Synthesis and Transfer of Amylase in Pigeon Pancreatic Microsomes*

C. M. Redman,† P. Siekevitz, and G. E. Palade
From The Rockefeller University, New York, New York 10021

SUMMARY

A pigeon pancreas microsomal system supplemented with microsomal supernatant fluid or pH 5 enzyme incorporates radioactive amino acids into purified amylase. Several parameters of the incorporation system are presented. Tryptic digestion of the isolated radioactive amylase shows that the amino acids are incorporated into peptide linkage within the protein, as in amylase synthesized in vivo. The radioactive amylase is first found on the attached ribosomes and with time is transferred to the deoxycholate-soluble microsomal subfraction, which corresponds largely to the content of microsomal vesicles. Only about 30% of the released amylase is found as soluble protein in the incubation medium. The system in vitro thus comprises the vectorial mechanism involved in the transfer of newly synthesized secretory protein across the endoplasmic reticulum membrane into the cisternal space.

The exocrine pancreatic cell synthesizes a variety of proenzymes (zymogens) and enzymes for export. The intracellular pathway, from the site of synthesis of the enzymes on attached ribosomes to their storage site in zymogen granules, has been elucidated in part (1). The postulated pathway involves first a transfer of the newly synthesized protein from the ribosomes, through the membranes of the endoplasmic reticulum to the Golgi complex, where they are progressively concentrated into, and finally fill up, the condensing vacuoles of the complex. When completely filled, these vacuoles become mature zymogen granules (2).

To obtain more information on the mechanisms involved in the first step of this process, to wit, the release of nascent protein from the ribosomes and its subsequent passage across the membrane of the endoplasmic reticulum, we have investigated a subcellular system capable of protein synthesis in vitro.

Weiss, Ae, and Lipmann (3) have already obtained in vitro amino acid incorporation into the trichloroacetic acid-insoluble protein of young pigeon pancreatic microsomes, and Dickman and Bruenger (4) and Gazzinelli and Dickman (5) have shown that dog pancreas microsomes and beef pancreas ribosomes can incorporate amino acids into TCA-insoluble proteins, although in the latter system the label was not recovered in a known finished protein (6). It is shown in this paper that a microsomal fraction prepared from pigeon pancreas and consisting primarily of closed vesicles derived from the rough endoplasmic reticulum is capable of incorporating amino acids not only into TCA-insoluble proteins but also into a specific protein, amylase. After short periods of incorporation, most of the labeled amylase is found associated with attached ribosomes, but after longer intervals, the majority of the enzyme is recovered in a deoxycholate-soluble fraction, which represents the microsomal content. The system is apparently capable of transporting newly synthesized secretory protein to the cisternal space.

EXPERIMENTAL PROCEDURE

Materials—dl-Leucine-1-14C, 16 mC per mmole; L-leucine-4,5-3H, 5 C per mmole; and a 14C-algal protein hydrolysate, 1.39 mC per mg, were obtained from New England Nuclear Corporation. Pyruvate kinase was obtained from C. F. Boehringer and Sons. ATP and GTP were purchased from Sigma; sodium phosphoenolpyruvate and deoxycholic acid were purchased from Calbiochem and the Wilson Laboratories, respectively. The latter was converted to the sodium salt at pH 7.8 before use. Hog pancreatic amylase, two times crystallized, was obtained from Worthington.

Preparation of Cell Fractions—Pigeons, 6 to 8 weeks old, were used in these experiments. The animals were killed by decapitation; the pancreatic glands were removed, minced with scissors, and passed through a hand-operated, stainless steel tissue press (1.0-mm holes). The ensuing pulp (1 g) was homogenized in 5 ml of 0.44 M sucrose and centrifuged at 2,000 × g for 15 min. The supernatant solution was diluted with 2 volumes of 0.44 M sucrose and centrifuged at 13,000 × g for 15 min to yield a mitochondrial fraction. The corresponding supernatant was recentrifuged for 90 min at 105,000 × g to obtain a microsomal pellet and a final supernatant. The microsomal pellet was

* This work was supported by United States Public Health Service Grant AM 01635 to P. Siekevitz from the National Institute of Arthritis and Metabolic Diseases. A preliminary report of this work was presented at the meeting of the American Society for Cell Biology, Philadelphia, November 1965.
† Postdoctoral Fellow of the National Institute of General Medical Sciences of the United States Public Health Service.
‡ Present address, Addiction Research Center, National Institute of Mental Health, Lexington, Kentucky 40501.
examine the electron microscope (cf. Reference 7 for preparation procedures) and was found to consist primarily of healed segments of the rough endoplasmic reticulum with about 20% of the vesicles having ruptured membranes (Fig. 1a).

**Incubation Procedures**—The microsomal pellet was resuspended in 0.25 M sucrose, and aliquots corresponding to about 8 mg of microsomal protein were added to each incubation vessel, which contained 1 mg of microsomal supernatant protein, 1 μmole of ATP, 0.5 μmole of GTP, 10 μmole of freshly prepared P-enolpyruvate, 160 μg of pyruvate kinase, 20 μmole of KCl, 5 μmole of mercaptoethanol, 5 μmole of MgCl₂, 50 μmole of Tris-HCl (pH 7.4), and 0.1 μmole of L-leucine-1-³⁴C. The final volume was 1 ml, and all incubations were carried out at 37°C. In some experiments, microsomes were recovered by centrifugation at the end of the incubation procedure and processed for electron microscopy in the same way as freshly isolated microsomal segments of the rough endoplasmic reticulum with about 20% examined in the electron microscope (cf. Reference 7 for preparation procedures) and was found to consist primarily of healed segments of the rough endoplasmic reticulum with about 20% of the vesicles having ruptured membranes (Fig. 1a).

When determining the incorporation of radioactive amino acid into total protein was measured, the reaction was stopped by the addition of an equal amount of 10% TCA, and the precipitated protein was isolated and washed by the method of Stievelitz (8). When the incorporation of radioactive amino acids into total amylase was determined, the reaction was stopped by the addition of DOC to a final concentration of 0.5% and then cold ethanol was added dropwise with constant stirring to a final alcohol concentration of 40%. After 15 min, this mixture was centrifuged at 105,000 × g for 1 hour. The amylase in the 40% alcohol supernatant solution was then precipitated by the addition of glycogen (9). Yields of over 80% could be obtained, however, by repeated precipitation with glycogen when the supernatant of the first precipitate still showed amylase activity.

When determining the incorporation of radioactive amino acid into amylase bound to ribosomes, or that present in the DOC-soluble fraction or released into the incubation medium, the reaction in vitro was stopped by adding 10 ml of cold 0.44 M sucrose, and the microsomes were recovered by centrifugation at 105,000 × g for 1 hour. These microsomes (8 to 10 mg of protein) were resuspended in 1.8 ml of 0.44 M sucrose, treated with 0.2 ml of 5% DOC, and recentrifuged at 105,000 × g for 2 hours to separate the ribosomes from the DOC-soluble fraction. The ribosomal fraction consisted mainly of particles with minimal contamination by residual microsomal membranes (Fig. 2). The sedimented ribosomes were resuspended in 1.2 ml of water. Ethanol extracts were prepared from both microsomal fractions by adding cold ethanol dropwise, with stirring, to a final concentration of 40%. The ribosomal fraction was centrifuged at 105,000 × g for 1 hour, and the DOC-soluble fraction at 14,000 × g for 30 min to remove the ethanol-insoluble protein. Amylase was precipitated from the corresponding supernatant fractions by the addition of glycogen (9). Since the incubation mixture was diluted with 10 ml of 0.44 M sucrose in order to recover the incubated microsomes, the amylase concentration in the medium was low, and low yields were obtained. To remedy this situation, 2,000 units of hog pancreatic amylase were added as carrier to the supernatant fraction obtained after microsome recovery. Cold ethanol was then added slowly, with stirring, to a final concentration of 40%, and the mixture was centrifuged at 105,000 × g for 1 hour. Amylase was isolated from the ensuing supernatant by glycogen precipitation.

**Preparation of Amylase Labeled in Vivo**—Each of two pigeons was treated by intraperitoneal injection with 10 μC of a ¹⁴C-algal protein hydrolysate. The pigeons were killed 2 hours after injection, and the pancreas was removed and homogenized in 30 ml of water. Sufficient absolute ethanol was added dropwise, with stirring, in the cold to make a 40% ethanol mixture which was subsequently centrifuged for 90 min at 105,000 × g. The amylase was isolated from the ensuing supernatant by glycogen precipitation (9).

**Column Chromatography**—The isolated glycogen-amylase complex was suspended in 2 ml of 20 mM phosphate buffer, pH 6.9, containing 7 mM NaCl and 0.5 mM CaCl₂, and the amylase was allowed to digest the glycogen for 2 hours at room temperature. The reaction mixture was then diluted with water to a final phosphate concentration of 5 mM and applied in the cold to a DEAE-cellulose column (1 × 12 cm) previously equilibrated with 5 mM phosphate, pH 6.9, containing 7 mM NaCl and 0.5 mM CaCl₂. The amylase was eluted by gradient elution with the use of 5 mM phosphate buffer (pH 6.9) with 0.5 mM CaCl₂ in the mixing vessel and 0.2 M phosphate with 0.6 mM CaCl₂ in the reservoir. Fractions of approximately 2 ml were collected.

**Trypsin Digestion and Electrophoresis of Peptides**—Amylase labeled either in vitro or in vivo with ¹⁴C-algal protein hydrolysate was denatured by boiling in water for 10 min and oxidized with performic acid (10). Trypsin hydrolysis and partial separation of the peptides were performed according to Campbell and Hochst (11), that is, by electrophoresis on Whatman No. 3MM paper with a pyridine-acetate buffer at pH 6.4.

**Analytical Methods**—Amylase activity was determined by the method of Bernfeld (12). A unit of amylase, as described by Loyter and Schramm (9), is that amount which liberates 1 mg equivalent of maltose in 3 min at 30°C. Protein was determined by the method of Lowry et al. (13) with the use of crystalline serum albumin as standard. Phosphate was measured by the method of Fiske and SubbaRow (14). ³⁴C was counted on planchets in a thin window, gas flow Nuclear-Chicago counter, while tritium was counted in a Packard Tri-Carb liquid scintillation counter with the use of the phosphor described by Bray (15).

**RESULTS**

**Characteristics of Incorporation System**—Previously (3) a pH optimum of 8.1 was reported for the incorporation of radioactive amino acid into TCA-insoluble protein in a similar pigeon pancreas system. Under the conditions used in our laboratory, the incorporation decreased at pH values higher than 7.4 (Fig. 3). There is no activity in the absence of Mg²⁺ (Table I), the optimal concentration of which is 5 mM (Fig. 4). No requirement for a sulfhydryl source was noticed since the addition of mercaptoethanol had no effect (Table I). KCl activates slightly at 10 mM and inhibits at higher concentrations; NH₄Cl gives similar results. An energy source is required for any incorporation of amino acids into protein to occur, and GTP is needed.
FIG. 2. Pellet of ribosomes isolated from a microsomal fraction treated with 0.5% DOC. The pellet consists primarily of ribosomes which occur in chains ($r_1$) or clusters ($r_2$). Contamination by residual membranes is negligible and limited to small, ribosome-free vesicles which occur only at the bottom of the pellet (not shown). $\times 100,000$. The pellet was fixed in situ in 1% OsO$_4$ in 1 M sucrose for approximately 15 hours at about 0° and processed thereafter as indicated in the legend for Fig. 1, a and b.

For optimal incorporation (Table I). The system is saturated with 0.1 mM 1-$^4$C-leucine (Fig. 5). A mixture of amino acids (minus leucine) has no effect on the incorporation of $^4$C-leucine into protein, probably because there are sufficient endogenous amino acids present. The incorporation of leucine-$^4$C into TCA-insoluble protein is linear with increasing amounts of microsomes (Fig. 6) until a concentration of 20 mg of microsomal protein per ml is reached.

While the addition of microsomal supernatant activates the incorporation, the optimal concentration being 1 mg of supernatant protein per 8 mg of microsomal protein, larger amounts for optimal incorporation (Table I). The system is saturated with 0.1 mM 1-$^4$C-leucine (Fig. 5). A mixture of amino acids (minus leucine) has no effect on the incorporation of $^4$C-leucine into protein, probably because there are sufficient endogenous amino acids present. The incorporation of leucine-$^4$C into TCA-insoluble protein is linear with increasing amounts of microsomes (Fig. 6) until a concentration of 20 mg of microsomal protein per ml is reached.

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FIG. 1. a, pellet of microsomes freshly isolated from a pigeon pancreas homogenate. The micrograph shows a small field in the upper half of the pellet in which the microsomal vesicles are well isolated from one another, and most of them appear intact. In the lower half of the pellet (not shown), the microsomes are tightly packed, and many of them are ruptured. In this figure, the microsomal membrane (m) and the attached ribosomes (r) are clearly visible. The apparently free clusters of ribosomes (fr) may represent grazing sections of microsomal vesicles or contamination by free ribosomal particles. The lower half of the pellet is contaminated by negligible amounts of mitochondria and condensing vacuoles (elements of the Golgi complex). Total contamination of the microsomal fraction is estimated at less than 5% of the mass of the entire pellet. $\times 50,000$. b, pellet of pancreatic microsomes (pigeon) recovered from the incorporation system after a 20-min incubation at 37°. The micrograph shows cause a marked inhibition (Fig. 7). The pH 5 enzyme, derived from that amount of microsomal supernatant which causes inhibition, does not by itself inhibit amino acid incorporation into TCA-insoluble protein, but the pH 5 enzyme supernatant, derived from 2 mg of protein of microsomal supernatant, is inhibitory (Fig. 8).

Isolation and Purification of Labeled Amylase—To determine whether leucine was incorporated into a specific secretory protein, amylase was extracted from incubated microsomes and purified. To obtain sufficient radioactivity in the isolated amylase, the volume of the incubation system was increased a small field in the upper half of the pellet in a region comparable to that in a. Most microsomal vesicles appear well preserved; their limiting membrane (m) and attached ribosomes (r) can be easily recognised. Some microsomes appear swollen; others show invaginations (i) or evaginations (e) of their membrane; finally, a few are ruptured (arrows). The percentage of ruptured microsomal vesicles is considerably higher in the lower half of the pellet. The contaminants of the fraction are little affected by the incubation. $\times 50,000$. Both pellets were fixed in situ in 1% OsO$_4$ in 0.1 M phosphate buffer, pH 7.4, for approximately 15 hours at about 0°, postfixed in 0.5% uranyl acetate for 2 hours at approximately 25°, dehydrated in ethanol, and embedded in Epon. The sections were doubly stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop I electron microscope.
Incorporation in vitro of 1-\(^{14}\)C-leucine into pigeon pancreatic microsomal protein

The complete system contained 10 mg of microsomal protein, 1 mg of supernatant protein, 1 \(\mu\) mole of ATP, 0.5 \(\mu\) mole of GTP, 10 moles of \(^{23}\)P-enolpyruvate, 166 pg of pyruvate kinase, 20 \(\mu\) moles of KCl, 5 \(\mu\) moles of 2-mercaptoethanol, 5 \(\mu\) moles of MgCl\(_2\), 50 \(\mu\) moles of Tris at pH 7.4, and 0.1 \(\mu\) mole of 1-\(^{14}\)C-leucine (16 \(\mu\)Ci per \(\mu\) mole), in a final volume of 1 ml. Incubation was conducted at 37° for 20 min. The ATP-generative system consisted of ATP, \(^{23}\)P-enolpyruvate, and pyruvate kinase.

<table>
<thead>
<tr>
<th>Systems</th>
<th>Incorporation of 1-(^{14})C-leucine (cpm/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, boiled microsomes</td>
<td>6</td>
</tr>
<tr>
<td>Complete system</td>
<td>306</td>
</tr>
<tr>
<td>Omissions</td>
<td></td>
</tr>
<tr>
<td>ATP-generative system</td>
<td>5</td>
</tr>
<tr>
<td>GTP</td>
<td>206</td>
</tr>
<tr>
<td>KCl</td>
<td>276</td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>307</td>
</tr>
<tr>
<td>Microsomal supernatant</td>
<td>123</td>
</tr>
</tbody>
</table>

10-fold over that described in “Experimental Procedure.” Although easily countable, the radioactivity in the isolated amylase was only 0.5 to 1.5% of the incorporation into the total microsomal protein, and its specific activity (counts per min per mg of protein) was only slightly higher than that of the total TCA-precipitable protein (Table II).

Loyter and Schramm (9) have shown that the specific enzymatic activity of amylase isolated by their glycogen precipitation method from pancreatin and rat parotid was as high as that of the crystalline enzyme. Hence, the radioactivity in the amylase isolated by us is probably due to leucine-1-\(^{14}\)C incorporated into amylase molecules. Yet it is conceivable that a small amount of highly radioactive contaminant coprecipitates with the amylase-glycogen complex. To test this possibility, the isolated amylase was carried through another step of purification, namely, chromatography on DEAE-cellulose. Both the specific enzymatic activity and specific radioactivity of the amylase did not change with chromatography (Table III), and radioactivity, amylase activity, and protein concentration were eluted together from the column (Fig. 9). Hence we assume that the system in vitro is capable of incorporating amino acids into a finished protein, amylase.

Comparison between Amylase Synthesized in Vivo and in Vitro—To determine whether the radioactive amino acid was incorporated into peptide links throughout the protein chain, a trypsin digest of amylase labeled in vitro was prepared as previously described, except that a \(^{14}\)C-algal protein hydrolysate was used as the radioactive precursor instead of leucine-1-\(^{14}\)C. This trypsin digest was compared to a trypsin digest of amylase labeled in vivo by injecting a \(^{14}\)C-algal protein hydrolysate intraperitoneally and isolating the pancreatic amylase 2 hours after injection. In each case, the isolated amylase was digested with trypsin, and the peptides were partially separated by paper electrophoresis as described in “Experimental Procedure.” However, the low radioactivity of amylase made possible only a partial separation. Autoradiography was performed with the use of Kodak x-ray “no screen” film, with an exposure time of 6 weeks. A long, dark, radioactive spot was seen running toward the anode, while toward the cathode there was a small, discrete spot close to the point of origin and other very faint spots which migrated further. When the separated peptides were localized with ninhydrin stain, it was seen that all the ninhydrin-positive regions corresponded to radioactive spots, but the anionic peptides had little radioactivity. The patterns of radioactive and ninhydrin-positive peptides obtained from the amylase labeled in vivo and in vitro were very similar to each other.

Site of Synthesis and Subsequent Transport of Amylase—To determine the relation of newly synthesized amylase to microsomal structure as a function of time, the microsome system was separated at chosen intervals after the beginning of incubation.
Fig. 6 (left). Effect of increasing amounts of pancreatic microsomes on labeling of TCA-insoluble protein. Conditions were as described under "Experimental Procedure," except that the amount of microsomes per vessel was varied. The sucrose concentration was kept constant.

Fig. 7 (right). Effect of postmicrosomal supernatant on the incorporation of 1-14C-leucine into the TCA-insoluble protein of pigeon pancreatic microsomes. Incubation was carried out for 20 min as described in "Experimental Procedure," except that the amounts of supernatant per tube were varied. The concentrations of sucrose in each vessel were kept constant.

Table II
Incorporation in vitro of dl-leucine-1-14C into amylase by pancreatic microsomes

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Incorporation of dl-leucine-1-14C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td>Microsomes</td>
<td>mg  units/mg cpm</td>
</tr>
<tr>
<td>Amylase-glycogen precipitate</td>
<td>264  19</td>
</tr>
</tbody>
</table>

Table III
Specific activity of amylase labeled in vitro before and after DEAE-cellulose chromatography

The incubation of the microsomes and the isolation of amylase were carried out as described in "Experimental Procedure" except that dl-leucine-4,5-3H (5 C per mmole) was used at a concentration of 0.04 mM, and the volume of the incubation mixture was increased 10-fold. Amylase was isolated as the amylase-glycogen precipitate as described in "Experimental Procedure."

Time of measurement | Amylase activity | Protein | Total radioactivity | Specific activity of amylase |
---------------------|-----------------|---------|---------------------|-----------------------------|
Before DEAE-cellulose chromatography | 766  0.695 | 3220 | 1100  4.2 | 4640 |
After DEAE-cellulose chromatography | 430  0.461 | 1088 | 935  4.3 | 4320 |
The specific radioactivity of amylase is higher in ribosomes than in any other fraction at all intervals examined. For instance, after 10 min of incubation, the corresponding values were 29,400 cpm per mg of amylase and 1,370 cpm per mg of amylase for the ribosome and the DOC-soluble fraction, respectively. The specific radioactivity of the amylase in the DOC-soluble (microsome content + solubilized membrane) increases continuously with time, while that of the ribosomal enzyme remains unchanged after 3 min.

The total radioactivity of the mixed microsomal proteins followed a different pattern. The ribosomes retained 90% of the counts even after 10 min of incubation. From the small percentage of radioactivity released (approximately 10%), more was recovered in the DOC-soluble fraction than in the final supernatant fraction. The discrepancy could be explained by assuming that only completed protein molecules are released into the cavities of microsomal vesicles, whereas unfinished molecules remain attached to the ribosomes and contribute substantially to their total radioactivity.

Since the DOC-soluble fraction represents both the content and the solubilized membrane of the microsomes, a series of attempts was made to separate one from the other in order to clarify the location of amylase after longer incubation intervals. The sonic treatment of microsomes recovered from the incubation medium was not successful since it also released the ribosomal enzyme. Hypotonic shock of the same preparation released a variable, usually large amount of amylase and did not cause the loss of ribosomal enzyme, but such treatment had the

The inherent disadvantages of diluting the amylase of the ribosomal content and rendering its recovery difficult. Microsomal fractionation at low DOC concentrations, according to the technique developed by Ernster, Siekevitz, and Palade (16) for liver microsomes, gave less satisfactory results with pancreatic microsomes. It allowed, however, at least a partial separation of membranes from content. Results obtained with this procedure are shown in Table IV. The ribosomal pellet has an RNA to protein ratio of 0.64 and only 10% of the microsomal phospholipid. The loose

![Figure 9](http://www.jbc.org/) Chromatography of amylase labeled in vitro on DEAE-cellulose. The incubation of the microsomes and the isolation, chromatography, counting, and assay of the amylase were done as described in "Experimental Procedure."

![Figure 10](http://www.jbc.org/) Time course of incorporation of L-leucine-4,5-3H into amylase and total proteins of ribosomes, DOC-soluble fraction, and incubation media. The incubations were as described under "Experimental Procedure," except that 0.04 mM L-leucine-4,5-3H (5 Ci per mmole) was used as label. At the end of the incubation, the system was fractionated, and the amylase in each fraction was isolated and counted as previously described. Total protein is that protein which precipitates with 5% TCA.

**Table IV**

**Distribution of radioactive amylase in microsomal subfractions after 5-min incubation in vitro**

<table>
<thead>
<tr>
<th>Microsomal subfractions</th>
<th>RNA (mg)</th>
<th>Phospholipid (mg)</th>
<th>Protein (mg)</th>
<th>Radioactive protein (cpm)</th>
<th>Amylase (units)</th>
<th>Radioactive amylase (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal pellet</td>
<td>12.3</td>
<td>1.3</td>
<td>19.2</td>
<td>51,800</td>
<td>19.5</td>
<td>53</td>
</tr>
<tr>
<td>Sedimentable membrane fraction</td>
<td>2.2</td>
<td>4.2</td>
<td>8.6</td>
<td>6,120</td>
<td>10.4</td>
<td>13</td>
</tr>
<tr>
<td>Clear supernatant</td>
<td>0.7</td>
<td>8.4</td>
<td>26.6</td>
<td>3,050</td>
<td>696.0</td>
<td>150</td>
</tr>
</tbody>
</table>

Pigeon pancreatic microsomes were incubated as previously described with leucine-1,4-C for 5 min at 37°C. The incubated microsomes were subfractioned according to the method of Ernster, Siekevitz, and Palade (16).
membrane layer, above the ribosomal pellet, has 30% of the phospholipid and 15% of the microsomal RNA. Both of these subfractions have little amylase. The clear supernatant, which represents the contents of microsomal vesicles, has the majority of the endogenous amylase, but it also contains 60% of the microsomal phospholipid, presumably in micellar form. The majority of the radioactive amylase is in the clear supernatant fraction, 27% is attached to ribosomes, and only 7% is found in the membrane fraction. Since the clear supernatant contains, in addition to approximately 65% of the radioactive amylase, about 60% of the microsomal membrane in DOC-soluble form, the possibility must be considered that the enzyme was in the membrane rather than in the content of the microsomal vesicles at the end of incubation. But the small amount of radioactive amylase and radioactive protein in the sedimentable membrane fraction makes this possibility unlikely, especially since the small amount of radioactivity found in this fraction could be ascribed to residual ribosomes. Again, as previously mentioned (Fig. 10), most of the TCA-precipitable protein separates with the ribosomes, and only a small percentage is recovered in the clear supernatant. The experiment suggests that the secretory protein amylase is released from the ribosomes upon completion and appears in the content of the microsomal vesicles, without being attached to, or otherwise retained within, the microsomal membrane.

**DISCUSSION**

There are only few reports concerning pancreatic microsomal systems capable of incorporation in *vitro* of amino acids into proteins (mixed microsomal proteins). Weiss et al. described such a system based on pancreatic microsomes isolated from young pigeons (3), and Dickman and Brienger (4) and Gazzinelli and Dickman (5, 6) subsequently found that dog pancreatic microsomes and beef pancreatic ribosomes can label protein with radioactive amino acids. The high amounts of RNase generally present in the pancreas were assumed to account for the usual lack of activity; for instance, beef pancreatic ribosomes had to be repeatedly washed free of RNase before showing any activity in incorporation (5). The pigeons used in this study were between 6 and 8 weeks old, and the pancreas of each contained 5 to 7 \( \mu \)g of RNase per g of pancreas, wet weight, which is nearly 100 times less than the pancreas of the adult guinea pig. The inhibitory effect of high concentrations of final supernatant protein (Fig. 7) may be due to the fact that approximately one-fifth of the pigeon pancreatic RNase is found in the microsomal supernatant. The pH 5 enzyme, which contains little RNase, did not inhibit incorporation, while the supernatant of the pH 5 enzyme, which does contain RNase, proved inhibitory. These results support the view that incubation with fractions rich in RNase damage the protein-synthesizing capability of the microsomes and provide an explanation for the fact that concentrated pancreatic homogenates are not capable of incorporating amino acids into proteins. Synthesis in *vitro* of amylase has been investigated by Straub, Ulmann, and Venetianer (17, 18), who were unable to obtain glycine-\(^{14}\)C incorporation into the enzyme in a cell-free system derived from pigeon pancreas, but who obtained positive results by reincubating a cell-free system prepared from pancreatic slices previously incubated with the same label. To explain their findings Straub (17) has postulated that the microsomes produce an amylase precursor which is converted into the finished enzyme in the mitochondria. Our results do not support this postulate because we have shown that our microsomal fractions, which consist almost exclusively of microsomes (Fig. 1, a and b), are capable of producing labeled amylase.

After short labeling, the enzyme with the highest specific radioactivity is found on the ribosomes, which are thus identified as the site of synthesis of this exportable protein. Nonspecific adsorption of soluble amylase on the ribosomes can be ruled out as an important factor in the interpretation of our results, since the enzyme present in the other fractions of the incubating system has lower specific radioactivity. The results obtained *in vitro* with our system confirm the earlier findings of Siekevitz and Palade (19), who showed that a pancreatic enzyme, \( \alpha \)-chymotrypsinogen, is synthesized *in vivo* on attached ribosomes.

The system described is capable of incorporating amino acids internally into the polypeptide chain of amylase as shown by the investigation of the trypptic digests of the isolated and purified enzyme. The peptides separated from these digests were radioactive and gave a similar labeling pattern for the enzyme labeled *in vivo* and *in vitro*. Complete separation of trypptic peptides was not achieved, however, primarily because of the low specific radioactivity of the enzyme. Hence, we cannot determine whether the system is capable of initiating the synthesis of new enzyme, or of completing only those molecules for which synthesis has been started *in vivo*.

In addition to amylase synthesis, the pancreatic microsomal system described is capable of transporting a large fraction (approximately 52%) of the enzyme produced from ribosomes to DOC-soluble fraction, which represents mainly the microsomal content. Little or no enzyme activity seems to be associated with the microsomal membrane proper, but about 30% of the enzyme released is found in the incubating medium. Since we have shown that the microsomal fraction used is not significantly contaminated by other components, it follows that the isolated microsomes, supplemented by pH 5 enzyme, contain all the equipment needed to synthesize protein on the attached ribosomes and to transfer this protein across the endoplasmic reticulum membrane into the lumen of the microsomal vesicles, *i.e.* the equivalent of the cisternal space *in situ*.

The significance of the enzyme released in the medium is difficult to assess. It may represent an artifact due to the presence of a relatively large percentage (approximately 25% by the end of the incubation) of ruptured microsomal vesicles in our preparations. Enzyme transported across the membrane of such vesicles is expected to diffuse into the medium. It should be pointed out, however, that in cell fractionation studies of pigeon pancreas, about 30% of the total amylase of the homogenate is found in the postmicrosomal supernatant, and that there is no agreement about the significance of this finding; it has been ascribed to leakage from open ducts, broken zymogen granules, and ruptured microsomes, and, conversely, it has been taken to represent the actual distribution of the enzyme *in situ*. The situation is further complicated by the fact that the system contains a large amount of nonradioactive, endogenous amylase introduced by the microsomal content and by added supernatant protein. The latter is expected to appear in the medium upon fractionation of the incubation system. The specific radioactivity of the amylase in the 0.5% DOC-soluble fraction is 2.2 times higher than that of the amylase in the medium at the end of 10 min of incubation, but under the conditions mentioned it cannot be decided whether or not the enzyme in
the medium comes from the microsomal content. We can conclude, however, that the newly formed amylase is not first released into the medium and then taken up by the vesicles, for in that case the specific activity of the enzyme in the medium would be higher than that of the enzyme in the 0.5% DOC-soluble fraction.

Taken together, our results support the conclusion that protein newly synthesized for export by attached ribosomes is unidirectionally released across the membrane of the endoplasmic reticulum into the cisternal space. They also indicate that the system in vitro contains, still intact, the mechanism involved in the transfer and release of such proteins.

REFERENCES

Synthesis and Transfer of Amylase in Pigeon Pancreatic Microsomes
C. M. Redman, P. Siekevitz and G. E. Palade


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