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Protein Synthesis by Beef Pancreas Slices*

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Beef pancreas slices constitute a very active amino acid incorporating system. Rate of uptake of valine-C^{14} and tryptophan-C^{14} into protein indicates that ribosomes are the site of synthesis. Valine-C^{14} has also been shown to be incorporated into the chromatographed proteins trypsinogen, chymotrypsinogen A, and ribonuclease during short (2 and 15 minute) incubations. The specific radioactivities of the latter two purified proteins in the microsomal solubilized portion were much greater than that of total protein in this fraction after a 2-minute incubation. This large difference in specific radioactivity was not found in the ribosomes themselves. The data suggest either that "export" proteins are synthesized many times more rapidly than total protein or that the sites of synthesis of the two types of protein are different.

Recent research has resulted in a marked increase in our understanding of some of the reactions of protein biosynthesis (Harris, 1961). The manner in which individual proteins are synthesized, however, remains unknown. In pursuit of this objective it is essential that the biosynthesis of specific proteins or polypeptides of known amino acid sequence be studied. The pancreas generally is recognized as an organ which is capable of a high rate of protein biosynthesis. In addition, the bovine gland is the source of a number of highly purified proteins (Northrup *et al.*, 1948). The amino acid sequences of several of these proteins (insulin [Sanger and Thompson, 1951, 1953; Ryle *et al.*, 1955], glucagon [Bromer *et al.*, 1957], and RNase [Hirs *et al.*, 1958]) have been determined, and those of the proteolytic zymogens presently are being investigated (Desnuelle, 1960).

In the present work the time course of incorporation of L-valine-C^{14} and DL-tryptophan-C^{14} into the proteins of intracellular fractions of beef pancreas was measured. Some of the factors which affect the rate of incorporation also have been studied. Finally, the uptake of L-valine-C^{14} into the specific proteins trypsinogen, chymotrypsinogen-A, and ribonuclease of two microsomal subfractions has been determined at two time periods.

Experimental Procedures

Bovine pancrease was obtained as soon as possible after the death of the animal and was trimmed and cut into 2-cm cubes and placed in ice-cold Krebs III solution (Krebs, 1950). Holstein tissues were preferred, since they generally are less fatty than those from other breeds, and slicing and homogenization are thereby facilitated. A

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TABLE I

FACTORS AFFECTING INCORPORATION OF C^{14}-VALINE INTO PROTEINS OF BEEF PANCREAS SLICES

Five g of slices were incubated for 1 minute in 30-ml Krebs III medium to which 8.3 μc L-valine-C^{14} was added. The slices were well rinsed and homogenized with 0.25 M sucrose and intracellular fractions obtained as described in the text. Each fraction was submitted to a Schneider (1957) fractionation, and the final residue was dissolved in formic acid and plated and the radioactivity counted. All counts are corrected for self-absorption.

Preincubation Conditions			C^{14} Incorporated in Tissue Fraction (cpm/mg)			
Time[a] (min.)	Storage Temp. (C)	Incubation Temp. (C)	Homogenate	Microsomes	Ribosomes	Microsomal Membranes
5	25	30	24	65	97	50
10	2–4	30	34	112	150	75
15	2–4	30	33	84	118	85
20[b]	2–4	30	45	120	148	90
25	2–4	37	68	215	270	175
30	10–12	30	26	75	105	45
90	2–4	30	14	35	45	30
95	2–4	37	42	120	165	110

[a] Interval between obtaining tissue and start of incubation. [b] This sample was prepared with the Stadie-Riggs slicer.

satisfactory gland readily furnished up to 40 g of slices in individual experiments.

Preincubation Treatment.—Slaughterhouse procedure limited the time between death of the animal and obtaining the tissue to a minimum of 10 minutes. Consequently, the effect of preincubation temperature on the subsequent capacity of the tissue to incorporate amino acids into protein was studied. As indicated by the data in Table I, tissue maintained at room temperature (25°) rapidly declined in its capability in this regard compared to tissue which was immediately chilled.

Slicing and Incubation.—A thin razor blade slicer was constructed according to McIlwain (1961) and was employed for most of this work. Five g of slices were weighed into 30 ml chilled Krebs III buffer contained in a 300 ml tall form beaker. The beaker was agitated in a hot water bath until the solution attained 37°, then the isotopic amino acid was rapidly added and shaking continued in a 37° water bath. At the end of the incubation the flask contents were poured onto a 7-cm Buchner funnel and the slices immediately well rinsed with 3 portions of cold 0.25 M sucrose (100 ml total). The slices were stored in tubes immersed in ice water until homogenized.

Homogenization and Separation of Intracellular Fractions.—The sliced tissue from each incubation was cut into small pieces with scissors and homogenized with 9 volumes of ice-cold 0.25 M sucrose in a Potter-Elvehjem (1936) type homogenizer equipped with a Teflon pestle. Four intracellular fractions were separated by a differential centrifugation procedure modified from that developed for mouse pancreas by Van Lancker and Holtzer (1959); (1) unbroken cells, debris, and nuclei, which were obtained at 600 × *g* for 10 minutes, (2) large granules (zymogen granules, mitochondria), which were sedimented at 15,000 × *g* for 13 minutes, (3) endoplasmic reticulum (microsomal fraction), which was centrifuged at 78,000 × *g* for 43 minutes and was washed once in sucrose, (4) the high-speed supernatant solution.

Preparation of Ribonucleoprotein Particles (Ribosomes).—A variety of procedures have been described for the preparation and isolation of ribosomes from microsomes (Harris, 1961). In the application of these treatments to beef pancreatic microsomes, it was thought advisable to evaluate the effects of detergents and the *p*H of the solution by means of chemical determinations of protein (Nayyar and Glick, 1954), RNA-ribose (Schneider, 1957), and phospholipid-phosphorus (Gomori, 1942). These data are included in an accompanying paper (Dickman *et al.*, 1962). It was found that 0.5% lubrol WX, *p*H 7.6, solubilized appreciable amounts of protein and phospholipid and minimal amounts of RNA. The washed microsomal fractions from the incubated slices were accordingly treated in this manner and the ribonucleoprotein particles centrifuged at 90,000 × *g* for 74 minutes.

Chromatography.—The extracts were prepared for chromatography by the procedure of Hirs *et al.* (1953). The *p*H 5.6 supernatant solution was dialyzed against H_2O for an hour in a Craig-type dialyzer (Craig *et al.*, 1957) and then lyophilized. The residue was dissolved in 3 ml of H_2O and an aliquot was placed on a column of XE-64 resin (0.9 × 30 cm) equilibrated with 0.2 M phosphate buffer, *p*H 6.02 (Keller *et al.*, 1958). It was essential to maintain the column at 4 ± 0.5° to obtain satisfactory resolution and recovery of the major components in the extracts. The proteins were eluted with this buffer and were collected in 1-ml fractions by means of an automatic fraction collector.

Enzyme Assays.—(*a*) Chymotrypsinogens A and B were activated according to Hirs (1953). Under these conditions trypsinogen is converted to an inactive protein. For the positive identifi-

cation of trypsinogen in certain peak fractions it was found that the commercial preparation Clarase could be satisfactorily substituted for the mold protease in the procedure of Nakanishi (1959) to convert trypsinogen to trypsin. (*b*) Proteolytic activity was determined for much of this work by the procedure of Chow and Peticolas (1948), which employs casein as substrate. When more definitive identification was required the specific substrates tosyl arginine methyl ester and benzoyltyrosine ethyl ester for trypsin and chymotrypsin were utilized as described by Hummel (1959). RNase was determined by the procedure of Dickman *et al.* (1956) as modified by Madison (1962) (see also Dickman *et al.*, 1962).

C^{14}-Determination.—(*a*) Crude proteins were subjected to a Schneider-type fractionation (1957) and the final residue dissolved in a small volume of concentrated formic acid. An appropriate aliquot was placed on a tared aluminum planchet and, after evaporation was complete, placed in a Nuclear-Chicago automatic, windowless gasflow counter. All counts have been corrected for self-absorption. (*b*) Aliquots of column eluates were placed directly on planchets, evaporated, and counted as above.

Protein Determination.—The "total protein" was determined by direct weighing after the sample had been submitted to a Schneider (1957) fractionation. This treatment successively removes (1) acid-soluble materials, (2) lipids, and (3) nucleic acids. The residue is considered to consist of protein.

Materials

Crystalline RNase, chymotrypsinogen, and trypsinogen were purchased from Worthington Biochemical Corporation. Clarase was kindly furnished by Miles Laboratories, Clifton, N. J. L-Valine-C^{14} and DL-tryptophan-C^{14} were obtained from the California Corporation. The cation exchange resin XE-64 was purchased from Rohm and Haas and was purified by the procedure of Hirs *et al.* (1953).

Results

The incorporation of L-valine-C^{14} into proteins of various intracellular fractions of beef pancreas slices was first investigated. In agreement with data previously obtained with mouse pancreas (Morris and Dickman, 1960) the microsomal fraction incorporated considerably more valine than any other organelle in the interval 5 to 15 minutes (Fig. 1). The specific activities of the ribonucleoprotein particles at each sampling, however, were significantly less than the activities of the microsomes from which they were prepared. If ribosomes are the site of protein synthesis in pancreas, their specific activity should be higher than that of any other fraction at some time period. Accordingly, shorter incubations were run, with samples being taken at 0.5, 1.0, 2.0, and 5.0 minutes. These data are included in Figure 2 and demonstrate that at 0.5 and 1.0 minutes ribonucleoprotein particles exhibited the highest specific activity of any intracellular fraction. After this time the membranous components (lubrol supernatant) surpassed them. The data from a comparable experiment, showing the incorporation of DL-tryptophan-C^{14}, are presented in Figure 3. Here the specific activity of the lubrol supernatant surpassed that of the ribonucleoprotein particles after 0.5 minute of incubation. These data demonstrate quite conclusively that beef pancreas slices can incorporate amino acids into protein very rapidly. Furthermore, as in other tissues, the ribosomes appear to be the primary site of incorporation. It is also obvious that the newly labeled protein does not remain long in the ribonucleoprotein particles, but is

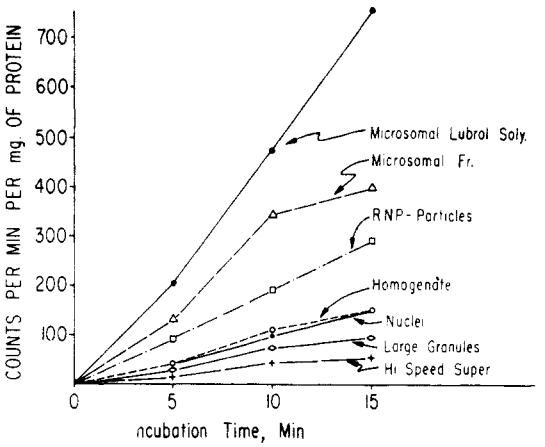

FIG. 1.—Incorporation of L-valine-C^{14} into proteins of intracellular fractions by beef pancreas slices. Five g tissue was incubated at 37° for 5, 10, or 15 minutes in 30 ml Krebs III medium containing 8.3 μc L-valine-C^{14}. The washed slices were homogenized and centrifuged as described in the text. Each fraction was subjected to a Schneider-type extraction (1957). The final residue was dissolved in formic acid and dried and the radioactivity counted. RNP, ribonucleoprotein.

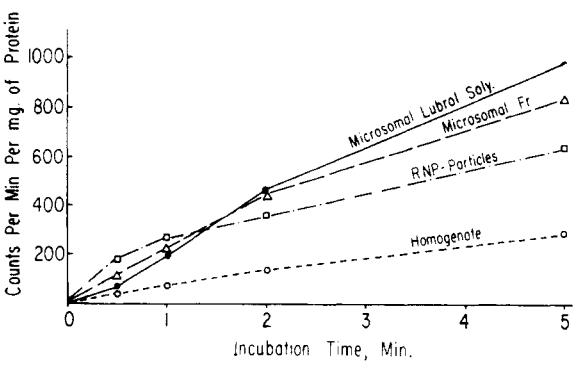

FIG. 2.—Incorporation of L-valine-C^{14} into proteins of microsomal fractions by beef pancreas slices. Incubation conditions and procedures as given in Figure 1. RNP, ribonucleoprotein.

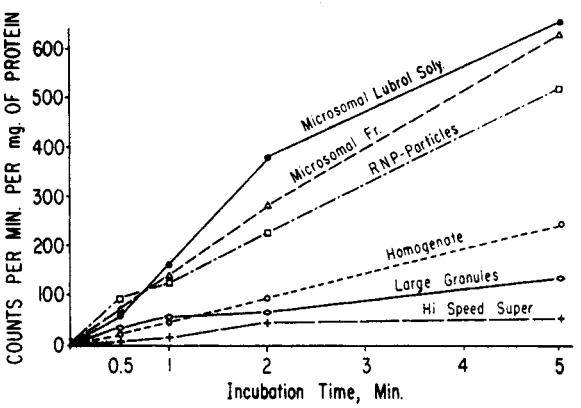

FIG. 3.—Incorporation of DL-tryptophan-C^{14} into proteins of intracellular fractions by beef pancreas slices. Incubation conditions and procedures as given in Figure 1 except that 16.6 μc DL-tryptophan substituted for L-valine-C^{14}. RNP, ribonucleoprotein.

rapidly transferred to the membranous component of the microsomal fraction. In contrast to the data obtained with mouse pancreas (Morris and Dickman, 1960), C^{14}-amino acid was incorporated into protein of the high-speed supernatant fraction less rapidly than into any other intracellular fraction. Beef pancreas slices and guinea pig pancreas are thus similar in their incorporation patterns, although the rate of valine and tryptophan incorporation in the slices exceeds that observed in the guinea pig with leucine (Siekevitz and Palade, 1958).

The treatment of the slices before the incubation was found to exert a considerable effect on the incorporation of labeled valine. The data included in Table I demonstrate that chilling the slices before incubation preserved their incorporation capability to a greater extent than when they were maintained at 25°. As was expected, greater incorporation occurred at 37° incubation temperature than at 30°. Slices obtained by means of the Stadie-Riggs slicer were as active as those secured with the McIlwain thin blade with free hand cutting. After 90 minutes of storage at 2–4°, the tissue retained considerable incorporation capacity.

It was of interest to determine whether a stimulation of tryptophan-C^{14} incorporation into protein could be achieved by the addition of an amino acid mixture and pilocarpine to the incubation medium. As shown in Figure 4, neither addition exerted a significant effect in incubations up to 30 minutes duration. The presence of the amino acid mixture stimulated microsomal incorporation of tryptophan-C^{14} by about one third over the control in a 60-minute incubation. With this supplement incorporation continued at a constant rate for the entire incubation period. The addition of pilocarpine to the medium had little effect on incorporation. Similar relationships were evident in the large granule fraction, except that incorporation was more rapid during the last half of the incubation than in the first half. This result is to be expected if the large granules are the repository of newly synthesized proteins in this tissue.

Experiments were next undertaken to study the incorporation of valine-C^{14} into specific proteins. Incubations were carried out for 2 minutes and for 15 minutes at 37° and ribonucleoprotein particles and lubrol supernatant prepared from each sample. Each solution was treated with H_2SO_4 as described by Hirs *et al.* (1953). The *p*H 5.6 supernatant solution was dialyzed and lyophilized. The residue was dissolved in a small volume of H_2O and chromatographed over XE-64 resin equilibrated with 0.2 M phosphate, *p*H 6.02, at 4° (Keller *et al.*, 1958). The proteins were eluted with the same buffer. The elution patterns are shown in Figures 5 and 6 for 2-minute and 15-minute incubation periods respectively.

Three radioactive peaks are evident in both elution patterns from the ribonucleoprotein particles. The peak at 20-ml elution volume represents chymotrypsinogen A and that at 51 ml, RNase. The large radioactive peak at 16 ml actually represents the protein trypsinogen, which was converted to an enzymically inactive protein under the conditions in which chymotrypsinogen was activated. These peaks also are present in the elution patterns obtained with the microsomal lubrol supernatant solution. In addition, a large

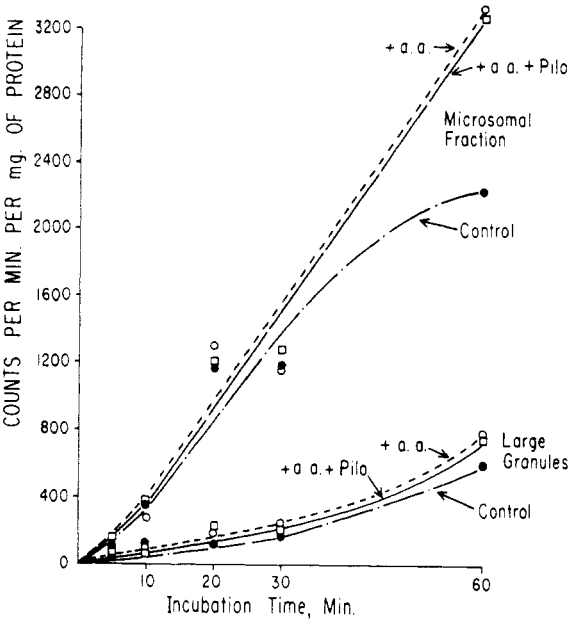

FIG. 4.—Incorporation of DL-tryptophan-C^{14} into proteins of microsomal and large granule fractions by beef pancreas slices. One g tissue was incubated for the period indicated at 37° in 6 ml Krebs III buffer to which 3.2 μc DL-tryptophan-C^{14} was added. The amino acid mixture contained the common L-amino acids with the exceptions of cystine and tryptophan. The final concentration of each amino acid was 0.5 μmole/ml and that of pilocarpine was 10^{-6} M. Further procedures as in Figure 1.

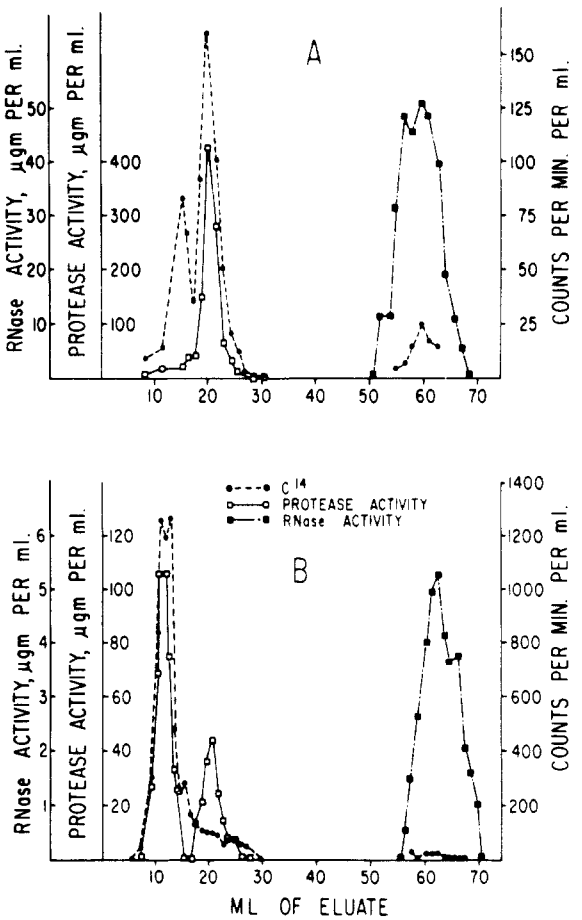

FIG. 5.—Chromatographic elution patterns of microsomal proteins derived from beef pancreas slices incubated 2 minutes with L-valine-C^{14}. Incubation conditions and tissue treatment given in Table II. *A*, ribonucleoprotein particles. *B*, microsomal lubrol-solubilized material.

breakthrough peak is evident in this solution which exhibited both radioactivity and proteolytic activity against casein substrate. The position of this peak suggests that it is composed of acidic proteins. The proteolytic activity probably is due to the presence of chymotrypsinogen B in this portion of the elution volume (Keller *et al.*, 1958). The occurrence of acidic proteins in the lubrol supernatant solution and their absence from the ribonucleoprotein particles is in marked contrast to the distribution of the basic proteins in this and similar systems. Keller and Cohen (1961) previously have demonstrated the adsorption of chymotrypsinogen A by beef pancreas ribosomes, and Madison (1962) has added crystalline pancreatic RNase to rat liver ribosomes and measured a significant adsorption of the added enzyme. This adsorption of basic proteins in pancreas homogenates must occur during the isolation of the microsomal fraction, since this pellet was washed once before the ribonucleoprotein particles were separated from the microsomal membranes by the lubrol treatment.

The similarity in the elution patterns of the 2 and 15 minute samples is marked. The specific activities of the individual proteins, however, demonstrate some striking differences (Table II). There was a large increase in the specific activity of total particle-bound proteins between 2 and 15 minutes. In the lubrol supernatant solution the proteins RNase and chymotrypsinogen A exhibited a much higher specific activity at 2 minutes than did the particles. After 15 minutes, the difference in specific activity between solubilized and particle-bound enzymes was considerably less. It is of interest also that RNase, which had attained as high a specific activity as chymotrypsinogen A in 2 minutes, was not as highly labeled after 15 minutes' incubation as the latter in either fraction. This result is in accord with that of Keller *et al.* (1961), who found the RNase of bovine pancreatic juice to be less highly labeled by C^{14}-arginine than the proteolytic enzymes in the interval 15–60 minutes.

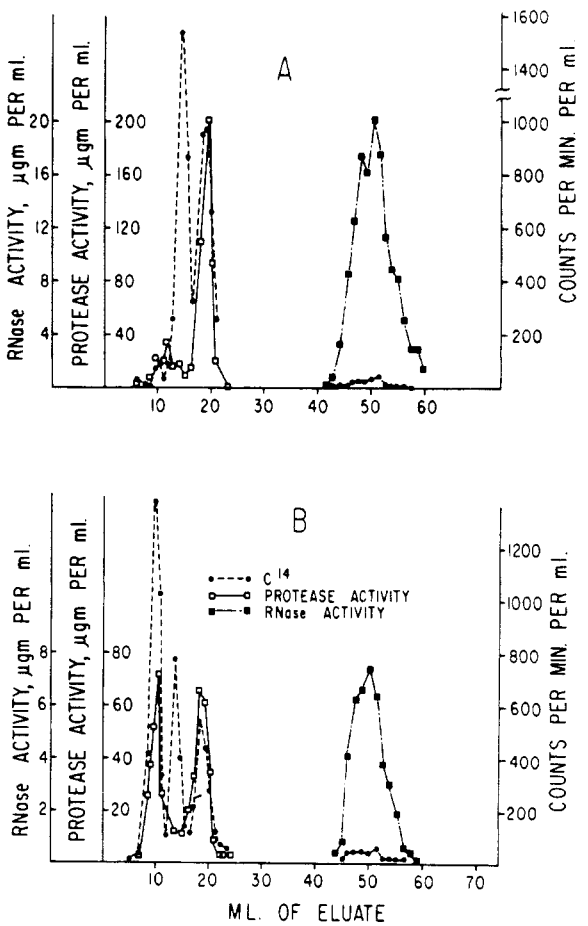

FIG. 6.—Chromatographic elution patterns of microsomal proteins derived from beef pancreas slices incubated 15 min. with L-valine-C^{14}. Incubation conditions and tissue treatment given in Table II. *A*, ribonucleoprotein particles. *B*, microsomal lubrol-solubilized material.

TABLE II

SPECIFIC ACTIVITY OF MICROSOMAL PROTEINS

A total of 20 g of pancreas slices was incubated at 37° for 2 minutes or 15 minutes with 33 μc L-valine-C^{14} in 4 flasks, each containing 30 ml of Krebs III medium. The ribonucleoprotein particles were prepared by treatment of washed microsomal fraction with 0.5% lubrol WX, pH 7.6, followed by centrifugation for 75 minutes at 90,000 × g. The tissue extracts were chromatographed and enzyme activities and C^{14} determined as described in the text. The specific radioactivity was determined for individual points on each peak, and the standard deviation was calculated for a minimum of 4 points.

	Specific Radioactivity (cpm/mg)	
	Incubation Time	
Sample	2 min.	15 min.
Ribonucleoprotein particles		
Total protein	330	1800[a]
Chymotrypsinogen peak	565 ± 193	7000 ± 2100
RNase peak	374 ± 68	2050 ± 460
Microsomal-solubilized		
Total protein	480	3000[a]
Chymotrypsinogen peak	3290 ± 857	7480 ± 575
RNase peak	4980 ± 975	5300 ± 410

[a] Extrapolated value from a 5-minute experiment.

DISCUSSION

Rate of Uptake of Amino Acids.—In a relatively early study of the rate of incorporation of labeled amino acids into rat liver intracellular fractions *in vivo*, Littlefield *et al.* (1955) reported that the deoxycholate-insoluble pellet was most rapidly labeled. The specific activity of these ribonucleoprotein particles attained a maximum in 3 minutes, then rapidly declined. In a similar study in the guinea pig, Siekevitz and Palade (1960) found that these particles from the pancreas were likewise most rapidly labeled by leucine-1-C^{14} and had attained a maximum specific activity in 3 minutes.

Rabinovitch and Olson (1959) demonstrated a very rapid uptake and removal of tracer doses of L-phenylalanine-C^{14} and L-leucine-C^{14} into the microsomal fraction and ribonucleoprotein particles of rabbit reticulocytes *in vitro*. With higher concentrations of L-leucine-C^{14}, however, the specific radioactivity of microsomal proteins was higher after 10 minutes of incubation than after 5 minutes. Thus the shape of the specific radioactivity-time curves of intracellular fractions in any given experiment is largely determined by the concentration of labeled amino acid in the medium. In comparison with these other systems, beef pancreas slices are outstanding in two respects: (*a*) the rate of incorporation of C^{14} amino acids into protein is very high, and (*b*) the interval before the specific activity of the microsomal membranous components surpasses that of the ribonucleoprotein particles is very short. These two associated observations furnish further experimental support to the widely held view that pancreas tissue is capable of a high rate of protein synthesis (Allfrey *et al.*, 1953).

The much higher specific radioactivity at 2 minutes of the individual solubilized enzymes compared to those which were ribosomal-bound is noteworthy. This difference is so much greater than that obtained with total protein that an explanation is called for. The data suggest that the "export" proteins are synthesized and turned over more rapidly in the ribosomes than the ribosomal structural proteins. On the other hand, the possibility that final steps in the synthesis of enzymatically active proteins may take place in association with the membranous components should be carefully evaluated. These results extend those of Siekevitz and Palade (1960), who measured the uptake of leucine-C^{14} into guinea pig pancreatic intracellular fractions, as well as into chromatographed chymotrypsinogen *in vivo*. In their experiments the specific activity of total ribosomal proteins required 3 minutes to reach a maximum, whereas the ribosomal-bound enzyme attained its maximum in 1 minute. It is unfortunate that their value for the specific activity of chymotrypsinogen of the microsomal contents at 1 minute is missing, making other comparisons with our data impossible.

Adsorption of nonlabeled basic proteins from the cell sap by microsomes or ribosomes would tend to lower the specific radioactivity of the bound enzymes to a greater extent than that of the solubilized proteins, especially during the initial phases of an incubation. While the quantitative importance of this factor cannot be calculated from these data, comparable distribution studies of the specific radioactivity of an acidic protein would be very interesting in this connection. The standard deviations of chymotrypsinogen specific radioactivity at 2 minutes are relatively larger than those of RNase. This situation probably is due to C^{14} trailing in this portion of the eluate and suggests that these specific radioactivities are higher than might be found with more purified samples. Further purification and measurement of basic and acidic proteins in short-term incubated samples is continuing.

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Chemical Studies on Amino Acid Acceptor Ribonucleic Acids.* I. Some Properties of Yeast Amino Acid Acceptor Ribonucleic Acid and Mapping of the Oligonucleotides Produced by Ribonuclease Digestion

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In order to investigate the distribution of uracil-5-ribosyl phosphate and of the other "additional" nucleotides in yeast acceptor RNA preparations, digests of the RNA by pancreatic ribonuclease have been fractionated by gel filtration and characterized by fingerprinting. The separation of mixtures of oligonucleotides has been improved by removing terminal phosphate groups. Analysis of a number of oligonucleotides indicates that the "additional" nucleotides of acceptor RNA are not confined to sequences common to all the acceptor RNA molecules. Three dinucleotides containing "additional nucleotides" have also been isolated. Gel filtration and ion-exchange cellulose papers have been used in simplified methods for the determination of the labeling of acceptor RNA with C^{14} amino acids.

Low-molecular-weight acceptor ribonucleic acids have been the subject of recent study both because of their ability to accept activated amino acids in systems carrying out protein synthesis and because of the occurrence in them of nucleotide components other than the four common ribonucleotides (Dunn *et al.*, 1960; Otaka *et al.*, 1959). The presence of these additional nucleotides will be of great value in sequence determination when preparations of a single molecular species of an acceptor RNA[1] are available. Experiments leading toward isolation of

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