

## SYNTHESIS OF PROTEIN IN THE PANCREAS

### II. THE ROLE OF RIBONUCLEOPROTEIN IN PROTEIN SYNTHESIS

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#### INTRODUCTION

The observations and experiments presented in this paper are concerned with the role of the protein component of ribonucleoprotein in synthesis of protein. This work shows that the nucleoprotein is part of the cell's equipment for protein synthesis and provides evidence that the protein component of nucleoprotein functions as precursor material for proteins synthesized in the cell.

It is well known that the uptake of isotopically labelled amino acids is more rapid in the protein of the microsome fraction (a nucleoprotein-containing material) of the liver than in other protein fractions of the tissue (1-3). Although such high incorporations into microsomes suggest that they play a role in protein synthesis, it is important to determine whether such activity is limited to a replacement of microsome protein or whether it bears on the general problem of protein synthesis in the cell. In the experiments about to be described, using the pancreas, it has been possible to make this distinction and to demonstrate that the microsome protein is concerned with the synthesis of the secretory enzymes of the cell.

The pancreas is the tissue to which we have directed our attention. Our reasons for selection of the pancreas should be given because in the numerous investigations on protein synthesis that are now appearing from so many different laboratories very little has been done on the pancreas. The exocrine portion of this tissue is highly specialized for protein synthesis, much in the way that striated muscle is specialized for contraction, so that cell components concerned with protein synthesis would be expected to be more prominent in the pancreas than in the generality of tissues. Protein synthesis is also a prominent aspect of growth and for this reason growing cells and tissues are frequently used as material for investigations of protein synthesis. Synthesis in a gland is, however, probably less complicated by other processes which occur in cell growth and division. Furthermore, in a digestive gland, such as the pancreas, the investigator can readily control conditions which influence the rate of the synthetic process. After a copious secretion there follows a period of active synthesis to replenish the supply of secretory enzymes. By inducing or inhibiting secretion the rate of synthesis can indirectly be influenced; and it can, in

fact, be shown by isotope incorporation experiments that synthesis proceeds more rapidly after secretion.

The pancreas we have used is that of the mouse. To obtain a sufficient quantity of tissue it has been necessary to use about six animals for each experiment. There is, however, a certain advantage gained by using a number of small animals in place of a single large animal: experiments are more reproducible. Another advantage is that the total weight of mice used in an experiment is much less than the weights of rats, guinea pigs, or rabbits that would be required for an equal quantity of pancreas, because the size of the pancreas in relation to total size of body is far greater in the mouse than in larger animals.

Results of experiments on the pancreas are more readily understood if corresponding experiments on other organs, such as liver and kidney, are done at the same time. Synthesis of protein in the liver is less active than in the pancreas, and in the kidney it is less than in the liver. It has been estimated that plasma protein production in the rat liver is at the rate of 0.002 mg. protein per 1 mg. of dry liver per hour (4). Our experiments on the pancreas of the mouse show that amylase synthesis is about 0.005 mg. per 1 mg. dry pancreas per hour. This estimation is based on the amount of enzyme accumulated by the gland after depletion by pilocarpine. Total protein synthesis in the pancreas, if all secretory enzymes are considered, is, therefore, more than ten times faster than in the liver. No process on a scale comparable to secretion of digestive ferments by the pancreas or of plasma proteins by the liver is known to occur in the kidney so that synthesis in its tissues is probably at a rate far less than that of the liver. These marked differences in rates of protein synthesis should constantly be borne in mind when comparing results of experiments on the pancreas, liver, and kidney.

It was such comparisons with respect to ribonucleic acid content and capacity for protein synthesis in different types of cells that led Brachet and Caspersson to suggest that ribonucleic acid is in some way concerned with the process of protein synthesis. A similar correlation between ribonucleic acid content and protein synthesis has also been observed in microorganisms (24-26). Attempts to proceed beyond this circumstantial evidence implicating ribonucleic acid in protein synthesis have hardly been successful so far. Indeed, Caspersson's contention that the quantity of ribonucleic acid in the pancreas varies during the cycle of synthesis and secretion has been shown to be incorrect (5, 6). Even so the mass of circumstantial evidence compels one to persist in searching for the role of nucleic acid in protein synthesis. In this way we have been led to examine the protein component of ribonucleoprotein and the part it plays in protein synthesis.

*The Nucleoprotein Pellet Prepared by Centrifugation at 40,000 R.P.M.*—Most of the ribonucleic acid of a tissue is in the so called microsome fraction, which sediments at 40,000 R.P.M. in a sucrose medium to form a pellet. In this paper it will be referred to as the pellet material. In a study of the protein component

of the pellet material it is important to know whether the various components of the pellet have merely been brought together by centrifugation at a certain speed for a certain time or whether the components are associated in sufficiently definite form to permit us to presume that they were also associated with each other in the living cell. The properties of the pellet material, to be described now, indicate that protein and nucleic acid are combined in a definite complex.

Most of these experiments have been done on pellet material of the liver rather than on that of the pancreas because of the intense activity in the latter of ribonuclease, which alters the composition of the pancreas pellet from the moment the tissue is disintegrated. A study of the action of ribonuclease on the pancreas pellet shows that combination of protein with the nucleic acid protects the latter to some extent from the enzyme; decomposition of free nucleic acid at pH 6.8 proceeds five times as fast and at pH 7.6 twice as fast as does decomposition of the nucleic acid of the pellet; but ultimately all the nucleic acid of the pellet is broken down. This is in marked contrast to the combination of protein and ribonucleic acid in the tobacco mosaic virus; in this case combination completely protects the nucleic acid from the action of ribonuclease. In the pancreas pellet combination with protein protects the nucleic acid no more than do the salt-like combinations formed when histone is added to ribonucleic acid, for experiments show that the breakdown of ribonucleic acid in presence of an excess of thymus histone proceeds at about the same rate as it does in the pancreas pellet at pH 6.8 and pH 7.6. (This comparison should not be taken to mean that the protein of the pellet is of a histone-type. At a later point in this paper a discussion of this problem is given.)

Electrophoretic experiments with pellet material show that in it protein and ribonucleic acid are combined in such a way that they do not migrate separately in an electric field. In Fig. 1 the results are given of a zone electrophoretic experiment on liver pellet material. The distribution of nucleic acid after electrophoresis in glass powder was followed by its characteristic absorption maximum at 258  $m\mu$ . Since nucleic acids have an absorption minimum at 230  $m\mu$ , where protein solutions have high extinctions, it was possible to measure the protein distribution after electrophoresis by measuring  $E_{230}$ . (This was supplemented by nitrogen analyses as explained in detail in the experimental section.)

In the figure both  $E_{268}$  and  $E_{230}$  are plotted against the distance of migration in centimeters. The pellet material shown in Figure 1 A was prepared in 0.25 M sucrose and the electrophoresis was done in 0.25 M sucrose. For comparison the migration of ribonucleic acid itself under similar conditions is given, showing clearly that the nucleic acid of the pellet material remains firmly attached to protein in the electric field.

That sucrose maintains the integrity of the pellet material is shown when the electrophoresis of material prepared in 0.25 M sucrose is carried out in the absence of sucrose. In Figure 1 B it is seen that in absence of sucrose there is a tendency for the nucleic acid of the pellet to move away from the protein.

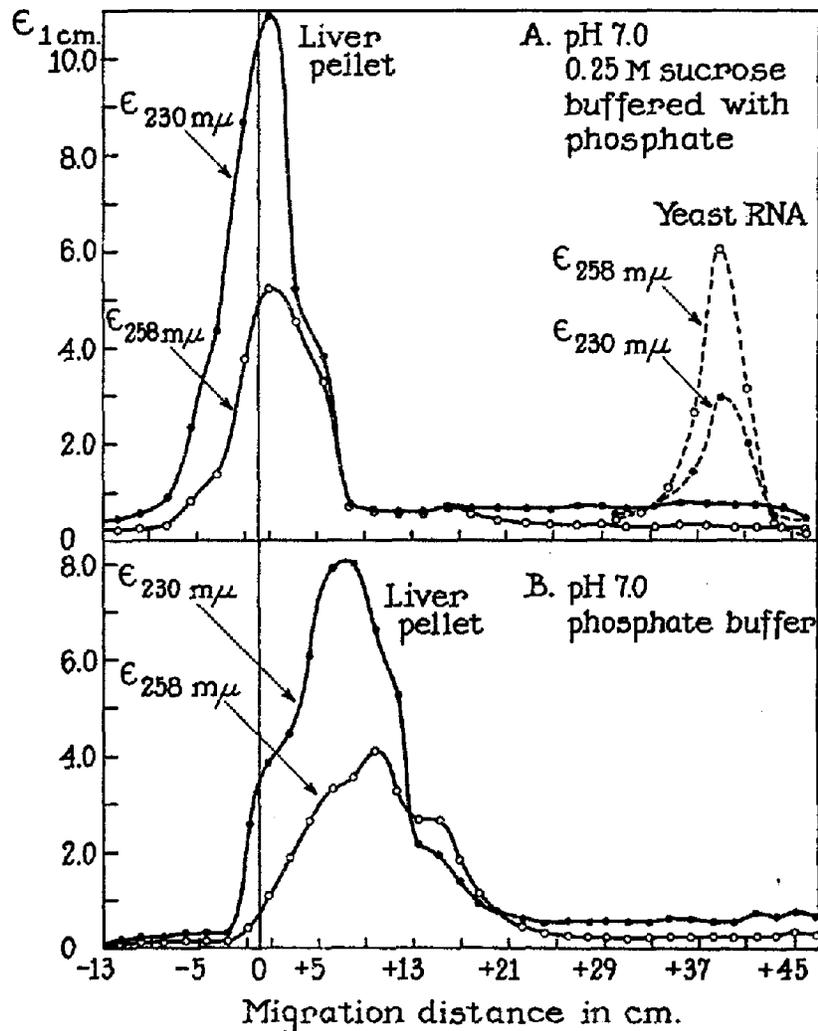


FIG. 1 A and 1 B. Fig. 1 A shows zone electrophoresis pattern of mouse liver pellet material after 100 hours' migration through glass powder in 0.25 M sucrose buffered with phosphate at pH 7.0. Nucleic acid distribution indicated by  $\epsilon_{258}$ . Protein distribution shown by  $\epsilon_{230}$ . The dotted curves show the electrophoretic pattern of yeast RNA under similar conditions. Fig. 1 B shows electrophoretic pattern of mouse liver pellet material prepared in 0.25 M sucrose but placed in 0.2 M phosphate buffer at pH 7.0 for electrophoresis.

When pellet material of pancreas, rather than that of liver, is subjected to electrophoresis the nucleic acid moves out far ahead of the protein, but when this fast moving nucleic acid is examined it is found to have been decomposed in

the long period required for this experiment by the ribonuclease present in the preparation.

When the pancreas pellet material is prepared, if done quickly, only a small part of its nucleic acid is broken off by ribonuclease. The high percentage of ribonucleic acid in this material is itself evidence that the combination of nucleic acid and protein is not an artifact formed during the preparation. Content of nucleic acid, varying slightly in different preparations, is about 23 per cent of the lipid-free pellet material. To this can be added the nucleic acid split off, about 10 per cent of the total. This amount is found by measuring the acid-soluble nucleotides present in the supernate after centrifuging at 40,000 R.P.M. and subtracting therefrom the amount of acid-soluble nucleotide found in pancreas treated at once with cold 2 per cent perchloric acid. Correcting in this way for decomposition occurring during preparation, the pellet material of the pancreas contains about 25 per cent ribonucleic acid. It hardly seems likely that protein capable of combining with so much nucleic acid at pH 6.8, the pH of the sucrose preparation, is not the protein that was combined with nucleic acid in the intact pancreas but was merely picked up in the course of preparation.

When the liver pellet is prepared two of the main conditions of the preparation, duration and speed of the high speed centrifugation, can be varied considerably so that several pellet samples can be prepared from the same mass of tissue and yet the samples with different sedimentation characteristics do not differ in the proportions of nucleic acid and protein. Experiments along these lines, done by two different investigators on rat liver, indicate that the nucleic acid and protein components of the pellet material have not become associated during preparation, but were originally combined in the cells of the liver (3, 7).

Constancy of the proportions of nucleic acid and protein in the liver pellet is also shown when this material (prepared from mice) is suspended in a sucrose solution buffered at pH 9.0 with borate and then centrifuged at 40,000 R.P.M. We found that although some 20 per cent of the material did not sediment, the ratio of nucleic acid to total nitrogen in the pellet was 0.10, the same as in the original pellet, which had been prepared at pH 6.8.

Another observation showing the definiteness of the combination between nucleic acid and protein in the pellet comes from a study of this material in the pancreas of fasting mice. After a 4 day fast the ribonucleic acid content of the pancreas decreased 39.6 per cent. Pellet materials prepared from the fasted mice and from control mice had, however, about the same composition. Lipid-free material of control mice had 17.35 per cent nitrogen and 2.3 per cent phosphorus (estimated both as total phosphorus and as nucleic acid phosphorus by determination of the ultraviolet extinction coefficient in the material released by 10 per cent perchloric acid at 70°C.); that of fasted mice had 17.4 per cent nitrogen and 2.2 per cent phosphorus.

In Table I the ribonucleic acid contents of the pancreas, liver, and kidney of

mice are given along with the nucleic acid contents of pellet materials of these tissues. The correspondence between RNA contents of these tissues and their activities in synthesizing protein is an example of the correlation first noted by Brachet (8) and Caspersson (9) and more recently observed in microorganisms (24-26). The data in this table show that there is also a correlation between the percentage of nucleic acid in the pellet material of a tissue and the activity of the tissue in protein synthesis.

The high nucleic acid content of the pancreatic pellet material raises the question of whether the protein of this pellet is especially rich in basic amino acids. A brief discussion of this matter must now be given, for it has, indeed, already been claimed that in such cells as those of the pancreas which contain high concentrations of ribonucleic acid there are proteins rich in diamino acids associated with the RNA, and much has been said about the production of such proteins by the nucleolus and the "nucleolus-associated chromatin" and

TABLE I  
*Ribonucleic Acid Contents of Mouse Tissue and Pellet Materials*

Tissue	RNA phosphorus	
	Tissue	Pellet material
	<i>per cent</i>	<i>per cent</i>
Pancreas	1.20	2.3 (2.5)*
Liver	0.389	1.11†
Kidney	0.237	0.690†

\* Total phosphorus.

† Orcinol.

their role in protein synthesis in nucleus and cytoplasm. In their first claims about these proteins Caspersson and his colleagues referred to them as of "histone type" because it was supposed by them that the ultraviolet absorption spectrum of a histone differs from that of other proteins, being shifted somewhat to longer wave lengths and this seemed to account for the absorption at these wave lengths which they reported in regions of cells rich in ribonucleic acid (10). When the absorption spectra of satisfactory histone preparations were determined, it was found, however, that the maximum of the ultraviolet absorption spectrum of a histone does not differ from that of the generality of proteins (11). When this became known the term "histone type" protein was dropped and these proteins associated with ribonucleic acid were referred to as proteins rich in diamino acids. Histones are in fact rich in diamino acids, but the evidence that the proteins associated with high concentrations of ribonucleic acid are rich in the basic amino acids is something one searches for in vain in the numerous papers from Caspersson's laboratory in which reference is frequently made to proteins rich in diamino acids (see also the recent book by Caspersson (9) and the book by Danielli (12)).

It is, of course, possible that the proteins associated with ribonucleic acid do have a high concentration of basic amino acids and for this reason the quantities of these amino acids in the proteins of pellet materials prepared from liver and pancreas were determined. The analytical results are given in Table II along with similar analyses of a histone and of mixed cytoplasmic proteins of the pancreas and liver. The diamino acids of the pellet proteins are no higher than in mixed cytoplasmic proteins and far lower than in a histone. The lack of an especially high content of diamino acids in pancreas pellet protein is noteworthy because this protein is associated with an exceedingly high concentration of ribonucleic acid. Absence of especially high concentrations of the diamino acids in liver and pancreas pellet materials is of interest in connection with what is known about the composition of the plant viruses, a group of ribonucleoproteins with nucleic acid contents varying from 5 to 41 per cent (13). The nitrogen contents of these nucleoproteins have been determined and if an accumulation of nucleic acid were correlated with a high content of diamino acids this would

TABLE II  
*Basic Amino Acid Composition of Pellet Proteins, Mixed Cytoplasmic Proteins, and Histone*

Amino acid	Moles amino acid per 100 moles nitrogen				
	Liver pellet	Pancreas pellet	Pancreas cytoplasm (horse)	Liver cytoplasm (calf)	Liver histone (calf)
Histidine	1.21	1.57	1.69	1.81	1.15
Lysine	4.50	4.42	5.08	5.20	6.25
Arginine	2.85	3.51	3.22	3.65	6.55

probably be shown by a high nitrogen content. The analyses show no such correlation. The nitrogen content of the nucleoprotein with 41 per cent nucleic acid is actually less than that of a nucleoprotein with only 5 per cent nucleic acid. Even if phosphoric acid groups of nucleic acid combine with amino groups of the diamino acids of the plant virus nucleoproteins and of the proteins in pancreas pellet material there is no need to suppose that the diamino acid contents of these proteins are unusually high.

*Comparison of the Rates of Uptake of Glycine Containing N<sup>15</sup> by Pancreas, Liver, and Kidney.*—In this way some insight can be gained concerning protein metabolism and protein synthesis. In Table III the results are given of rates of incorporation of N<sup>15</sup>, administered to mice as glycine, into the mixed proteins of the pancreas, liver, and kidney, into the protein of the pellet materials prepared from these tissues, into the protein of the supernatants remaining after sedimentation of the pellets at 40,000 R.P.M., and into the ribonucleic acid of the pellet materials. The figures for liver proteins in Table III showing that rate of incorporation of N<sup>15</sup> into protein of the pellet is markedly greater than that into other liver protein fractions confirm the results obtained by previous investigators (1-3).

Comparison of rates of  $N^{15}$  uptake by proteins of the three tissues studied shows that they follow the sequence: pancreas, liver, kidney—the first being about twice that of the second and the second twice that of the third. Uptake by proteins of the pellet material is likewise highest in the pancreas, less in the liver, and lowest in the kidney. This is also the sequence of rates of protein synthesis in the three tissues. The high rates of protein synthesis in liver and to even a greater extent in pancreas are due to the massive protein secretion by these tissues. There is no simple way of collecting the protein secreted by the liver and it is not at present possible, therefore, to distinguish sharply in this tissue between protein synthesized as part of the internal metabolism of the cell and protein synthesized for secretion. Since the secretion of the pancreas flows out through a duct it can readily be collected. Examination of the pancreatic secretion shows that no nucleoprotein is present. The nucleoprotein of

TABLE III  
*Incorporation of Glycine- $N^{15}$  into Proteins and Ribonucleic Acid of Cell Fractions*

Tissue	$N^{15}$ excess 30 min. after glycine administration in			
	Mixed protein	Pellet protein	Supernate protein	RNA of pellet
	<i>atom per cent</i>	<i>atom per cent</i>	<i>atom per cent</i>	<i>atom per cent</i>
Pancreas	0.105	0.183	0.067	0.006
Liver	0.059	0.107	0.055	0.038
Kidney	0.025	0.046		

Each animal received 30 mg.  $N^{15}$ -glycine (33.3 atom per cent excess) in 0.2 ml.  $H_2O$  intraperitoneally at zero time.

All animals fasted 20 hours prior to  $N^{15}$ -glycine administration.

the pellet material is, therefore, definitely not one of the substances synthesized to be itself secreted by the gland. With this possibility excluded, there are grounds for considering the pellet material to be concerned with the synthetic process itself. In a tissue, such as the pancreas, highly specialized for protein synthesis a protein fraction with both an unusually high content of ribonucleic acid and an unusually rapid uptake of  $N^{15}$ -containing glycine must be intimately involved in synthesis of the secretory proteins. Experiments designed to show the part played by the pellet material will presently be described.

In Table III figures are given for the uptake of  $N^{15}$  by the nucleic acid of the liver and pancreas pellet materials. Quite the contrary to the results obtained for uptake by the proteins of these tissues, rate of incorporation of  $N^{15}$  into ribonucleic acid is far higher in the liver than in the pancreas. Quantity of ribonucleic acid in a pellet seems to be correlated with uptake of  $N^{15}$  by its protein (as can be seen by comparing the data in Tables I and III) but uptake of glycine- $N^{15}$  into the purines of the nucleic acid is not correlated with uptake

into the protein attached to it or into other proteins of the cell. These observations are similar to those made by Abrams in experiments in which he studied the effect of x-rays on the incorporation of  $N^{15}$ -glycine into proteins and also into adenine and guanine of RNA and DNA of the intestinal epithelium (14). The rates of incorporation into RNA and DNA were markedly reduced by x-rays but incorporation into protein was relatively unaffected.

When an animal is fasted, the acinar cells of the pancreas synthesize and store digestive ferments which are rapidly secreted after ingestion of food. The presence of the accumulated enzymes in the fasted pancreas is recognized by microscopic observation of zymogen granules or by enzymatic assays of the tissue. After secretion both microscopic observation and chemical assay show that the stores of enzymes are depleted. When digestion is finished the stores of enzymes in the gland are built up again. Even in the "resting" pancreas, the pancreas of a fasting animal, a slow, steady secretion goes on. The amount of this varies in different animals. In the rabbit, for which precise measurements are available, total enzyme content of the constant secretion of the resting pancreas is about  $\frac{1}{3}$  that of an actively secreting gland (15). Since the quantity of enzymes in a resting pancreas remains fairly constant, synthesis is constantly in progress to replenish the steady secretion that occurs in periods of rest.

Such a steady state, in which the rate of synthesis of enzymes is balanced by their rate of secretion is well suited for tracer studies which test the relationship between a precursor and a product (16). The following experiments were designed to test the hypothesis that the protein of the pancreas pellet serves as precursor material for the synthesis of the secretory proteins. At different intervals after a single massive injection of  $N^{15}$ -glycine, measurements were made on the uptake of  $N^{15}$  by the mixed protein of the tissue, the protein of the pellet, and the protein remaining in the supernate after centrifugation at 40,000 R.P.M. The data for the time course of  $N^{15}$  uptake are given in Table IV and plotted in Fig. 2.

A comparison of the curves for pellet protein and for mixed tissue protein shows that the pellet fulfills two requirements of a precursor, that its  $N^{15}$  uptake be higher than that of the product before the latter reaches its maximum incorporation and that the isotope content of the precursor be lower than that of the product after that time.

This relationship between the pellet protein and the total mixed protein of the tissue indicates that the pellet protein probably lies on the direct pathway of protein synthesis. It does not follow, however, that the pellet protein is simply the next to the last step in the synthesis of an enzyme. Indeed, the latter viewpoint is most unlikely since the pellet protein prepared in our experiments is the mixed protein of the microsome fraction of the tissue. Recent electron micrographs of microsomes (17) reveal a fairly definite structural entity, a particle about 10  $m\mu$  in diameter. Our experiments indicate that these particles

are a site of protein synthesis. If this is true one would expect that the microsome or pellet protein is a combination of synthetic machinery and many stages in the synthesis of the tissue proteins. The rates of uptake of  $N^{15}$ -glycine by individual proteins in the pellet may be expected to vary considerably and, indeed, part of the pellet protein may be relatively inert. There are two lines of experimental evidence which indicate that the pellet proteins differ widely in

TABLE IV  
Time Course of  $N^{15}$  Incorporation into Proteins of Cell Fractions

Tissue	Time	$N^{15}$ excess in cell proteins		
		Mixed tissue protein	Pellet protein	Supernate protein
	<i>hrs.</i>	<i>atoms per cent</i>	<i>atoms per cent</i>	<i>atoms per cent</i>
Pancreas	0.5	0.105	0.183	0.067
	1	0.278	0.440	0.255
	1.5	0.373	0.532	0.545
	2	0.418	0.438	0.587
	3	0.362	0.339	0.416
	4	0.308	0.298	0.377
	5	0.278	0.254	0.378
Liver	0.5	0.059	0.107	0.049
	1	0.110	0.210	0.100
	1.5	0.133	0.223	0.122
	2	0.173	0.259	0.157
	3	0.205	0.270	0.189
	4	0.186	0.243	0.176
Kidney	0.5	0.025	0.046	
	1	0.051	0.087	
	2	0.099	0.128	
	3	0.110	0.162	
	4	0.111	0.150	

Each animal received 30 mg. of  $N^{15}$ -glycine (33.3 atom per cent excess) in 0.2 ml.  $H_2O$  intraperitoneally at zero time.

All animals were fasted 20 hours prior to  $N^{15}$ -glycine administration.

their capacities for isotope incorporation. The first indication that part of the protein of the pellet is relatively inert is given by a comparison of the "time- $N^{15}$  uptake" curves for supernate and pellet proteins. Since the supernate contains about  $\frac{1}{4}$  of the protein nitrogen of the tissue and about  $\frac{1}{2}$  of its amylase and protease, uptake into it may be considered to give a better indication of what the uptake is in the secretory proteins than is given by the mixed proteins of the whole tissue. (Experiments are now being undertaken in which a secretory protein is isolated.) The curves indicate that the rate of  $N^{15}$  incorporation

by pellet protein far exceeds that of the supernate protein up to 1 hour after glycine administration. The maximum uptake into the pellet occurs at  $1\frac{1}{2}$  hours, that of the supernate occurs  $\frac{1}{2}$  hour later. However, it is significant that the maximal concentration of isotope in the supernate exceeds that of the pellet proteins. Although many complicating factors may exist, this would be expected if part of the pellet were relatively inert.

There is a second and more direct test of this hypothesis; namely, the fractionation of the pellet proteins. In preliminary experiments this fractionation

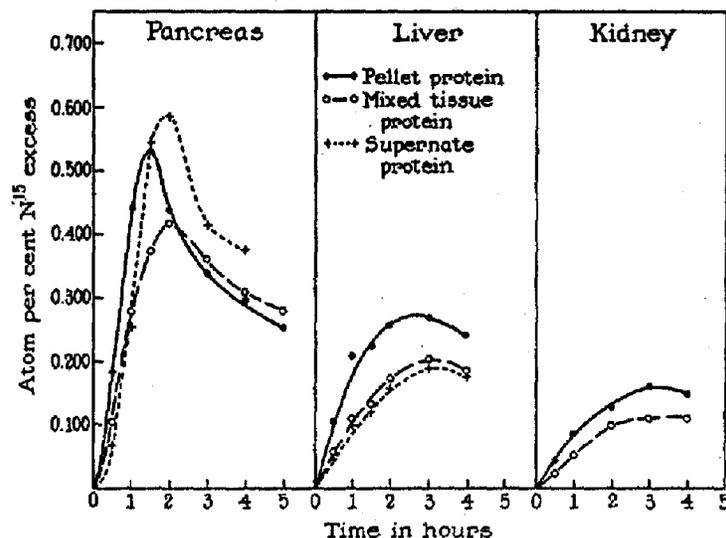


FIG. 2. Time course of  $N^{15}$  incorporation into proteins of cell fractions of the pancreas, liver, and kidney. Ordinate, atom per cent  $N^{15}$  excess in protein fraction. Abscissa, time in hours after intraperitoneal injection of 30 mg.  $N^{15}$ -glycine (33.3 atom per cent excess) into each animal. All animals fasted 20 hours prior to glycine administration.

has been accomplished by incubating the pellet with ribonuclease and subsequently centrifuging at 40,000 R.P.M. After treatment with ribonuclease about 20 per cent of the total N of the pellet remains in solution; the remaining 80 per cent is sedimentable. The  $N^{15}$  concentrations of the different pellet fractions are quite different. In one experiment the over-all  $N^{15}$  concentration in the pellet proteins was 0.545 atom per cent excess. The sediment obtained after treatment with ribonuclease had an isotope concentration of 0.405 atom per cent excess. The non-sedimentable protein had an  $N^{15}$  concentration nearly three times as high, 1.189 atom per cent excess. This figure is nearly twice the maximal uptake observed in the pancreas supernate protein and further strengthens the case for part of the pellet protein serving as precursor material

for the secretory proteins of the cell. From the over-all  $N^{15}$  concentration of the pellet proteins and the  $N^{15}$  contents of the pellet fractions it is possible to calculate the relative amounts of each of the fractions. Such a calculation shows that the non-sedimentable portion is 20 per cent of the total pellet protein; in complete agreement with direct chemical analysis of the fractions.

The experiments just described have tested conditions in the pancreas of a fasting animal, in which a "steady state" prevails, balancing synthesis and secretion. Further evidence for a role of pellet protein in enzyme synthesis is obtained when rates of uptake in the steady state are compared with  $N^{15}$  incorporations by an actively secreting pancreas or by a pancreas in process of building up its enzyme reserves.

The pancreas can be made to secrete by the ingestion of food, or by the injection of pilocarpine. Under these conditions the stored enzymes are secreted and the enzyme content of the tissue is diminished. If the pancreas is no longer stimulated to secrete, it gradually fills up with enzymes. It is important to know whether the enzymes accumulate in the pancreas simply because secretion slows down, or whether they accumulate also because synthesis is accelerated. Long term isotope incorporation experiments show that the *total synthesis* in an active pancreas is, in fact, greater during a prolonged period of synthesis and secretion than it is in a resting pancreas. In some of our experiments described in a recent paper (18) mice received injections of  $N^{15}$ -glycine every  $1\frac{1}{2}$  hours for 8 hours. One group was fed just before the experiment began and received no more food, so that in these animals the enzyme content of the pancreas gradually increased during the period of the experiment. A second group was fed continuously throughout the experiment providing several cycles of pancreatic synthesis and secretion. The mixed cytoplasmic proteins were examined for their  $N^{15}$  contents 2 hours after the final injection of glycine.  $N^{15}$  contents of liver proteins of the two groups of animals were the same, but in the pancreas they were quite different—0.735 atom per cent excess in those fed before the experiment and 0.330 in those fed continuously (and, it may be added, 0.423 in steady-state animals that were fasting both before and during the experiment). The figures show that more protein was passing through the glands of the continuously fed mice and, therefore, that more synthesis occurred. The duration of the experiment was the same in the two groups and it follows that the rate of synthesis is accelerated by secretion.

The effect of secretion on  $N^{15}$  uptake is also evident in short term experiments. In the resting pancreas the  $N^{15}$  concentration of supernate proteins 30 minutes after glycine injection is 0.067 atom per cent  $N^{15}$  excess. In the secreting pancreas of animals fed immediately before the experiment the corresponding figure is almost 42 per cent higher, 0.095 atom per cent excess. (No difference is found in the  $N^{15}$  contents of mixed tissue or supernate proteins in the livers of fed and fasted animals.) Similarly the  $N^{15}$  concentration in the pancreas pellet protein of animals fed before the experiment is 0.258 atom per cent excess while

that of the pellet from fasting animals is only 0.183. (Again  $N^{15}$  uptakes into liver pellet proteins are identical in the two groups.) Thus, secretion accelerates  $N^{15}$ -glycine incorporation into both pellet and supernate proteins. To the extent that such incorporation represents synthesis, secretion accelerates synthesis. It should be pointed out that this difference in  $N^{15}$  incorporation into the pancreas pellet proteins of fed and fasted animals is a direct demonstration that the pellet protein is concerned, not only with its own replacement or turnover, but with the synthesis of other proteins in the cell. The increased synthesis of digestive enzymes which follows secretion is matched by a higher rate of  $N^{15}$  incorporation into the pellet. No similar differences are observed in liver pellet proteins. This illustrates the advantage of dealing with a gland like the pancreas in the study of the general problem of protein synthesis.

It is known that the administration of pilocarpine to a fasted animal results in copious pancreatic secretion. The effect of pilocarpine injection on  $N^{15}$ -glycine incorporation is currently under investigation and preliminary results are compatible with those obtained in feeding experiments. A related problem has to do with the inhibition of pancreatic secretion by drugs like atropine. This, too, is being investigated using tracer techniques.

Table IV also includes data for the time course of  $N^{15}$  incorporation by mixed tissue protein, supernate and pellet proteins of the liver, and for the mixed tissue and pellet proteins of the kidney. This information is plotted in Fig. 2. In all cases the  $N^{15}$  uptakes into pellet proteins are far greater than the uptakes into supernate or mixed tissue proteins. The over-all protein synthesis in both liver and kidney is quite small compared to that which occurs in the pancreas, and the  $N^{15}$  concentrations observed reflect these differences. A comparison of the time- $N^{15}$  uptake curves of liver and kidney proteins with those obtained from pancreas proteins reveals a major difference; at no time in the period investigated did the  $N^{15}$  concentration in the liver or kidney pellet fall below the  $N^{15}$  concentration in the corresponding mixed tissue or supernate proteins. This difference between the pattern of pancreas protein incorporations on one hand and of liver and kidney uptakes on the other is reasonable if one considers the comparatively small amount of protein synthesis occurring in the latter tissues, and the likelihood that many of the proteins in the mixed tissue or supernate fractions of liver and kidney are relatively inert. Therefore, although the role of the pellet in protein synthesis in liver and kidney is probably the same as it is in the pancreas the detection of its function is not so readily accomplished.

An interesting point emerges when one measures the effect of fasting on glycine- $N^{15}$  uptake in the liver. This is shown in Table V. It can be seen that fasting for periods up to 96 hours leads to a progressive diminution in the rate of  $N^{15}$  incorporation into the mixed tissue, supernate and pellet proteins, and also into the RNA. No such striking effects of fasting are seen in pancreas proteins, although some decline in RNA- $N^{15}$  concentrations is observed.

The evidence given thus far points to a direct role of the pellet protein in

the synthesis of other proteins. In a fine investigation Siekevitz has shown that the incorporation of  $C^{14}$ -alanine into the microsome fraction of liver represents peptide bond formation and, furthermore, that the energy required for this incorporation is derived from oxidative phosphorylation (19). Our work indicates that this incorporation of amino acids into the microsome is a stage in the synthesis of the other proteins of the cell. The evidence presented in this paper is not taken to mean that all the steps in protein synthesis occur in the microsome, but rather that one of the important stages in the process directly involves the ribonucleoprotein of the microsome fraction.

*The Role of Ribonucleic Acid in Protein Synthesis.*—Siekevitz (19) has described an *in vitro* system of microsomes + mitochondria which will incorporate

TABLE V  
Effect of Fasting on  $N^{15}$  Incorporations into Proteins and RNA of Cell Fractions

Tissue	Duration of fast	$N^{15}$ excess 30 min. after glycine administration			
		Mixed protein	Pellet protein	Supernate protein	RNA
	<i>hrs.</i>	<i>atom per cent</i>	<i>atom per cent</i>	<i>atom per cent</i>	<i>atom per cent</i>
Liver	20	0.059	0.120	0.055	0.038
	45	0.052	0.093	0.051	
	72	0.041	0.074	0.040	0.020
	96	0.037	0.061	0.039	0.019
Pancreas	20	0.105	0.228	0.067	0.006
	45	0.138	0.256	0.100	0.010
	72	0.114	0.231	0.072	0.004
	96	0.102	0.237	0.072	0.003

Each animal received 30 mg. of  $N^{15}$ -glycine (33.3 atom per cent excess) in 0.2 ml.  $H_2O$  intraperitoneally at zero time.

radioactive alanine, and he has presented evidence for such incorporation being a measure of peptide bond formation. It occurred to us that such a system is well suited to a study of the role of ribonucleic acid in protein synthesis. More specifically it permits a direct test of the dependency of amino acid incorporation upon the presence or intactness of the nucleic acid in the system. In preliminary experiments using essentially the same procedures described in detail by Siekevitz (19) a rat liver microsome fraction was preincubated with added crystalline ribonuclease for 15 minutes at  $37^\circ$ . A control portion of the microsome preparation was incubated under the same conditions without added enzyme. Following incubation these two fractions were added to sucrose suspensions of mitochondria (which had been kept at  $0^\circ$ ) and the mixtures incubated at  $37^\circ$  for 30 minutes in the presence of DL-alanine-1- $C^{14}$ . The specific activity of the "RNAase-treated" microsome protein was only 46 per cent of

that observed in the control preparation. It is evident that preliminary treatment with RNAase impaired the ability of the system to incorporate alanine. A similar impairment of amino acid incorporation by tissue homogenates is observed when they are incubated in the presence of RNAase. That the incorporations measured do not represent an adsorption of isotopic amino acid is evident from the dependence of the system on the presence of an oxidizable substrate, for in the absence of  $\alpha$ -ketoglutarate no appreciable incorporation of amino acid is observed. To the extent that the incorporation of  $C^{14}$ -alanine by the microsome protein represents protein synthesis, the above experiments show that synthesis is dependent upon the presence of ribonucleic acid in the microsomes.

#### EXPERIMENTAL

Adult animals of the Rockefeller Institute colony of Swiss mice were used in these experiments. Animals which were used for studies of the incorporation of  $N^{15}$ -glycine were first fasted for the periods of time indicated in the tables then injected with glycine and killed at designated intervals after the injection. In experiments designed to study the effect of feeding on isotope uptake the mice were allowed to feed for 30 minutes starting 20 minutes before the injection and were killed 30 minutes after the injection.

Mice used for other studies of the pellet material were fasted only when specifically indicated.

In the isotope experiments injections of  $N^{15}$ -glycine were given intraperitoneally. The animals received 1 mg. glycine (33.3 atom per cent  $N^{15}$  excess) per gm. of body weight.

Animals were killed by decapitation following ether anesthesia; the organs were removed and placed immediately in a container chilled in ice or in an ice cold sucrose solution.

*Preparation of the Pellet Material.*—Samples of pancreas were homogenized for 3  $\frac{1}{2}$  minutes in a glass tube fitted with a teflon pestle in 1 gm. portions with 7 ml. of a solution containing sucrose 0.25 M, citrate 0.01 M, and soy bean trypsin inhibitor 0.1 mg./ml. Citrate was added to inhibit desoxyribonuclease activity, and the soy bean inhibitor to eliminate the activity of traces of active trypsin which might be present.

Small samples of liver tissue were homogenized in 0.25 M sucrose in the same manner as the pancreas, but when larger quantities of tissue were used, it was more convenient to homogenize the tissue in a small metal blender run at 35 v. for 2  $\frac{1}{2}$  minutes as suggested by Petermann *et al.* (20).

Kidney samples were homogenized first in the blender for 2  $\frac{1}{2}$  minutes and then for a few seconds in the teflon homogenizer to break up bits of tissue that were not disintegrated in the blender.

Microscopic examination of these homogenates showed that few of the cells were unbroken. For preparation of the pellet material the homogenates were centrifuged at 5000  $\times$  g for 10 minutes, the residues were washed once with 0.25 M sucrose, and the combined supernatants and washings were centrifuged again for 10 minutes at

5000  $\times$  g, and the final supernatant centrifuged in the Spinco model L ultracentrifuge for 30 minutes at 40,000 R.P.M. (105,400  $\times$  g). The pellet was washed by resuspension in sucrose and centrifuged for 1 hour at 40,000 R.P.M.

In the isotope incorporation experiments the pellet material was then treated with ribonuclease in order to separate the nucleic acid from the protein. The pellet was suspended in water with the addition of a little alkali or buffer to bring the pH to 7.5 and treated with ribonuclease for 1 hour at room temperature. The pellet proteins were precipitated with trichloroacetic acid and washed several times with trichloroacetic acid, hot alcohol, and ether and dried at 110°C. for 10 minutes. When the uptake of  $N^{15}$  into the RNA of the pellet material was to be measured the trichloroacetic acid supernatant, which contained the ribonucleotides, was saved. The nucleotides were separated from the small amount of protein remaining in solution by adsorption on a column of dowex-2 (chloride form). Before adsorption, the solution was extracted with ether to remove the trichloroacetic acid and adjusted to pH 9 with alkali. The solution was passed through the column, the column washed with water and sodium acetate 0.01 N at pH 6, and then nucleotides were eluted with 1 N HCl.

The isotope incorporation into the mixed proteins of the tissue and into the proteins of the supernatant obtained after the first centrifuging at 40,000 R.P.M. was also measured. Samples of the homogenate and supernatants were precipitated with trichloroacetic acid, washed with trichloroacetic acid, alcohol, and ether, and dried at 110°C. for 10 minutes. The samples of supernatant proteins from the experiments described in Table IV, however, were prepared in a slightly different manner. Before precipitation of the proteins with trichloroacetic acid the supernatants were brought to pH 5.4 by the addition of a little acetic acid in order to remove small amounts of nucleoprotein which did not sediment at 40,000 R.P.M.

*$N^{15}$  Analyses.*—The  $N^{15}$  concentrations of the proteins and RNA were determined in the mass spectrometer (Process and Instruments Company model) after the usual conversion of organic nitrogen to gaseous  $N_2$  by Kjeldahl digestion and treatment with hypobromite.  $N^{15}$  concentrations of samples were determined to within  $\pm 2$  per cent (average deviation) and compared with tank nitrogen as standard.

*Chemical Analyses.*—Nitrogen, phosphorus, and RNA contents of the pellet materials were determined on samples prepared as described above except that after the final centrifugation at 40,000 R.P.M. the pellets were washed immediately with hot 95 per cent alcohol several times, then with ether, and finally dried at 110°C. for 10 minutes.

(a) *Nitrogen and Phosphorus.*—After Kjeldahl digestion of the samples the nitrogen content was determined by nesslerization and the phosphorus by Allen's method (21).

(b) *RNA.*—Samples of pellet material were washed once with cold 2 per cent perchloric acid and then heated at 70°C. for 20 minutes to extract the nucleic acid. RNA was estimated by the orcinol reaction, or by the ultraviolet extinction coefficient of the perchloric acid extract. For the pancreas pellet material values for RNA content obtained in this way are in good agreement with the total phosphorus. In the liver pellet preparations there was a certain amount of DNA P (10 per cent of the RNA P): therefore, the orcinol figures for liver pellet are reported in Table III.

*Amino Acid Composition.*—The pellet materials were treated with ribonuclease, and then precipitated and washed with trichloroacetic acid, alcohol, and ether. The proteins were hydrolyzed by refluxing with 6 N HCl for 18 hours, and the basic amino acids determined by the ion exchange procedure of Moore and Stein (22).

*Zone Electrophoresis.*—The pellet material prepared from mouse liver was resuspended in 0.25 M sucrose to give a final nitrogen concentration of 5 mg./ml. To 4 ml. of this suspension was added 1 ml. of 1 M phosphate buffer at pH 7.0. The mixture was carefully poured into a small rectangular well 30 cm. from one end of a moist glass powder slab 70 cm. long, 8 cm. wide, and 1 cm. deep. The preparation of this glass powder and the technique of applying a potential across it are described by Taylor, du Vigneaud, and Kunkel (23). In the experiment pictured in Fig. 1 A the glass powder was suspended in a mixture of 9 parts 0.25 M sucrose and 1 part 1 M phosphate buffer at pH 7.0. The same mixture was used in the electrode vessels. The potential applied across the electrodes was 320 v.—the current passing in the system was 10 milliamperes. Duration of the experiment was 100 hours. In the experiment shown in Fig. 1 B the glass powder was suspended in 0.2 M phosphate buffer at pH 7.0. The applied potential was 320 v.—the current was 12 milliamperes. Duration of the experiment was 100 hours. For purposes of comparison an electrophoretic pattern is shown for a solution of yeast ribonucleic acid (5 mg./ml.). This was a sample of commercial yeast RNA which was purified by acid precipitation and subsequent dialysis. In this case 5 ml. of RNA solution was placed on a starch slab, 70 cm. long, 10 cm. wide, and 1 cm. deep. The applied potential was 320 v.—the current passing in the system was 11 milliamperes. Duration of the experiment was 100 hours.

Following electrophoresis, the slabs were cut at 2 cm. intervals to give 35 zones 2 cm. across by 1 cm. deep by 8 cm. wide. The glass powder in each zone was suspended in 8 ml. cold H<sub>2</sub>O and the suspension was filtered through a coarse sintered glass filter to remove the glass powder. The filtrate was then analyzed in one of two ways: (a) When the filtrate was clear, extinctions at 258 m $\mu$  and at 230 m $\mu$  were determined in the Beckman spectrophotometer. These were used as an indication of nucleic acid and protein distribution. (b) When the filtrate was turbid (this was always the case for zones near the origin), nitrogen analyses were performed. In order to maintain the single parameter,  $E_{258}$ , throughout the plot of the electrophoresis pattern, the nitrogen content thus determined was converted to  $E_{230}$  in the following way. The ratio  $E_{230}/N$  was determined for zones adjacent to the turbid areas but which gave clear filtrates. This ratio was then used to compute the equivalent  $E_{230}$  for turbid zones. The values plotted near the origins of the curves in Fig. 1 were obtained in this way. The values of  $E_{258}$  for turbid solutions were obtained by heating in 5 per cent trichloroacetic acid for 15 minutes at 90°C. to remove protein and release the nucleic acid. After centrifugation  $E_{258}$  was determined on the clear supernate. These values were then corrected for an increase in the  $E_{258}$  caused by a reaction between sucrose and hot 5 per cent trichloroacetic acid.

#### SUMMARY

1. The ribonucleoprotein of the microsome fraction which sediments at 40,000 R.P.M. as a pellet (and which is referred to as the *pellet material*) has been studied with reference to its role in protein synthesis in the pancreas.

2. In pellet material nucleic acid and protein form a definite complex as shown by its electrophoretic behavior and unchanging composition under various conditions.

3. Protein of pellet material is not especially rich in the diamino acids.

4. Evidence is brought forward indicating that the protein component of pellet material takes part in the general process of protein synthesis in the cell.

(a) The well known correlation between quantity of RNA and rate of protein synthesis in a tissue implicates the protein of the pellet material, for most of the RNA in the pancreas and other tissues is in this material.

(b) Uptake of isotopically labelled glycine by the pellet material, confirming results of previous workers, is for short periods greater than in other protein fractions.

(c) Comparing the pellet materials of pancreas, liver, and kidney—three tissues with vastly different rates of protein synthesis, in the sequence given—there is a correlation between the quantity of RNA in the pellet and the rate of protein synthesis in the tissue; a similar correlation between quantity of RNA in the pellet material and rate of  $N^{15}$ -glycine uptake by the protein component of the pellet; and finally, the level of uptake by total protein varies with the tissue and is related to the uptake of  $N^{15}$ -glycine by protein of the pellet.

5. In the pancreas a distinction can be made between proteins synthesized for secretion and the nucleoprotein of the pellet (not found in the secretion) which, however, takes part in the synthetic process, as shown by the fact that the  $N^{15}$  uptake by protein of the pellet is increased when the synthesis of digestive enzymes is stimulated by secretion.

6. The time course of  $N^{15}$  uptake by proteins of the pancreas indicates that pellet protein serves as precursor material in the synthesis of the secretory proteins.

7. Rate of uptake of  $N^{15}$ -glycine by the purines of RNA of the pellet material is not correlated with uptake by the protein.

8. The uptake of  $C^{14}$ -alanine by an *in vitro* system of microsomes + mitochondria is impaired by preincubation of the microsomes with ribonuclease. This is direct experimental evidence for the dependence of protein synthesis upon the presence or intactness of ribonucleic acid in the microsomes.

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