both regions.

Conclusion

It is therefore concluded that with amino acids such as leucine and methionine, the greatest proportion of the radioactivity originates from proteins; a small quantity originating from the nucleic acids. With glycine, the amount of radioactivity in the nucleic acids may be higher, but nevertheless was not sufficiently high to cause a detectable difference in the radioautographic picture.

The Nature of the Binding of Labelled Amino Acids to Proteins

Introduction

When a labelled amino acid, such as leucine, is injected into an animal, about 90 to 97% of the resultant radioactivity seen in histological sections by radioautography can be attributed to proteins. However, this does not necessarily prove that the amino acids are acting as tracers of newly formed proteins. Several possibilities, other than the utilization in protein synthesis, exist regarding the process of the uptake of labelled amino acids. Two explanations, alternate to de novo synthesis of proteins are as follows:

1) Exchange of labelled for unlabelled amino acid residues of pre-existant protein molecules.
2) Adsorption of labelled amino acids to protein molecules by bonds other than peptide linkage.

Although these alternatives do not constitute the only explanation, they are the most frequently used arguments claiming that amino acid

incorporation does not necessarily involve a synthesis of new proteins.

The Phenomenon of Exchange

The following quotation from the discussion of Schoenheimer et al (1939) on the dynamic rather than static state of the tissue proteins, outlines the basis for the exchange of amino acid residues.

> "It has been shown that nitrogenous groupings of tissue proteins are constantly involved in chemical reactions; peptide linkages open, the amino acids liberated mix with others of the same species of whatever source, diet or tissue. This mixture of amino acid molecules, while in the free state, takes part in a variety of chemical reactions; some reenter directly into vacant positions left open by the rupture of peptide linkages;"

The evidence upon which this is based stemmed from feeding rats a diet which contained deuterium labelled leucine. The interpretation of the results indicated that at least 24% of the leucine originally present in the proteins of the liver had been replaced by dietary leucine, and that at least 32% of the carbon chain of the isotope was introduced as leucine into the proteins of the animals.

The postulated mechanism whereby the replacement of amino acids occurs requires the removal of an amino acid from the protein linkage prior to the introduction of a new identical molecule. Thus, the reaction would proceed in two steps; stage one involves opening of the peptide linkage for liberation of the molecule, and stage two, involves the introduction of a similar species of amino acid, with

the closing of the peptide bond. In addition, Schoenheimer et al (1939) suggest two general reactions which may lead to amino acid replacement. One is the complete breakdown of proteins into its units, followed by resynthesis, the other involves a partial replacement of units.

The striking lack of a third postulated mechanism, namely de novo synthesis of proteins, is worthy of particular notice.

Gale and Folkes (1955), on the basis of the experimental results to be described below, concluded that the incorporation of amino acids is not an indication of protein synthesis. The experiment consisted of incubating disrupted staphyloccus cells with a single labelled amino acid in the presence of chloramphenicol, a substance known to completely inhibit protein synthesis. They found no net increase in protein, but the label was incorporated for at least two hours. Since the incorporation of one amino acid is known to be more resistant to chloramphenicol than total protein synthesis, they concluded that the incorporation of amino acids does not indicate a synthesis of proteins. They suggested that an exchange occurs between labelled molecules and corresponding unlabelled residues in the proteins, but attempts to prove this experimentally on intact cells have been unsuccessful.

However, in contrast to the results described above, Mandelstam and Rogers (1958) found that in intact Escherichia coli, incorporation of glycine and leucine is totally stopped by chloramphenicol. This was also found in Bacillus cereus with methionine and leucine. In addition, it was found that staphylococcus is resistant to

chloramphenicol in the case of amino acids found as constituents of its cell wall. Thus, it is possible that single amino acids, such as glycine or glutamic acid, are incorporated into synthesis of cell wall, which in staphylococcus consists of glycine, alanine, glutamic acid, lysine, serine and two amino sugars.

Their results on intact staphylococcus show the following:

Counts/min. at infinite thickness

Amino Acid	Cell wall + protein	Protein	Cell wall	% Increase in cell wall
Glycine	145	3	662	85
DL-Glutamic acid	17	3	65	33
L-Lysine	16	2	61	30

From the above results, they concluded that cell wall synthesis occurs in the presence of chloramphenicol, and that this accounts for the incorporation, as seen by Gale and Folkes, of amino acids into the so-called protein fraction, which includes the cell wall. To test whether the incorporation into the cell wall involved an actual synthesis or was merely an exchange, the percentage increase in the cell wall was compared to the expected increase, with the conclusion that the incorporation definitely reflected a synthesis of cell wall.

The experimental data of Mandelstam and Rogers (1958) tend to disprove Gale and Folkes' (1955) experimentally based postulate of

exchange of residues as a principle mechanism of amino acid incorporation; however, the phenomenon of exchange, as theorized by Schoenheimer et al (1939) deserves further consideration. Assuming that exchange constitutes the main mechanism of amino acid incorporation, then this, by virtue of the fact that there is no known control system, is subject to the laws of chance, in that one residue has as much chance of exchanging as another residue, the other conditions remaining constant. Thus, if a random exchange were the only explanation, no reproducible pattern of distribution of radioactive molecules would be expected. However, the entire section of pancreas from every animal in the radioautographic experiments, with each amino acid used, showed a completely reproducible, clearly defined distribution of radioactivity characteristic of each time interval.

It is therefore concluded that if the exchange phenomenon does occur when tracer dosages are used, it does so to such a small extent that it may be neglected from consideration.

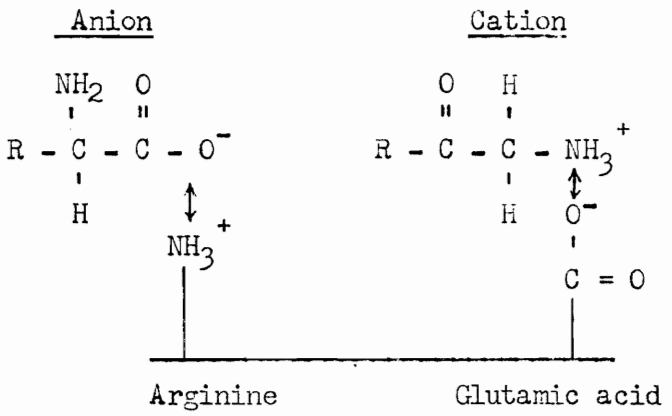
Adsorption of Labelled Amino Acids onto Protein Molecules

There is some evidence to show that when individual amino acids are incubated in simple amino acid-protein systems, binding of amino acids to the protein molecule results.

Levin et al (1956) incubated fresh human serum proteins with differing amounts of methionine-S^{35} under conditions which exclude the possibility of protein synthesis. They found that one to five percent of the S^{35} was carried with the proteins after TCA precip-

itation. From this, they concluded that some degree of adsorption of methionine to proteins could occur.

Recently, Godin (1960) reported evidence showing that the free amino groups and the free carboxyl groups of a protein molecule are partly responsible for this binding. The amino acids would then act as anions or cations capable of ionic bonding to the ionized, free amino or carboxyl groups of the protein, as shown below;



To examine whether or not this phenomenon occurs in radioautography, where tracer doses are used, the protein residue, obtained from the histological sections of leucine-C^{14} injected mice, was extracted with 1 N NaOH (see Methods). The NaOH, being a strong base, is capable of replacing ionic bound amino acids from the proteins, thus, liberating the radioactive amino acids bound in this way. It was found that the NaOH fraction contained a small quantity of radioactivity; this amount, when expressed as a percentage of the total radioactivity, indicated that a maximum of 5% of the label bound to proteins is NaOH labile. Approaching the problem from a

different direction, the results of a recent experiment (B. Droz, personal communication) tend to confirm the existence of adsorption in vitro. In this experiment, non-labelled sections of various tissues were incubated at 37° C for 30 minutes, in a solution of leucine-C^{14}. The slides were thoroughly washed in water, dried and counted under a Geiger Müller tube; the background count, obtained over the same slide before treatment, was subtracted. In all cases, the radioactivity after incubation had increased markedly from the background. However, when these sections were treated with Bouin's fixative, washed in water, dried and recounted, the radioactivity was completely removed; the counts again dropping to the background level. This experiment clearly indicates that Bouin's fixative is capable of completely removing adsorbed amino acids from proteins. In the case of the in vivo experiments with leucine-C^{14} the question arises as to why the original Bouin's fixation did not remove the adsorbed amino acids. Again, the possibility that the radioactivity in the NaOH fraction is not due entirely to liberated amino acids, but in part at least to contamination by proteins, must be considered. Extracting the protein residue with alkali, involved complete dissolution of the proteins. The addition of TCA, to reprecipitate the proteins, and of HCl to neutralize the solution added further complications. Thus, three possible causes of contamination exist within the extraction medium. One, is that the NaOH itself may cause a partial hydrolysis of the protein, thereby liberating a small fraction of proteins or polypeptides. The second possiblity is a partial hydrolysis with the HCl which was used to neutralize the solution. A third, and

perhaps more important consideration is that of an incomplete TCA precipitation from the NaOH medium.

Therefore, it is concluded that the amount of radioactivity adsorbed by the proteins in vivo probably is much less than 5%, and that this small amount does not appreciably influence the radioautographic results.

General Conclusion

Of the several possibilities alternate to de novo synthesis of proteins, as an explanation of amino acid incorporation, all were found to be inadequate in explaining the occurrence of over 90% of the total radioactivity in the proteins after labelled amino acid injection. On this basis, it is concluded that the labelled amino acid molecules are incorporated into newly synthesized proteins, and do in fact form specific tracers of these proteins. The detection of the labelling by radioautography at early time intervals after injection allows the cytological sites of synthesis to be localized; at later time intervals, the subsequent fate of the proteins can be followed at the cytological level.

The Biological Nature of the Proteins Studied Radioautographically

Having concluded that the radioactivity originating from the tissue sections indicates the location of newly synthesized proteins, the nature of the proteins formed in the pancreas must be determined.

Although no direct evidence was obtained in this study, the circumstantial evidence, together with the results of other studies reported in the literature, indicates that a large proportion of the proteins synthesized are enzymatic in nature.

Basically, the proteins synthesized by a cell contribute to two main functions; the structural elements in the cell, that is, the proteins involved in the renewal and maintenance of the cellular structures; and enzyme production. The latter may be divided into cellular enzymes, involved in the metabolism of the cell, and enzymes produced for secretion, as is the case in pancreatic cells, known to produce enzymes for digestion.

It is well known that zymogen granules of the pancreas contain, as inactive precursors, all the enzymes which are demonstrated in the pancreatic fluid (Babkin, 1950). The main enzymes are trypsin, chymotrypsin, carboxypeptidase, lipase, amylase and ribonuclease. Several of the above mentioned enzymes have been isolated and found to contain radioactivity after in vivo experiments with labelled amino acids. Following injection of leucine-C^{14} into guinea pigs, Siekevitz and Palade (1958) have extracted labelled chymotrysinogen and ribonuclease from the microsomal and zymogen granule fractions. Morris and Dickman (1960) have extracted labelled ribonuclease from the microsomes and zymogen granule fraction of mouse pancreas after valine-C^{14} injection.

In addition to these, incorporation of amino acids in vivo have been used to demonstrate the synthesis of ribonuclease (Keller, 1959), trypsinogen and α -chymotrypsinogen (Hansson, E. 1959; Keller, 1959).

Straub (1958) has shown that pancreatic slices labelled in vitro yielded labelled amylase.

Thus, the incorporation of radioactive amino acids has been used to demonstrate the synthesis of most of the digestive enzymes of the pancreas.

It is also known that proteins which leave their sites of synthesis are turned over much more rapidly than proteins which remain as intracellular components (White et al, 1959). The results of this study show that at 36 hours after the injedtion of labelled amino acids most of the labelling is lost from the acinar cells, only few scattered grains remaining; concommitant with this loss, the ducts of the pancreas at 4 hours and at 36 hours after injection contain heavily labelled material within their lumena. This rapid loss of radioactivity is greater for the pancreas than for any other tissue studied radioautographically (Leblond et al, 1957). This has been confirmed biochemically with leucine-C^{14}. The results of the latter experiment show that the pancreas, seven to eight times more labelled than the liver at 30 minutes, drops at 24 hours to the same level as that of the liver (Table 2). In addition, when fresh tissue aliquots of pancreas and liver were extracted following the same procedure as with the histological sections, 30 minutes after the injection the protein fraction of the pancreas is about 10 times more labelled than that of the liver. This has also been reported by Allfrey et al (1953).

Thus, it is concluded that most of the labelled proteins seen in the radioautographs, which turnover within about 24 hours, are digestive enzymes, produced by the cell for secretion. The reactions

remaining after 36 hours probably represent the structural material replaced by the cell.

Interpretation of the Radioautographic Results

Of the three morphological entities included in the radioautographic grain distribution drawings, viz. the nuclei, ergastoplasm and zymogen granule regions, the latter two were further subdivided as described on page 57 . The subdivision of the ergastoplasm showed that both proximal and distal portions of this region were equally labelled at all time intervals, indicating that no difference exists between the activity of the two subdivisions, and that the entire ergastoplasm behaves as a unit.

However, the subdivision of the zymogen granule region demonstrated a marked difference between the proximal and distal portions with respect to time after administration of the labelled amino acids.

Ergastoplasm

Having shown that the detection by radioautography of the label introduced on amino acid molecules indicate the sites of newly synthesized proteins, it follows that the localization of the label at the earliest time interval after injection must indicate the site of protein synthesis.

At 10 minutes, the earliest time interval after the injection of leucine-H^3 the ergastoplasm has almost reached its maximum concentration of silver grains per 100 μ^2 area. It reaches the peak at

30 minutes and falls off slowly thereafter. The concentration over the proximal zymogen granules at 10 minutesis equal to, or slightly higher than that over the ergastoplasm (Table 5, Fig. 5).

Five minutes after the injection of glycine-H^3 into mice, the ergastoplasm contains the highest concentration of radioactivity (Table 8, Fig. 12); with methionine-H^3 again, the ergastoplasm and proximal zymogen granules are equally labelled (Table 9, Fig. 13).

From these results, it is not clear whether both the ergastoplasm and proximal zymogen granule regions, or only one of the two synthesizes protein. It was, therefore, decided to work at even earlier intervals and the following experiment was performed. Eight rats conforming exactly to the specifications described previously, were given a single intraperitoneal injection of 2.5 µc per gram of body weight of leucine-H^3 (New England Nuclear Corporation, Boston, Mass; specific activity 35.7 mc/mM), and killed in pairs at 2, 5, 10 and 30 minutes after the injection. The pancreas was removed as described in Material and Methods, fixed in Bouin's fluid, and H & E stained, 5 µ sections were radioautographed.

At 2 minutes after the injection, only those acini which are in close proximity to blood vessels show reactions. Of those acini which are labelled, it is clearly seen that the silver grains are almost exclusively localized over the ergastoplasm with only several grains over the zymogen granules (Figs. 18 and 19). At 5 and 10 minutes (Figs. 20 and 21) after the injection, all the acini are labelled, the grains being localized over the ergastoplasm; the scattered grains over the proximal zymogen granules increase in number, partic-

PLATE 8

Radioautographic localization of leucine-H^3 in the pancreatic acini of the rat at very early time intervals after injection. (x 1000,35 day exposure, H & E staining)

Fig. 18, 19 Pancreatic acini at 2 minutes after injection.

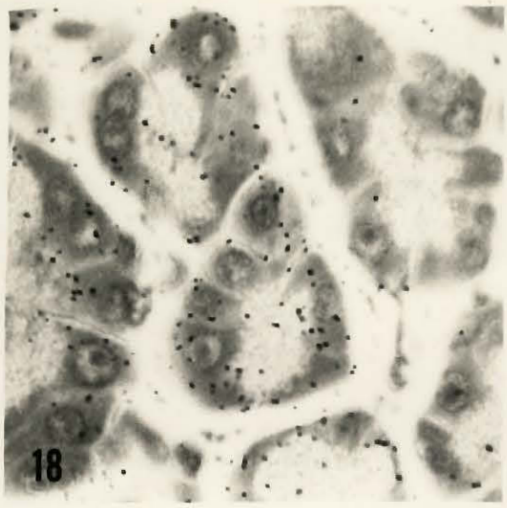
It is clearly seen that the reactions are almost exclusively confined to the basophilic ergastoplasm (see center of both figures). Evidently, at this early time interval, the labelled amino acid has not diffused throughout the entire pancreas; consequently, only those acini close to a blood vessel are labelled, the majority of acini containing little or no radioactivity (right, in both figures).

Fig. 20 5 minutes,
Fig. 21 10 minutes after injection

Again the reactions are confined primarily to the ergastoplasm, with an increased number of grains visilbe over the zymogen granules.

Fig. 22 Acinus 30 minutes after injection.

The pattern obtained in the major experiments with leucine are confirmed with glycine and methionine, since the reactions are concentrated in clumps over the proximal zymogen granule regions. Curiously, fewer grains than at 10 minutes are seen over the distal zymogen granules, suggesting that the radioactivity present here at 5 and 10 minutes may also have migrated into the proximal zymogen granule region.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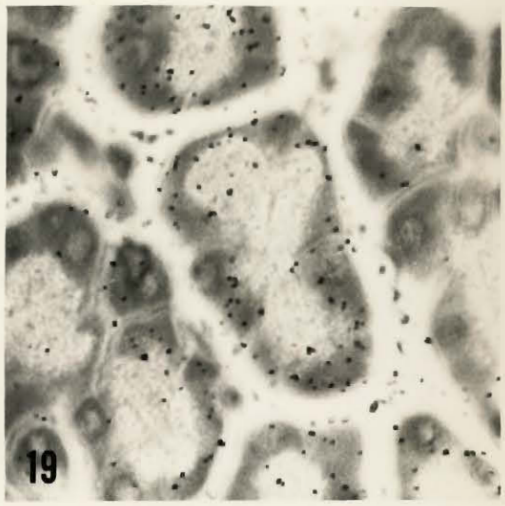
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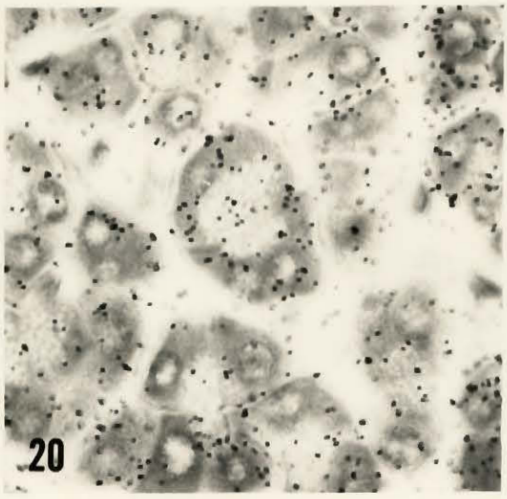
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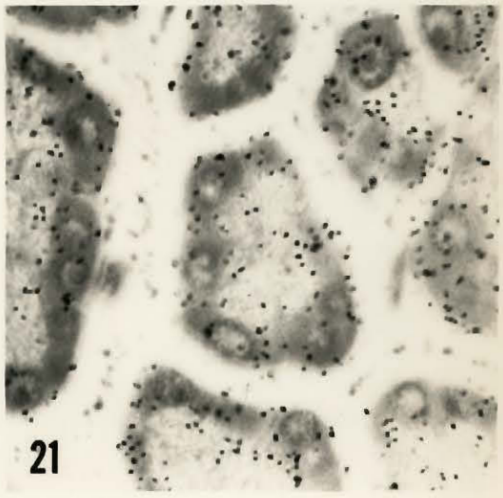
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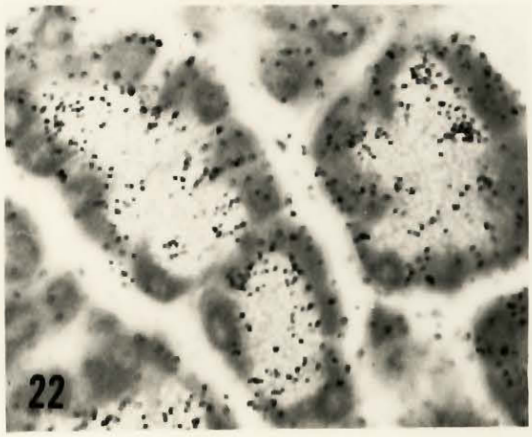
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ularly at 10 minutes. The pattern at 30 minutes (Fig. 22) is identical to that obtained in the other experiments; showing an accumulation of grains over the proximal zymogen granules in clumps corresponding in position to the Golgi apparatus.

The results obtained by Siekevitz and Palade (1960) and Morris and Dickman (1960), indicate that as early as 1 to 3 minutes after injection of labelled amino acids, biologically active, labelled enzymes are found in the microsomal fraction of pancreatic acinar cells. It has been shown that the microsomes represent disrupted elements of the endoplasmic reticulum, and with the electron microscope, the ergastoplasm consists of highly oriented elements of the endoplasmic reticulum associated with ribonucleoprotein particles (RNP). These particles are presumably responsible for the basophilia of the ergastoplasm; the basic hematoxylin combining with the acidic RNP granules.

Therefore, in agreement with the above mentioned results, it is concluded that the ergastoplasm is the site of protein synthesis in the acinar cells of the pancreas. The high concentration of silver grains over the proximal zymogen granules at 10 minutes can be explained as a rapid migration of the labelled proteins from the ergastoplasm into the proximal zymogen granule region.

The scattered reactions found at the early time intervals over the zymogen granules may be attributed to either the background fog, which was found to be of the order of less than 1 grain per 100 μ^2, or more probably, to the few elements of the endoplasmic reticulum found in this region.

Proximal Zymogen Granule Region

The maximal concentration of labelled protein accumulated at 30 minutes in the proximal zymogen granule region, is probably the intermediate stage between synthesis of the enzymes and their appearance in the zymogen granules.

Qualitative observations of the radioautographs at 30 minutes, from the four experiments, reveal more or less distinct clusters of silver grains over the proximal portion of the zymogen granules (Figs. 6 and 8, 14, 15, 22). The division of the zymogen granules into proximal and distal portions was specifically designed to segregate these grain accumulations into the proximal zymogen granule region. Since the region of the grain clusters closely approximates the location of the Golgi apparatus as defined by classical silver methods, the division of the zymogen granules also segregated most of the Golgi apparatus into the proximal zymogen granule region.

The Golgi Apparatus

Since its initial discovery by Camillo Golgi in 1898, the apparatus has been thought to participate in secretion. The appearance of groups of silver grains overlying the supranuclear position within the cell at 30 minutes after leucine-H^3 injection, gives the impression that the radioactivity originates from the Golgi apparatus. To confirm that the grain accumulations at 30 minutes do occupy the position of the Golgi apparatus, measurements of the extent of the silver impregnations from the Aoyama preparation, and the grain accum-

ulations from overexposed radioautographs, were made of the long and short axis of the Golgi apparatus and the silver grain mass, as well as the distances from the nucleus and cell apex. Comparing these measurements statistically, a fairly good correlation was found to exist between the position of the Golgi impregnation and the accumulation of silver grains (Plate 9).

That the concentration of radioactivity in the proximal zymogen granules at 30 minutes is not merely a step in the random diffusion of labelled protein from the ergastoplasm, is indicated by the preferred grouping of silver grains in definite and more or less discrete areas, plus the fact that this concentration probably lasts for some time, at least one half hour. Overexposing the radioautograph of the pancreas at 30 minutes after injection of leucine-H^3 produced very heavy, clearly defined blackening of the emulsion (Fig. 26). Confirmation of these accumulations were obtained from the pancreas of a rat which received trytophan-H^3 (M. Enesco, unpublished) and was killed one hour after the injection (Plate 10). (A recent experiment with tryptophan-H^3 at various time intervals confirms the findings of the other experiments in all respects.)

Sjöstrand and Hanzon (1954 b), studying the fine structure of the exocrine cells of the mouse pancreas by electron microscopy, have noted an intimate relationship between the Golgi apparatus and the zymogen granules. These results correspond with those obtained by Lacy and Challice (1956) using silver impregnation coupled with electron microscopy. They observed granules in this region which appear to represent a whole series of stages in the formation of zymogen granules.

PLATE 9

Localization of the Golgi apparatus with silver impregnation and radioautography using leucine-H^3.

Fig. 23, 24 Schematic drawing of pancreatic acinar cells made to scale from measurements of the distances indicated by the solid lines.

The stippled area in Fig. 23 represents the average limits of the Golgi apparatus after silver impregnation (Aoyama's Technique). Fig. 24 confirms the correlation of the silver grain accumulations at 30 minutes after leucine-H^3 injection, with the position of the Golgi apparatus.

In fact, the Golgi apparatus is not always in a position directly between the nucleus and the cell apex, but may face the lateral cell membrane. This explains why its average length is greater than the width of the cell in Fig. 23.

The nucleus was found to be invariably separated from the Golgi apparatus and the radioautographic grain accumulations by approximately 2 u.

Fig. 25 Silver impregnation of the pancreatic acini of the rat (x 1200, Aoyama's technique).

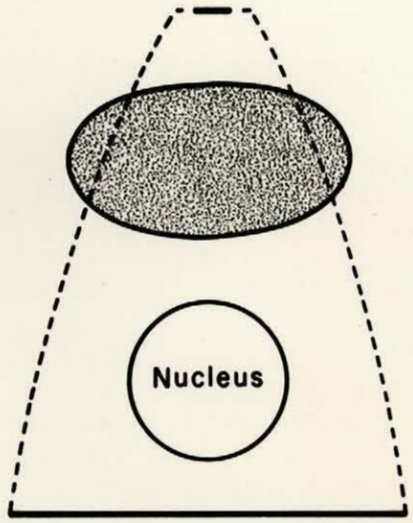
Within the central acinus, the cell in the upper right segment shows the Golgi apparatus typically orientated between the nucleus and the cell apex. However, in the lower cell, the apparatus appears between the nucleus and the lateral cell membrane.

The impregnation clearly indicates the chromophilic area which encloses the chromophobic portions.

Fig. 26 Pancreatic acinus 30 minutes after injection of leucine-H^3 (x 1200, 67 day exposure, H & E staining).

The grain accumulations over the proximal zymogen granules are clearly seen. The ergastoplasmic reactions are relatively slight and few reactions appear over the distal zymogen granules.

GOLGI APPARATUS
Silver Impregnation
by
Aoyama's Technique



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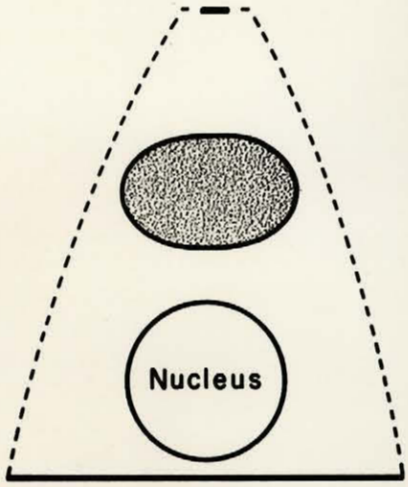
LEUCINE – H^3
Radioautograph
at 30 Min. after Injection
67 Days Exposure



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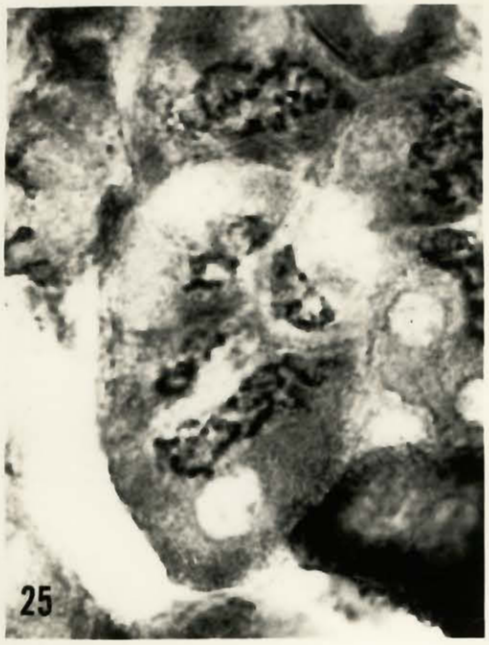
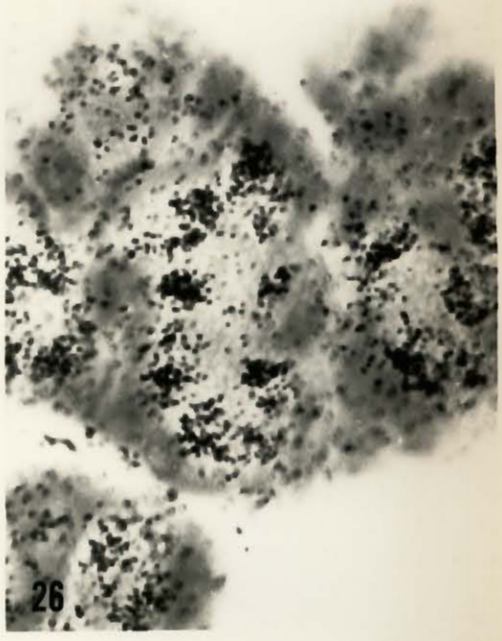
Scale: 2 μ



25



26

PLATE 10

Radioautographic localization of tryptophan-H^3 in the pancreatic acini of the rat (46 day exposure, H & E staining).

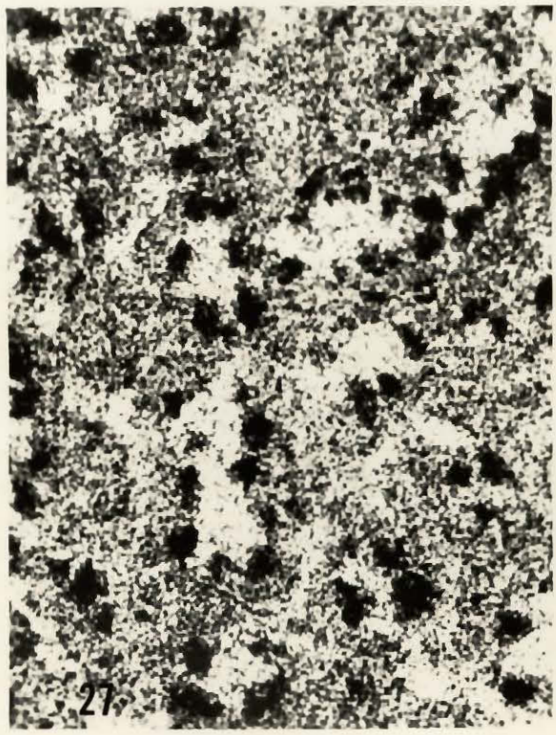
Fig. 27 (x 500) Low magnification of an area of the pancreas from a rat killed 1 hour after injection.

Dark aggregates of silver grains are seen throughout the field, usually arranged in a sequence outlining an elliptical or circular pattern. The area enclosed by such a pattern (zymogen granules) is lighter than the outlying regions (ergastoplasm), as shown in Figures 28 and 29.

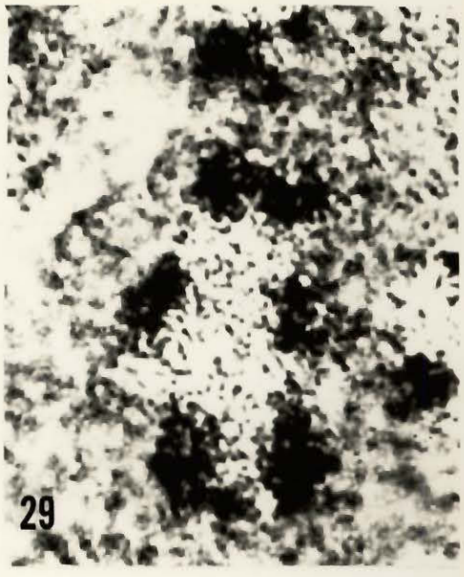
Fig. 28 (x 1300) Higher magnification of one of the elliptically arranged grain aggregates. This photograph, taken at the level of the tissue section shows the "shadow" of the grain clumps. It is clearly seen that the ellipsoidal arrangement is due to the accumulation of grains over the proximal zymogen granule region of the acinus. It is also clear that in each case the grain "shadow" is located between the nucleus and the cell apex; and that a slight space separates the grains from the distal pole of the nucleus.

Fig. 29 (x 1300) The same acinus as in Fig. 28, but the photograph was taken at the level of the silver grains. This confirms that the "shadows" are due to masses of silver grains, and that the light area of the circles or ellipses correspond to the zymogen granule region.

The grain aggregates are thought to originate from the Golgi apparatus.


27


28


29

Farquhar and Wellings (1957) studying the formation of zymogen granules in the mouse pancreas have repeatedly observed material resembling zymogen in density and composition within the otherwise virtually empty appearing Golgi vacuoles.

The evidence cited above is only a small part of the literature showing the involvement of the Golgi apparatus in the process of secretion. Most of these are based on the classical methods of interpreting function from morphological observations by the apparent relationship between several independently occurring structures or phenomenon. The pitfalls of this purely morphologic method are obvious. However, the radioautographic visualization of the accumulation of labelled protein (previously seen mainly in the ergastoplasm) in a region closely corresponding to the morphological position of the Golgi apparatus is direct evidence that the Golgi apparatus is the intermediary between synthesis of the protein and its appearance in the apical or distal zymogen granules.

In confirmation of the above, very strong evidence connecting the Golgi apparatus with the process of secretion has recently been published by Caro (1961) In a study combining the electron microscope with radioautography, he has clearly shown that 20 minutes following the injection of leucine-H^3 into a guinea pig, silver grains are precisely localized over the vesicles and vacuoles which constitute the electron microscopic picture of the Golgi apparatus.

Distal Zymogen Granule Region

With leucine-H^3, the maximum concentration of silver grains at

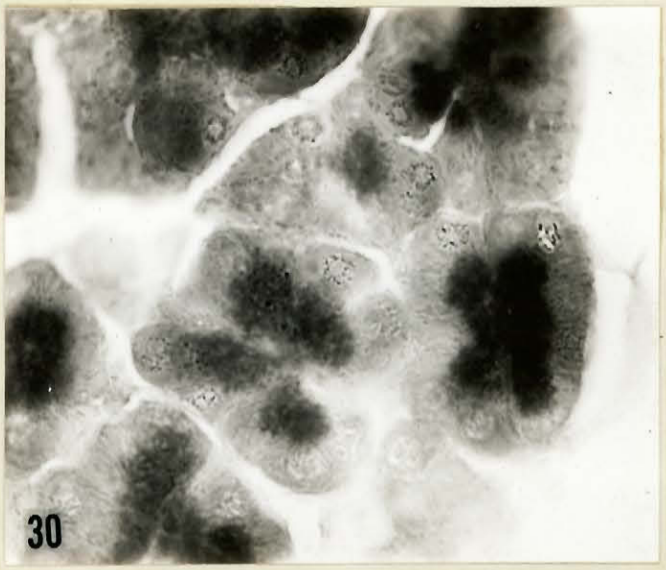
4 hours after the injection is over the distal zymogen granule region. Since the labelled amino acid is not present in the circulation after 1 hour, the radioactivity at this time must have originated from the ergastoplasm and proximal zymogen granules which were labelled previously. The steady rise in activity over the distal zymogen granule region from the very early time intervals, indicates that a slow rate of entrance of labelled material into this region occurs continuously. The loss of labelled proteins, as expelled zymogen material, which is seen in the glandular ducts as early as 30 minutes (Fig. 8), but particularly pronounced at 4 and (Fig. 9) and 36 hours, is taken to indicate that a continuous flow of labelled zymogen occurs from this region. The loss of labelling is gradual at first, then increases in intensity at 4 and 36 hours, presumably as more labelled zymogen granules reach the apex of the cell. By 36 hours, almost no labelled proteins remain in the acinar cells.

Allfrey et al (1953) have shown that in the pancreas of a fasting animal i.e. one whose pancreas is presumably in a "resting" state, a slow steady secretion goes on. Since the quantity of enzymes in a resting pancreas remains fairly constant, they concluded that there must be a constant synthesis to replenish the loss due to the steady secretion that occurs. This has been repeatedly confirmed by the observation that every acinus from the pancreas of all the animals used in the radioautographic experiments was seen to synthesize proteins after injection of labelled amino acids.

Consideration of Specific Activity

The quantitative results presented in the thesis are expressed in terms of concentration of radioactivity per unit area. Ideally, the best representation is obtained by expressing the results as a ratio of the amount of labelled amino acids to the total concentration of amino acids in the proteins of the acinus, that is, as specific activity. This would involve determining the total amount of leucine present per unit area in the proteins of the ergastoplasm and zymogen granule regions. However, there is no specific histochemical method for the determination of leucine in tissue sections. An alternate method is to measure the amount of protein per unit area of the ergastoplasm and zymogen granules, and to calculate the ratio of the amount of labelled protein per unit area to the total amount of protein. On the basis of quantitative radioautography, the amount of labelled proteins can be determined by counting the number of silver grains in the photographic emulsion overlying a given area of structure. These figures are available from the data presented. To determine the concentration of proteins in the various areas of the pancreas, Millon's reaction (Rasch and Swift, 1960) was used on Bouin's fixed section of pancreas. Millon's reaction has been shown to be specific for tyrosine residues of the proteins, when measured at a wave length of 500 mμ or greater. Tyrosine being a uniformly distributed residue in the protein molecule, this reaction can, therefore, be considered as specific for proteins generally. The picric acid remaining after Bouin's fixation was removed from the section with lithium carbonate, and the sections stained as described by Rasch and Swift.

Figure 30 shows an area of the pancreas of a rat as stained with


30

the Millon reaction (x 1000). The ergastoplasmic band surrounding each acinus is much paler than the darkly stained, central mass of zymogen granules. In addition, the reaction intensity within the zymogen granules is seen to vary somewhat from acinus to acinus. Microspectrophotometric measurements of the Millon's reaction intensity, using the two wave length method (Swift and Rasch, 1956) were made of 20 acini. In each acinus 10 readings were taken; 5 over the ergastoplasm and 5 over the zymogen granules (B. Droz, unpublished). The results obtained showed the following ratios:

$$\frac{\text{Protein concentration (zymogen)}}{\text{Protein concentration (ergastoplasm)}} = 2.77 \pm 0.43$$

To determine the specific activity of the proteins, the number of grains per unit area was divided by the concentration of proteins per unit area of the two regions measured. There was no detectable microspectrophotometric difference between the proximal and distal zymogen granule regions. Obtaining the specific activity involves dividing the zymogen region grain concentration by 2.77, and dividing the ergastoplasmic concentration by one.

The results obtained this way were plotted as specific activity against time after injection (Fig. 31).

Interpretation of the Specific Activity Time Curves

From the specific activity time curves, the relationship between a precursor and its product can be analyzed according to the method proposed by Zilversmit et al (1943). By this method, if a substance A is the precursor of a substance B, the relationship of the specific activity time curves of the two substances must satisfy the following conditions:

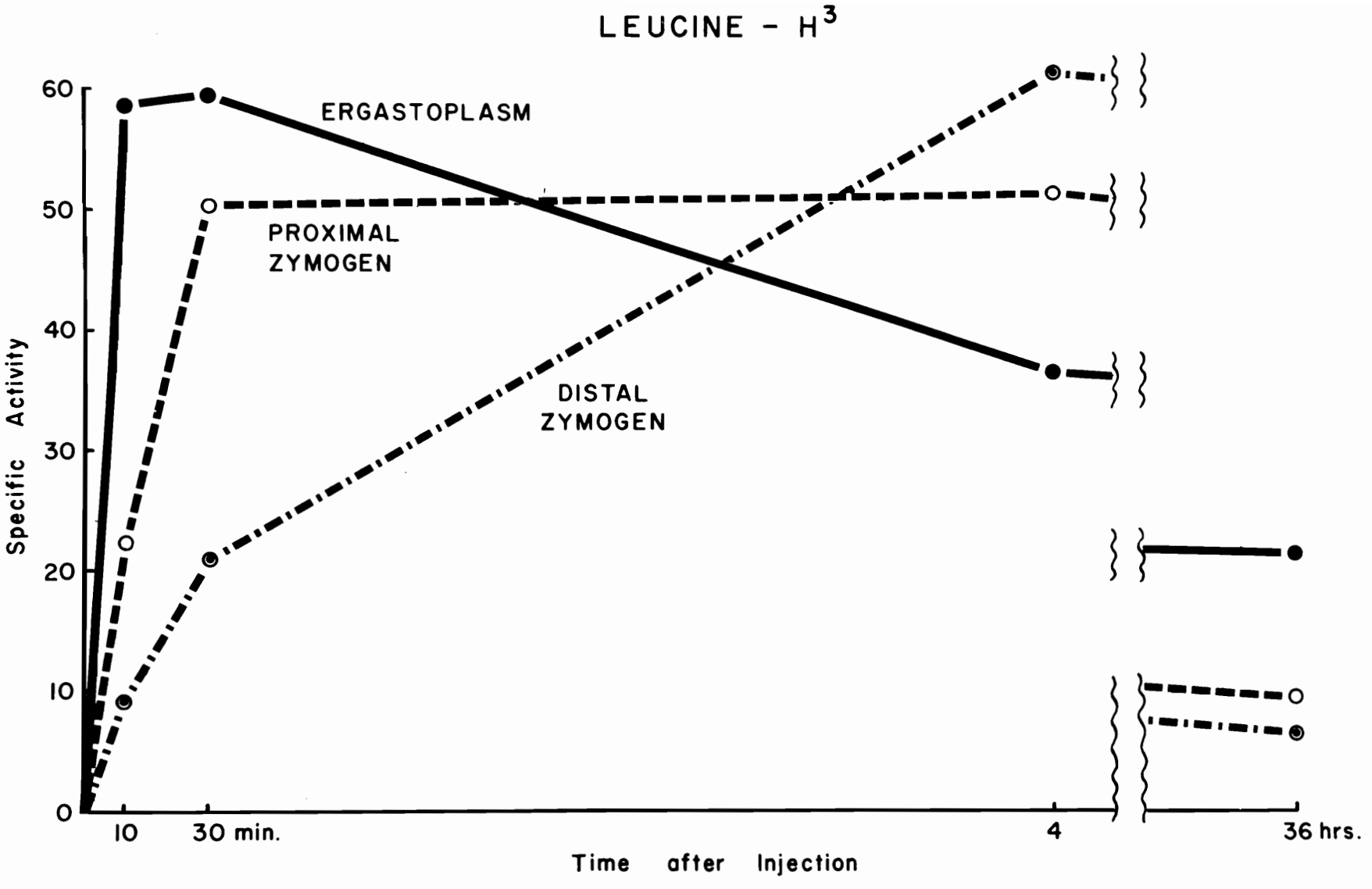

LEUCINE - H3
ERGASTOPLASM
PROXIMAL ZYMOGEN
DISTAL ZYMOGEN
Specific Activity
60
50
40
30
20
10
0
10
30 min.
4
36 hrs.
Time after Injection

Fig. 31

1) the specific activity of A must be greater than that of B at the early time intervals.
2) at the point of intersection of the two curves, the specific activity of B must be at its maximum.
3) the specific activity of B must thereafter be greater than that of A.

Examination of the specific activity curves (Fig. 31) indicates that the ergastoplasmic specific activity reaches a peak greater than that of the proximal zymogen granule region at 10 and 30 minutes. The curve of the proximal zymogen granule region intersects that of the ergastoplasm at what appears to be the maximum point of the proximal zymogen region. However, it is known that precursor in the form of labelled leucine, is available for at least one hour after the injection. Therefore, the maxima of both ergastoplasm and proximal zymogen granules, may be shifted somewhat to the right, with true maxima in the ergastoplasm occurring at perhaps one hour, and in the proximal zymogen granule region at about two hours (estimations based on qualitative observations of radioautographs of the pancreas taken from rats killed at these time intervals after injection of leucine-H^3). Nevertheless, it appears that the first and second of Zilversmit's criteria are fulfilled in the case of the ergastoplasm forming a precursor of proteins to the proximal zymogen granule region.

The continuous increase of specific activity in the distal zymogen granules to a point at four hours about equal to that of the ergastoplasm at 30 minutes, indicates that the labelled proteins from the

ergastoplasm and proximal zymogen granule regions migrate into this region, and that the proteins in the proximal region are probably the direct precursor of those in the distal zymogen granules. This is evident from the fact that the specific activity in the proximal zymogen region remains at a high level from 10 minutes to 4 hours; indicating an accumulation of labelled proteins in this region. The complete reversal of the magnitudes of specific acitivity in the three components from 10 minutes to 4 hours, that is, the ergastoplasm at 10 minutes containing the highest specific activity, and dropping to the lowest at 4 hours; the opposite occurring in the distal zymogen granules, clearly indicates that the labelled proteins formed in the ergastoplasm are precursors of the labelled proteins in the proximal zymogen granule region, which in turn are the precursors of the proteins in the distal zymogen granules.

SUMMARY AND CONCLUSIONS

The aim of this work is the localization of the sites of synthesis, and migration with time, of the proteins synthesized in the pancreatic acinar cells of rats and mice.

Preliminary experiments were designed to determine whether leucine is an adequate tracer of the proteins which are being synthesized. A large number of histological sections of 4 organs from adult mice injected with a tracer dose of leucine-C^{14} were prepared by routine methods. Proteins and nucleic acids were extracted from the sections and analyzed for radioactivity with a Geiger counter. Furthermore, to determine to what extent, if any, labelled amino acids are adsorbed to the proteins in vivo, the protein fraction was treated with NaOH in order to liberate these presumably ionic bound molecules. It was thus found that, following administration of labelled leucine, over 90% of the total radioactivity present in the 4 investigated organs was in the protein fraction of the tissue sections. Furthermore, the results of the NaOH extraction procedures indicated that the incorporation of labelled amino acids into proteins cannot be explained as either exchange or adsorption, but must represent a synthesis of new protein.

To localize the sites of synthesis and follow the labelled proteins, the main experiments consisted of injecting groups of rats with leucine-H^3, killing the animals at specific times thereafter, and radioautographing the histological sections. Similar experiments, designed to investigate the early intervals after injection, were performed in mice with the amino acids glycine and methionine-H^3. Quantitative analysis of the amount of radioactivity in the various parts of pancreatic acinar cells of these rats and mice were performed using drawing made to scale of the pancreatic

acini, and of the silver grains in the emulsion overlying them.

The proteins synthesized in the pancreas which turn over in 24 to 36 hours, represent the proteins synthesized for secretion as enzymes, while the labelled proteins remaining after 36 hours are probably due to the proteins synthesized for utilization by the cell.

The precise localization obtained using radioautography with tritium labelled amino acids at very early time intervals after injection reveals the sites of protein synthesis, which in the exocrine cells is chiefly the basophilic ergastoplasm, located at the base of the cells, where the electron microscope shows the presence of the ribosomes of the endoplasmic reticulum. A slight amount of synthesis also occurs in the zymogen granule regions, perhaps again in relation to the elements of endoplasmic reticulum found in this region.

The pattern of distribution at various times after injection allows the migration of the labelled proteins to be followed from the time of synthesis. From the ergastoplasm, the newly-formed proteins migrate very rapidly into the proximal portion of the zymogen granules, a region which may be shown to contain the Golgi apparatus. The proteins remain in the Golgi region for at least 1/2 hour, then diffuse slowly into the distal zymogen granule region near the lumen of the acinus. As early as 30 minutes, but particularly at 4 and 36 hours, labelled proteins are found in the lumen of the excretory ducts, indicating that a continuous secretion of enzymes, constantly replenished by synthesis, occurs in all acinar cells of the pancreas.

Identical results were obtained with other amino acids, glycine-H^3 and methionine-H^3, in another species - the mouse. Thus, in both the rat and the mouse, with the three different amino acids, the site of synthesis of pancreatic proteins was shown to be the ergastoplasm. From the ergastoplasm the labelled proteins migrate into a region containing the Golgi apparatus, emerging from here as the zymogen granules. These contain the

labelled proteins subsequently seen as extruded zymogen in the lumen of the pancreatic ducts.

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