

# A Cytochemical Study on the Pancreas of the Guinea Pig

## III. *In Vivo* Incorporation of Leucine-1-C<sup>14</sup> into the Proteins of Cell Fractions

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### ABSTRACT

DL-leucine-1-C<sup>14</sup> was administered by intracardiac injection to guinea pigs and its *in vivo* incorporation into the proteins of various pancreatic cell fractions followed over a period of 2 hours.

The pancreas was homogenized in 0.88 M sucrose and fractionated by differential centrifugation to give nuclear, zymogen, mitochondrial, microsomal, postmicrosomal, and final supernatant fractions. The proteins of these fractions, obtained by precipitation with trichloroacetic acid followed by washing, were counted.

The proteins of the microsomal fraction showed the highest early specific activity and were followed by those of the zymogen and mitochondrial fractions.

The microsomal fraction was broken up into two subfractions: one consisting of detached RNP particles, the other representing mainly the microsomal content and membranes.

The incorporation of labelled leucine into the proteins of microsomal subfractions and in those of postmicrosomal fractions was studied comparatively in the pancreas of fasted and fed guinea pigs as well as in the liver and pancreas of fasted animals.

A tentative cytological picture of protein synthesis and transport based on these findings is presented.

### INTRODUCTION

The pancreas is a particularly favorable organ for the study of protein synthesis because the exocrine cells, which form the bulk of its glandular tissue, have a high protein output (1, 2), their secretory activity is cyclic and can be triggered by various means (1, 3) including feeding (4, 2), and finally the proteins produced are enzymes, a circumstance which facilitates quantitative studies. These advantages might be diminished by the fact that most of the proteins concerned are produced for "export," not for intracellular use. They are indeed hydrolytic enzymes which are excreted by the gland into the intestinal lumen to carry out therein the digestion of the chyme. Hence it could be expected that some aspects of the synthesizing processes, particularly those connected with structural relationships, might be different from the general mechanisms involved in the synthesis of

proteins for intracellular use. Nevertheless, the advantages of the material are so great that we decided to use the pancreas of the guinea pig for the study of a number of problems connected with protein synthesis. To this end we have studied the morphology of the acinar cells (5, 6) and have devised methods for the fractionation of pancreas breis into fractions of known cytological significance (7). These include a nuclear fraction; a zymogen fraction which consists primarily of zymogen granules; a mitochondrial fraction; a microsome fraction which contains the fragments of that part of the endoplasmic reticulum associated with particles (5); one or two postmicrosomal fractions representing the "free" ribonucleoprotein (RNP) particles of the cytoplasmic matrix; and finally a supernatant fraction presumably corresponding to the "cell sap" or cytoplasmic matrix but containing also whatever materials have been extracted from the components of the preceding

fractions during the grinding and fractionation of the tissue.

In the first paper of this series (7), we studied the intracellular distribution of trypsin-activatable protease (TAPase) and ribonuclease (RNase) activities among pancreatic cell fractions and found that they are present in relatively large amounts and high concentration in the zymogen fraction. From Hokin's work on dog pancreas (8) it appears that this fraction contains also a large part of the lipase and amylase activities of the cell. These findings finally constitute direct evidence for the old hypothesis (9) that the zymogen granules are temporary intracellular depots of digestive enzymes and enzyme precursors.

In the second paper of the series (10), we showed that the TAPase and RNase activities of the microsomal fraction increase markedly 1 hour after feeding at the time when the cavities of the endoplasmic reticulum contain numerous intracisternal granules, and when the content of the microsomal vesicles shows a corresponding increase in density. By fractionating deoxycholate (DOC)-treated microsomes, we obtained a concentrate of such granules in which we found TAPase and RNase activities in concentrations similar to those encountered in purified zymogen preparations. From the same DOC-treated microsomal preparations, we also isolated ribonucleoprotein particles, presumably detached from the microsomal membranes as a result of the treatment, and we found that these particles also possess significant amounts of TAPase and RNase activities.

To explain these findings, we postulated that the postprandial increase in enzymatic activity is due to the synthesis of new enzymes by the microsomes and we assumed that these enzymes are temporarily segregated into the intracisternal granules which thus appear as precursors of the mature zymogen granules of the cell.

The first step in testing these views can be made by tagging the cell proteins *in vivo* with radioactive amino acids, by trying to detect the intracellular loci where labelled proteins are formed, and by following with time their subsequent intracellular migration, on the basic assumption that the labelling affects primarily newly synthesized proteins.

In the present article we report the results of such a study in which variations in the amount and concentration of radioactivity were followed with time in the proteins of the various cell frac-

tions and subfractions after labelling the proteins *in vivo* with leucine-1-C<sup>14</sup>.

#### EXPERIMENTAL

In some experiments, guinea pigs fasted for 40 to 48 hours received by intracardiac injection 0.5 ml. of a solution of DL-leucine-1-C<sup>14</sup> (0.5 mg. containing 7.3  $\mu$ c.) in isotonic saline. In other experiments, animals fasted for the same time were fed a meal of cabbage, received the same amount of radioactive leucine either 15 or 60 minutes after the beginning of their meal, and were returned thereafter to their feeding. At specified intervals after the injection, the animals were anesthetized, the pancreas removed, weighed, homogenized, and fractionated by simple differential centrifugation, as described in references 5 and 7, to give nuclear, zymogen, mitochondrial, microsomal, postmicrosomal, and supernatant fractions. In some experiments (Figs. 1 to 3), a single postmicrosomal fraction was separated by centrifuging the microsomal supernatant for 16 hours at 105,000 g. In other experiments (Fig. 4), two postmicrosomal fractions were obtained, the first (*PM*<sub>1</sub>) by centrifuging the microsomal supernatant for 3 hours at 105,000 g, and the second (*PM*<sub>2</sub>) by centrifuging the supernatant of the preceding fraction for 16 hours at 105,000 g. The microsomal fraction was subfractionated by differential centrifugation after treatment with 0.3 per cent deoxycholate as indicated in references 5 and 10.

As shown by previous morphological analysis (7), the heavy cell fractions were noticeably heterogeneous. The zymogen fraction, for instance, was heavily contaminated by mitochondria<sup>1</sup> whereas the mitochondrial fraction contained a number of zymogen granules and a relatively large proportion of microsomes. The lighter fractions, *i.e.* the microsomal and postmicrosomal (*PM*<sub>1</sub>, *PM*<sub>2</sub>) fractions were, by present morphological criteria, almost homogeneous. Both *PM*<sub>1</sub> and *PM*<sub>2</sub> were morphologically and chemically similar to preparations isolated previously from guinea pig pancreas (5) and found to consist of ribonucleoprotein particles,  $\sim 150$  A in diameter.

The proteins of the various cell fractions were obtained, washed, plated, counted, and weighed as previously described (11). Their specific activity was expressed in counts per minute (c.p.m.) per milligram protein, divided by the wet weight of pancreas pulp

<sup>1</sup> Except for the zymogen fraction in the experiment illustrated by Fig. 6. This fraction, isolated by centrifugation in a discontinuous density gradient (7), was noticeably more homogeneous.

(in grams), to render comparable values obtained from different animals. Another necessary correction involved the determination of the specific activity of the precursor amino acids. An approximation to this value was obtained by assuming that there is a pool of amino acids in the soluble phase (final supernatant) and that these amino acids are equally available to all cell structures. Although the assumption is probably not entirely true, it should hold enough to permit a better comparison of values obtained from different fractions and different animals at different times during the experiments. To this intent the free amino acids were determined in the acid-soluble part of the final supernatant, (after the proteins of this fraction were precipitated in 5 per cent trichloroacetic acid) by the colorimetric ninhydrin method of Moore and Stein (12). The carboxyl carbon was obtained by the method of Van Slyke *et al.* (13) with the addition of inactive leucine as a carrier. The  $\text{BaCO}_3$  samples were plated and counted as indicated in reference 11, and the specific activity of the free amino acids was equated with the number of counts per minute divided by the ninhydrin color value quantitated from a standard curve for leucine. The relative specific activity of the various fractions (Fig. 2) was made equal to the counts per minute, per milligram protein, per gram pancreatic pulp  $\times 10^2$ , divided by the specific activity of free (acid-soluble) amino acids in the final supernatant.

DL-Leucine-1- $\text{C}^{14}$  was obtained from the Isotope Specialties Company, Burbank, California, and sodium deoxycholate from the Wilson Company, Chicago.

#### RESULTS AND DISCUSSION

FIG. 1 shows the specific activities and Fig. 2 the relative specific activities (*cf.* above) of the various fractions at different times after the injection of labelled leucine. The curves were constructed with data obtained from 4 different animals, each sacrificed at the time indicated on the abscissa. In this experiment the guinea pigs were fed after a fast of 48 hours and injected with radioactive leucine 15 minutes after the beginning of the meal. It can be calculated that 5 minutes after injection, approximately 0.6 per cent of the labelled leucine is present in the pancreas in acid-soluble form and 0.6 per cent has already been incorporated into the proteins of the gland. By 45 minutes the corresponding figures are 0.4 and 2.4 per cent, respectively.

The relative specific activity (RSA) of the microsomal fraction rises rapidly before that of any other cell fraction, reaches a relatively high peak around

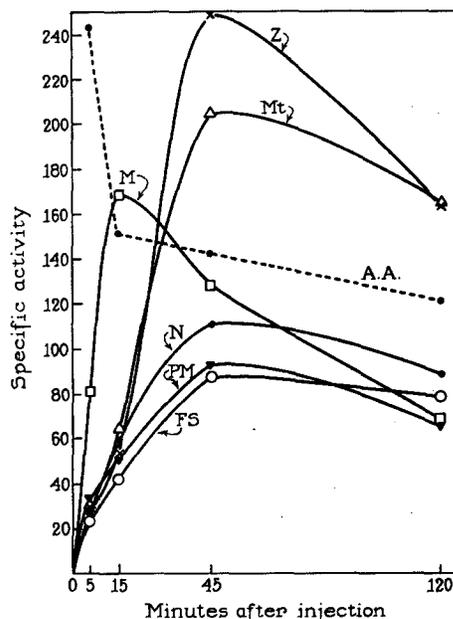


FIG. 1. Variation with time in the specific activities of proteins of guinea pig pancreas after injection of DL-leucine-1- $\text{C}^{14}$ . Z, zymogen fraction; Mt, mitochondrial fraction; N, nuclear fraction; M, microsomal fraction; PM, postmicrosomal fraction; FS, final supernatant fraction; A.A., Amino acids in the acid extract of the final supernatant. Methods and description of the fractions are given in the experimental section and in the text.

15 minutes, and then declines precipitously crossing the still ascending curves of most other fractions, beginning with those of the zymogen and mitochondrial fractions. From Fig. 2 it can be seen that this 15 minute peak is still present when the curves are corrected for variations in the specific activity of the free amino acids in the final supernatant. The finding suggests either that a steady state was not reached under the conditions of the experiment or, more probably, that the actual precursor of the microsomal proteins is not the free leucine of the final supernatant, but a derivative compound of higher specific activity (*cf.* reference 14).

The RSA curve of the microsomal fraction can be considered representative for the incorporative characteristics of the microsomes because of the relative homogeneity of this fraction. The same cannot be said, however, about the curves of the zymogen and mitochondrial fractions. It can be

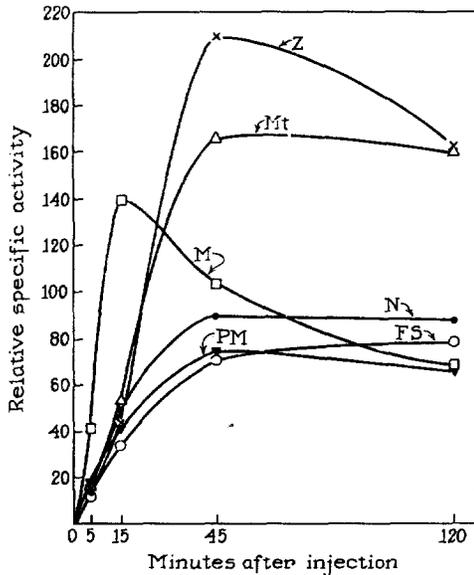


FIG. 2. Variation with time in the relative specific activities of proteins of various cell fractions of guinea pig pancreas. The data in Fig. 1 have been recalculated by dividing the specific activities of the various fractions by the specific activity of the acid-soluble amino acid fractions at the stated times.

safely assumed, for instance, that a representative curve for the zymogen granules would be higher than the one here recorded for the zymogen fraction, for the RSA of the latter is undoubtedly lowered by heavy mitochondrial contamination. As already indicated (7) the mitochondrial fraction is also heterogeneous, being contaminated by both zymogen granules and microsomes. An RSA curve representative for mitochondria would probably be lower than the one here recorded for the mitochondrial fraction.

The crossings in Figs. 1 and 2 are compatible with the concept of a transfer of labelled proteins from the microsomal to the mitochondrial and zymogen fractions, but admittedly they do not represent conclusive evidence on this point. If microsomal proteins are transferred to the zymogen granules and to the mitochondria, then the zymogen granules receive more than the mitochondria as suggested by the curves themselves and by the preceding considerations on fraction heterogeneity. Relations between the microsomes and the remaining fractions are even more difficult to interpret. In the absence of definite crossings, the curves neither suggest, nor completely exclude, the movement of proteins from the micro-

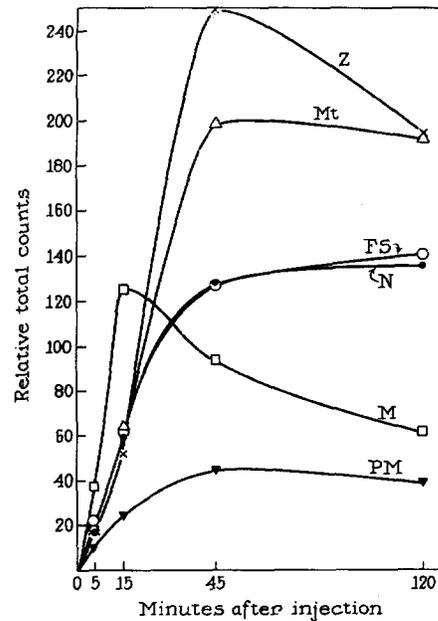


FIG. 3. Variation with time in the relative total counts of proteins of various cell fractions of guinea pig pancreas. The data in Fig. 1 have been recalculated by multiplying the specific activities by the protein weight of the respective cell fractions.

somes to these fractions. It should be emphasized, for instance, that the transfer of small amounts of highly radioactive microsomal proteins to a fraction with a large pool of non-radioactive proteins, like the final supernatant, would result in a small or negligible increase in the RSA of the recipient fraction. It is noteworthy that the incorporative ability of the free ribonucleoprotein particles of the cytoplasm (postmicrosomal fraction) is considerably lower than that of the microsomes. The corresponding RSA curve rises slowly and remains close to the curve of the final supernatant until the end of the experiment.

More information about the complex situation encountered can be obtained from Fig. 3 which records variations in the total radioactivity of the cell fractions. The curves in this graph have been calculated by using RSA values obtained in this study (Fig. 2) and previously published figures (7) for protein distribution among cell fractions isolated from animals fasted and then fed according to the same schedule. The curves in Fig. 3 are generally similar to those in Fig. 2, except that the total radioactivity curves of the nuclear and final supernatant fractions rise much higher than

the RSA curves of the same fractions. The total radioactivity curve of the microsome fraction still reaches values higher than those of any other cell fraction at 5 and 15 minutes and still crosses in its subsequent descent the ascending curves of the zymogen and mitochondrial fractions. If in this case also corrections be considered for the heterogeneity of the fractions, then a number of counts should be exchanged between the zymogen and mitochondrial fractions because of their mutual contamination. The mitochondrial fraction should also lose some counts to the microsomal fraction on account of its contamination by microsomes. As a result of these corrections, the total activity curve of a microsomal fraction containing all the microsomes derived from 1 gm. pancreatic pulp would undoubtedly be higher, the curve of a similar mitochondrial fraction lower, and finally the curve of a complete and homogeneous zymogen fraction probably higher than the corresponding curves recorded in Fig. 3. Here again the crossings are compatible with the idea of a transfer of labelled proteins from the microsomes to other cell fractions. But even with the corrections envisaged and with an assumed high rate of transfer it seems unlikely that the decrease in microsomal radioactivity could balance the increase in counts in all the other cell fractions. It follows that incorporation of amino acids into proteins, and presumably protein synthesis, is not necessarily restricted to the microsomes and that other cell components are likely to have similar abilities. Among these cell components the microsomes appear to be distinguished, however, by higher initial incorporative rates and closer relationship to the zymogen granules. That such a relationship actually exists is suggested by our previous finding (10) that the microsomes, like the zymogen granules, contain digestive enzymes in large amounts and high concentration especially after feeding.

Results similar to those illustrated by Figs. 1 and 2 were obtained with guinea pigs which received radioactive leucine directly after a 48 hour fast. In these cases, in which the meal preceding the injection of labelled leucine was therefore omitted, the number of total counts in the acid-soluble fraction of the supernatant was the same as in Fig. 1 and the specific activity of the proteins of the various fractions was only slightly higher than those recorded in Figs. 1 and 2. However, not enough animals were used to ascertain whether there was any significant difference in

incorporative ability introduced by feeding. The values so far obtained in the two conditions examined were of the same order of magnitude.

As already shown in ours (5) and other laboratories (15), deoxycholate in appropriate concentrations "solubilizes" the limiting membrane and the content of microsomal vesicles, while apparently inflicting little or no morphological damage<sup>2</sup> upon the RNP particles attached thereon. As a result, the latter are "detached" and can be isolated by subjecting the treated microsomal suspensions to differential centrifugation. For instance, after treatment with 0.3 per cent DOC a hepatic microsomal suspension yields, upon centrifugation for 2 hours at 105,000 *g*, a pellet consisting primarily of small, dense particles and containing ~80 per cent of the RNA and ~20 per cent of the protein of the original preparation (16). The morphologically similar pellets obtained from identically treated pancreatic microsomes account for ~60 per cent of the RNA and ~15 per cent of the protein of the original microsomal fraction (5). Under these circumstances, the search for the structural elements in which incorporation begins can be further pursued to find out whether the particulate or the membranous component of the microsomes can be identified as the primary incorporative site. Such inquiries, already carried out by Littlefield *et al.* (15) on liver microsomes, and by Littlefield and Keller (17) on fractions obtained from ascites tumor cells have shown that incorporation starts earlier in the DOC-insoluble part of the microsomal fraction, *i.e.* in the attached RNP particles, than in the DOC-soluble part. Similar results were obtained by Hultin (18) and by Simkin and Work (19) who, after bicarbonate extraction (18) or extraction with solutions of varied ionic strength (19), found the highest early specific activity in the proteins associated with the ribonucleic acid of liver microsomes. We were interested in finding out whether a similar situation prevails in the pancreas. In addition, we considered worthwhile comparing the activity of the attached RNP particles with that of the free RNP particles of the cytoplasm. As material for this inquiry, we chose the pancreas of fasted guinea

<sup>2</sup> Centrifugation of DOC-treated microsomal suspension yields a pellet of particles similar in size and density to those originally attached to the microsomal vesicles (5, 10). Although these particles show little or no morphological change at the present level of resolution, they are inactive in the *in vitro* incorporation of labelled amino acids into the proteins (17).

TABLE I

*Incorporation of DL-Leucine-1-C<sup>14</sup> into the Proteins of Various Cell Fractions Isolated from the Pancreas and Liver of Fasted Guinea Pigs*

For the 10 and 20 minute points, the animals received 0.5 mg. DL-leucine-1-C<sup>14</sup> (7.3  $\mu$ c.) by intracardiac injection; for the 3 minute point the amount of labelled leucine was increased to 1 mg. (14.6  $\mu$ c.). The pancreas and liver were excised at the specified times after injection, homogenized in 0.88 M sucrose solution, and centrifuged at 20,000 g for 30 minutes. The resulting supernatant was further centrifuged at 105,000 g for 60 minutes to yield a microsome pellet. The latter was resuspended in sucrose and enough deoxycholate (pH 7.5-7.8) added to give a final concentration of 0.3 per cent. The cleared suspension was then centrifuged at 105,000 g for 120 minutes to give a pellet of insoluble microsomal material. This pellet is composed primarily of attached RNP particles. The corresponding supernatant contains DOC-soluble, microsomal material representing mainly the membranes and content of microsomal vesicles. The supernatant of the original microsomal fraction was centrifuged at 105,000 g for 16 hours to give a common postmicrosomal fraction. In the case of the pancreas this fraction consists mainly of free RNP particles; in the case of the liver it is a mixture of particles and vesicles derived from the smooth surfaced part of the endoplasmic reticulum. The proteins of the various fractions were obtained and counted as described in reference 11. The figures are in C.P.M./mg. protein/gm. wet weight pancreas or liver.

Organ	Fraction	Time after injection		
		3 min.	10 min.	20 min.
Pancreas	Microsomal:			
	DOC-insoluble	67.8	32.3	40.2
	DOC-soluble	49.9	55.7	128.2
	Postmicrosomal	20.0	27.7	76.3
Liver	Microsomal:			
	DOC-insoluble	49.5	—	60.4
	DOC-soluble	11.9	—	39.7
	Postmicrosomal	7.6	—	19.5

pigs because intracisternal granules are not present in their microsomes, and consequently the attached RNP particles can be compared to the solubilized membranes with a minimum of interference by material contained within the microsomal vesicles.

First, we followed the *in vivo* incorporation of leucine-1-C<sup>14</sup> into pancreatic microsomal subfractions and into a single pancreatic postmicrosomal fraction (the pellet obtained by spinning the whole microsomal supernatant for 16 hours at 105,000 g) and compared their incorporative abilities with those of the corresponding liver preparations obtained from the same animals. The results of the experiments are given in Table I. They should be interpreted bearing in mind that the subfractions of the pancreatic and hepatic microsomes are morphologically similar, whereas the postmicrosomal fractions are not. The hepatic postmicrosomal fraction differs from its pancreatic counterpart, in being noticeably heterogeneous (16): it contains, in addition to free particles, numerous small vesicles apparently derived from the well developed, smooth surfaced parts of the endoplasmic reticulum of hepatic parenchymal cells.

In the case of the pancreas, the attached particles have a high specific activity at 3 minutes and a low one at 10 and 20 minutes after the injection of labelled leucine. Since the radioactivity of the soluble subfraction varies conversely, the data are compatible with a rapid transfer of highly labelled proteins from the attached RNP particles to either the membranes or the content of the microsomes. In the case of the liver, the specific activity of the attached particles is relatively high at 3 minutes and continues to increase slightly up to 20 minutes. The initially low specific activity of the DOC-soluble subfraction rises also in the meanwhile but, at variance with previous reports (15), no crossing occurs. The figures suggest, however, that one could be reasonably expected in an experiment covering a longer time period. It follows that in liver, microsomal proteins are probably transferred, as already reported (15), from the attached RNP particles to the membrane or content of the microsomes, but that the transfer is noticeably slower than in the pancreas.

The incorporation of leucine into the proteins of the free pancreatic RNA particles proceeds initially at a much slower rate but rises continu-

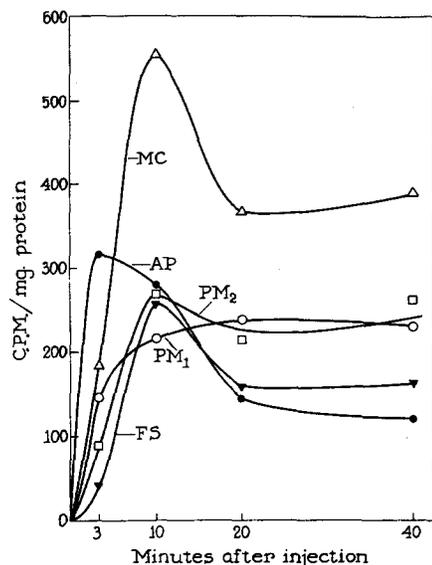


FIG. 4. Variation with time in the specific activities of the proteins of various cell fractions of guinea-pig pancreas. *AP*, attached particles; *MC*, microsomal content; *PM*<sub>1</sub> and *PM*<sub>2</sub>, first and second postmicrosomal fractions; and *FS*, final supernatant. Methods and description of the fractions are given in the experimental section and in the text.

ously and by 20 minutes is higher than that of the attached RNP particles. It cannot be ascertained from these data whether the labelling of the free RNP particles is due to local incorporation, or to the transfer of labelled proteins from the attached RNP particles. The incorporating activity of the hepatic postmicrosomal fraction remains low throughout the experiment, a finding in agreement with the general hypothesis that the RNP particles, not the membranes of the endoplasmic reticulum, represent the active element in amino acid incorporation and presumably in protein synthesis.

In another experiment on pancreas alone (Fig. 4), the incorporative ability of microsomal subfractions (DOC-soluble and -insoluble) was compared with that of two postmicrosomal fractions (*PM*<sub>1</sub> and *PM*<sub>2</sub>) and with that of the final supernatant. In agreement with data in Table I, the curves show that the attached RNP particles are initially more active than the free RNP particles of the cytoplasm in incorporating labelled leucine into proteins. Fig. 4 shows in addition that this finding applies for both *PM*<sub>1</sub> and *PM*<sub>2</sub>. Some differences exist between the two postmicrosomal

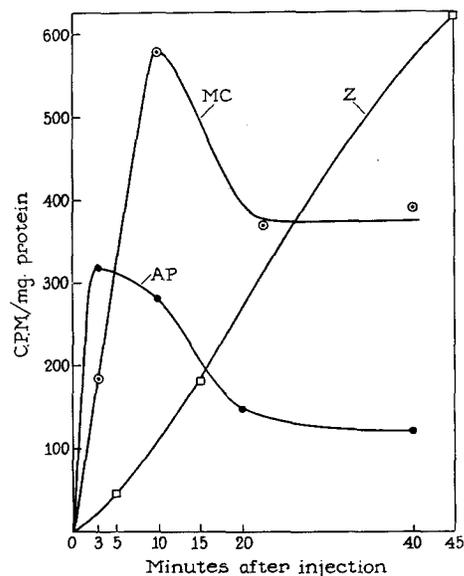


FIG. 5. Variation with time in the specific activities of protein of attached particles (*AP*), microsomal contents (*MC*) and zymogen granules (*Z*) of pancreas after injection of DL-leucine-1-<sup>14</sup>C into fasted guinea pigs.

fractions at the early points investigated, but with the evidence at hand it is not possible to ascertain whether the differences are significant or not.

The experiments thus far reported did not reveal any striking difference in incorporative ability between pancreas preparations obtained from fasted and fed animals. Since it has been reported in the literature that the incorporation of labelled amino acids into the total proteins of the pancreas of other animals (*e.g.* the mouse, rat, and pigeon) is stimulated by feeding or by treatment with carbamyl choline or pilocarpine (20–23), we decided to explore the course of incorporation at a later time after feeding. We chose 1 hour because at that time we had found large granules within the cavities of the endoplasmic reticulum and a concomitant increase in the enzymatic activity of the microsomes (6, 10). Both findings might be interpreted as signs of increased enzyme synthesis.

Fig. 5 represents the control of these experiments and compares the specific activities of the proteins of: (a) DOC-insoluble microsomal subfraction, *i.e.*, attached RNP particles, (b) DOC-soluble microsomal subfraction, *i.e.*, microsomal membranes and content, and (c) zymogen fraction isolated from fasted guinea pigs. In this case the zymogen fraction was isolated by usual differential

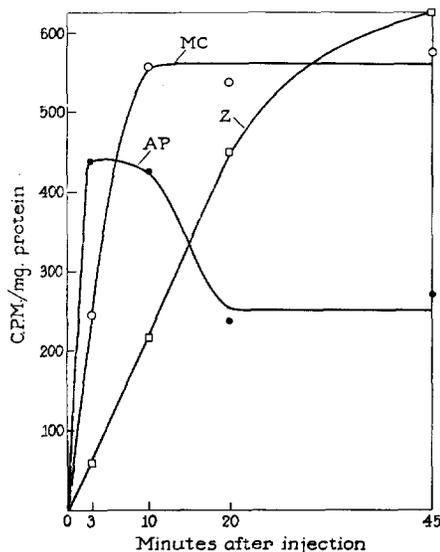


FIG. 6. Variation with time in the specific activities of protein of attached particles (AP), microsomal content (MC), and zymogen granules (Z) of pancreas after injection of DL-leucine-1-C<sup>14</sup> into guinea pigs 1 hour after refeeding.

centrifugation and was therefore highly heterogeneous. Fig. 6 presents the curves obtained with the corresponding cell fractions isolated from animals fed after a fast of 48 hours, injected with labelled leucine 1 hour after the beginning of the meal, and sacrificed after the injection at the times indicated on the abscissa. In this experiment the zymogen fraction was isolated by centrifugation in a discontinuous density-gradient and, as such, was noticeably more homogeneous (7). A comparison of the two figures shows that the specific activity of the microsomal subfractions is higher in fed animals, especially at later time points. At these points, the curves for all microsomal subfractions flatten out, but the activity level is twice higher in fed guinea pigs.

It is reasonable to assume that the shape of these curves is a function of both the rate of synthesis of new protein and the rate of protein transfer from one cell compartment to another. A levelling off in the curves probably represents a steady state between the two processes, since at this time (see Fig. 1) there is a relative constancy in the specific activity of the acid-soluble amino acids. At this steady state level, the specific activity of the attached particles is lower than that of the microsomal contents. If we assume a transfer of

newly synthesized protein from the former to the latter, we must further assume that highly labelled proteins (digestive enzymes) are preferentially transferred while the structural proteins of the RNP particles are retained.

Our results show that, within the time limits explored, the incorporation of labelled leucine by the attached RNP particles of the pancreas is a continuous process which proceeds at a more or less stable rate unaffected by feeding at least over a period of 60 to 75 minutes after food intake. The finding is in agreement with many morphological and physiological observations (*cf.* reference 1) which indicate that zymogen is continuously released by the gland even during prolonged fasting. A similar view was expressed more recently by Allfrey *et al.* (21) to explain their biochemical findings.<sup>3</sup> The rise in specific activity shown by the microsomal subfraction 80 minutes after feeding could result from a slowing down of protein transfer from the attached RNP particles to the microsomal content and finally to the zymogen granules. Such an interpretation is supported by the visible accumulation of a product within the cavities of the endoplasmic reticulum (intracisternal granules) and by the marked increase in microsomal enzymatic activity at about the same time; *i.e.*, 1 to 2 hours after feeding. A partial and temporary block affecting the transfer of protein from the endoplasmic reticulum to the zymogen granules could explain all these concomitant events. Conversely our results could reflect a rise in protein production occurring at the time mentioned and could be due to either an increase in rate or to an increase in the number of active synthetic sites (*i.e.*, attached RNP particles) participating in the process. The last explanation is particularly attractive, but with our present evidence we cannot decide among these various possibilities. It is interesting to note that Farber and Sidransky (23) also found that feeding produces a relatively late (30 minute) increase in the specific activity of the proteins of the whole pancreas of the rat. Our experiments suggest that in the guinea pig the response to the stimulation by feeding is even slower than in the rat and this circumstance could explain the apparent discrepancy between our results and those reported by Hokin (8).

We realize that the situation we attempted to

<sup>3</sup> According to Allfrey *et al.*, proteins are synthesized by the cell to compensate for any zymogen release and are later converted into digestive enzymes.

analyze is extremely complex and that accordingly few points in our work can be considered as well established, and few interpretations as certain. It appears reasonably well established, for instance, that under our experimental conditions the microsomes, and especially their attached RNP particles, are the sites of earliest incorporation, and that labelled proteins appear later in the other fractions and accumulate in large amounts in the zymogen granules. It also appears that there is a clear distinction between free and attached RNP particles, the former being considerably less active than the latter. Our results are in general agreement with those previously obtained on cell fractions of mouse and rat pancreas by Allfrey *et al.* (21) and by McLean *et al.* (24). In their studies, however, no distinct zymogen fraction was isolated and the microsomal fraction, or a derivative thereof (21), was compared with the whole tissue brei (21) or with the standard fractions of the usual fractionation procedure (24). A similar course of events, *i.e.* rapid incorporation into the "microsomes" followed by transfer to other cell sites, was reported in reticulocytes (25). There is, however, no agreement between our results and those presented by Khesin in an early report (26) in which he concluded that "pancreatic secretory granules" can synthesize amylase *in vitro* when fortified with some mitochondrial products. More recently (27) he reported an increase in amylase activity when mitochondria were incubated together with "light large granules" rich in ribonucleic acid.

The findings here presented are compatible with our general hypothesis according to which the digestive enzymes are synthesized by the attached RNP particles,<sup>4</sup> transferred across the limiting membrane into the cavities of the endoplasmic reticulum, segregated temporarily (under certain conditions) into intracisternal granules, and finally packed and stored in the form of mature zymogen granules. We recognize, however, that other interpretations are not excluded and that many points require further work for elucidation.

<sup>4</sup> Our earlier finding (10) that up to 15 per cent of the TAPase and up to 30 per cent of the RNase activity of the microsomes are associated with the attached nucleoprotein particles would indicate that this activity represents newly synthesized proteins which are still attached to their site of synthesis. Isolation of highly radioactive enzymes from the particles would settle this issue.

It appears, for instance, that the microsomes cannot be the only cell components engaged in amino acid incorporation (presumably protein synthesis) and that other active sites must exist in the cell. Considering the number of different proteins produced by the protoplasm, it is indeed to be expected that the synthesis of many proteins is proceeding simultaneously at different rates and possibly at different sites in the cell. The attached RNP particles may represent such a site; indeed, it may be the site concerned only with the synthesis of those proteins which are elaborated by the cell for export. Recent evidence suggests that the nucleus (28) and mitochondria (29) are other sites of protein synthesis. The zymogen granules might conceivably be on a third site (*cf.* reference 26). Indeed, in an alternative interpretation to that given on page 560; the curves in Figs. 1 to 3, 5 and 6 could be taken to show that proteins are simultaneously but independently synthesized in the zymogen granules and in the microsomes. Our results *per se* do not exclude this alternative, but the general cytological evidence is against it. It is known for instance that the zymogen granules are temporary structures that behave primarily as storage sites (9, 1). Our previous findings (10) indicate that in fed animals the microsomal and the zymogen fractions contain equal amounts of digestive enzymes (TAPase and RNase), and the results here reported show that the incorporation of amino acids initially proceeds at a considerably higher rate in the microsomes than in the zymogen fraction. It seems to us that all these findings can be better explained by a unitary hypothesis, according to which the digestive enzymes are synthesized in a single site, *i.e.* the microsomes or their attached particles, to be transferred thereafter to the zymogen granules. A multiple site hypothesis seems less likely when the group of digestive enzymes is singled out for consideration.

The main source of confusion in the interpretation of experiments of the type here reported is the fact that what is followed is not the synthesis of a single, specific protein, but the sum of a diversity of simultaneous synthetic processes. To clarify the situation we propose to restrict the incorporation studies to a single protein. By following with time the labelling of a single digestive enzyme in the microsomal and zymogen fractions we hope to provide an answer to some of the questions raised in this paper.

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