Biosynthesis of Ribonuclease in Mouse Pancreas*

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Investigations on the mechanism of protein synthesis can be classified according to (a) whether an isotopic amino acid is incorporated into a specific protein or into an undefined mixture of proteins, and (b) whether or not a net increase in protein has occurred. It is obvious that for a thorough understanding of the process, the net synthesis of a specific protein must ultimately be studied and the intermediates identified. In the investigations reported here the incorporation of L-valine-Cl4 into the pancreatic ribonuclease of the living mouse under conditions of rapid synthesis of this protein has been followed. The data have furnished information concerning the site of formation of ribonuclease in pancreatic cells and on the transfer of the newly formed protein within the cell. An estimation of the time required for the synthesis of a completed, enzymatically active protein molecule is also included.

EXPERIMENTAL

Treatment of Animals—An equal number of adult male and female mice (inbred strain, CBA) were fasted for 3 hours. Each animal was then given an intraperitoneal injection of 0.1 ml of freshly prepared aqueous pilocarpine hydrochloride solution (10 mg per ml). One hour later the mice were offered Rockland mouse pellets and water ad libitum. At specific intervals following pilocarpine injection each mouse was killed by snapping the spinal cord, and the pancreas was removed, weighed, and homogenized in cold 0.25 M sucrose.

In other groups exactly 18 hours after the pilocarpine had been injected, each mouse was given an intraperitoneal injection of 2 μ of uniformly labeled L-valine-Cl4 (8.0 μc per μmole, Nuclear Instrument and Chemical Corporation) in 0.1 ml of distilled water. The animals were then sacrificed at a specific interval.

Cell Fractionation Procedure—Thirty mice were injected with pilocarpine hydrochloride and radioactive valine as described above. The mice were killed at a specific interval following valine-Cl4 injection, and the pancreatic tissue was pooled and homogenized in 26 to 29 ml of cold 0.25 M sucrose. The homogenate was fractionated by differential centrifugation at 1° by a modification of the procedure of Van Lancker and Holtzer (3).

A nuclei fraction was separated by centrifuging the homogenate for 10 minutes at 600 × g. The pellets were washed three times by resuspension in 5 to 7 ml of sucrose solution. The combined washings and cytoplasmic fraction were centrifuged for 16 minutes at 11,125 × g to yield a combined zymogen granule and mitochondria pellet which was washed twice by resuspension in 3 to 5 ml of sucrose solution. Previous work has indicated that the mitochondrial portion of mouse pancreatic cells contains only minimal amounts of ribonuclease (4). A microsomal fraction was prepared by centrifuging the washes and supernatant from the zymogen granule fraction for 30 minutes at 105,000 × g. The microsomal pellet was washed twice by resuspension in 9 ml of sucrose. The resulting supernatant solution and washes are referred to as Supernatant1. In one instance the Supernatant1 from a study of mouse pancreas 15 minutes after isotope injection was further fractionated to yield two postmicrosomal fractions and a high speed supernatant (Supernatant2). These fractions were obtained by two centrifugations of the Supernatant1 fraction for 60 and 240 minutes, respectively, at 105,000 × g.

Extraction and Chromatography of Ribonuclease—A modification of the extraction procedure of Hirs et al. (5) was developed in order to prepare the ribonuclease of homogenates and cell fractions for chromatography. The homogenate or cell fraction was mixed with cold, dilute H2SO4 to a final concentration of 0.25 M. After standing for a few minutes at 0° the mixture was centrifuged at 600 × g for 10 minutes, and the supernatant was decanted. The solution was then adjusted to pH 5.7 with dilute sodium hydroxide and allowed to stand for 10 minutes. After centrifugation, the clear supernatant was decanted and used in the chromatographic procedures. For certain of the cell fractions, especially nuclei and supernatant solution, further purification was necessary. The pH 5.7 supernatant was passed through a 1.2 × 10 cm column containing XE-64 resin at pH 6.47. The extract was allowed to seep into the resin bed and was eluted with 60 ml of 0.2 M phosphate buffer at pH 6.47. A large portion of the impurities remained in the resin during this procedure while ribonuclease was eluted. The eluate was then dialyzed for approximately 7 hours against 20 liters of 0.001 M ethylenediaminetetraacetic acid, pH 7.0, in a constant flow rocking dialyzer. The dialyzed extract was lyophilized and the residue was then dissolved in a small volume of water and chromatographed on a 0.9 × 30 cm column of XE-64 resin at pH 6.47 (5).

Analysis of Pancreatic Components and Column Eluates—Ribonuclease activity of homogenates and column eluates was determined by the procedure of Dickman et al. at pH 5.0 (6). A unit of activity is defined as the difference between the experimental absorbancy and the inactive enzyme control (A280), multiplied by the dilution factor. Amylase activity was determined by the
Hokin modification of the Smith and Roe assay (7). Trichloroacetic acid precipitable protein was estimated by micro-Kjeldahl (8) analysis following extraction of the nucleic acids according to the procedure of Schneider (9), or total protein was determined by the procedure of Nayyar and Glick (10). The RNA concentration of the extract was determined by the orcinol method (8) and DNA by the cysteine-sulfuric acid method (11).

Column eluates were counted for C14 by plating 0.5 ml of the solution on a tared aluminum planchet. The solution was then dried under an infrared lamp. Radioactivity of the dried samples was determined with an automatic windowless gas flow counter. All values are corrected for self-absorption and background radiation. Each sample was counted for at least 2560 total counts.

The specific activity of the eluted ribonuclease is defined as the ratio of radioactivity to enzymatic activity:

\[
\text{Counts per min (0.5-ml eluate)} / A_{260} \times 100
\]

**RESULTS**

*Effects of Pilocarpine Injection on Pancreatic Components—*

Harper and Mackay (12) have shown that the exocrine secretion of the pancreas is controlled by two types of factors: hormonal and nervous. Parasympathomimetic agents such as pilocarpine and carbamylcholine have been used extensively to deplete the acinar tissue of zymogen granules and secretory enzymes both in vivo and in vitro (12-21). Whereas injection of pilocarpine into mice or rats results in maximal depletion of the zymogen granules and secretory enzymes of the exocrine pancreas in 1 to 3 hours (13, 16, 18), considerable variations in the rate of restoration of these components have been reported (19, 20, 22, 23).

The data of Fig. 1 show that the response of the pancreas following pilocarpine injection can be divided into four phases: (a) the initial drop in concentration of components due to extrusion of the zymogen granules (0 to 1 hour); (b) a period of relative constancy during which continued secretion is approximately balanced by synthesis (1 to 15 hours); (c) the recovery period in which synthesis exceeds secretion (15 to 21 hours); and (d) reversion toward steady state concentrations (21 to 24 hours). During Phase 1 amylase activity decreased by 55%, ribonuclease activity by 33% and total protein by approximately 10%. It is known from previous work that the zymogen granules contain a larger proportion of the total amylase than of ribonuclease (3, 4). Thus, the larger decrease of amylase activity on pilocarpine stimulation is not unexpected. The relatively small loss in total protein, measured by two different methods, indicates that the zymogen granules comprise only a small proportion of the total exocrine cell protein. It is interesting to note that the three constituents which were measured in this experiment return to the original values at different rates. Amylase activity recovered in about 10 hours, protein concentration in 7 or 18 hours, depending on the method of measurement, whereas ribonuclease activity required 21 hours.

The large overshoot in amylase activity at 21 hours is striking. Whether this phenomenon involves a physiological conversion of "bound" to "active" amylase in the microsome fraction, similar to that observed by Douglas and Munro (24) after various treatments of pigeon pancreas microsomes, cannot be decided from these data. These figures also suggest that an accelerated rate of protein synthesis commences immediately after injection and continues for 21 hours.

The concentrations of deoxyribonucleic acid and ribonucleic acid remained relatively constant throughout the experimental period (Table 1). The constancy of DNA indicates that variations in the water content or of solids in the pancreas were not responsible for the changes in enzyme activities shown in Fig. 1. The constancy of RNA composition provides further evidence for the view that net protein synthesis can occur independently of net RNA synthesis in the pancreas (20, 25). These data confirm other workers who have performed similar measurements on mouse pancreas after pilocarpine stimulation (16, 17, 20).

Studies were undertaken to determine whether a labeled amino acid could be introduced into pancreatic ribonuclease during conditions of net synthesis. Eighteen hours after pilocarpine treatment, nine mice were each given an intraperitoneal injection of 2 μc of uniformly labeled L-valine-C14. Two hours later the mice were killed, and the pancreas was extracted. The specific activity of the eluted ribonuclease is defined as:

\[
\text{counts per min (0.5 ml eluate)} / A_{260} (0.1 ml eluate) \times 100
\]

1 This definition of specific activity utilizes enzyme activity instead of mass in the denominator of the equation. This modification is justified by the previous demonstration that enzyme activity is directly proportional to the weight of ribonuclease (6).
were killed, the pancreatic tissue was removed and homogenized, and the ribonuclease was extracted according to Hirs et al. (5). The extract was chromatographed on XE-64 resin and the eluate fractions were analyzed for both ribonuclease activity and radioactivity. As shown in Fig. 2 most of the C\(^{14}\) was eluted rapidly. In the ribonuclease Peak III area (4, 25), however, the C\(^{14}\) elution pattern is essentially superimposable on that of ribonuclease activity. From these data the specific activity of ribonuclease Peak III can be calculated.

For the correct interpretation of specific activity data, however, it was necessary to determine whether ribonuclease "pool sizes" of the different intracellular fractions were varying during the course of the experiment. Consequently the ribonuclease activity of each fraction at 5, 10, 15, 30, and 120 minutes after valine-C\(^{14}\) injection was determined and these results are presented in Table II. The data indicate that the ribonuclease concentration of each fraction and of the total homogenate remained relatively constant throughout the experimental period.

The total radioactivity of each homogenate and cell fraction was also determined. Thus the rate of appearance of radioactivity in the pancreatic tissue could be plotted. As shown in Fig. 3 an appreciable quantity of radioactivity reached the pancreas during the first 5-minute interval. Maximal radioactivity of the homogenate was observed 10 minutes after injection and the C\(^{14}\) content remained fairly constant during the next 20 minutes.

Two hours after injection, total radioactivity in the pancreas fell to less than one-half of the maximal value.

It is interesting to note that approximately 10% of the total injected dose of radioactivity was found in the pancreas after 10 minutes. The pancreatic tissue of a mouse weighs approximately 125 mg and the total body weight of an adult mouse is 20 to 25 g. It can thus be calculated that the pancreatic tissue has concentrated the radioactivity 20-fold. Farber and Sidransky (18) have observed that uptake of L-valine-C\(^{14}\) in vitro by rat pancreas is doubled by pilocarpine treatment.

Most of the radioactivity incorporated into the pancreas during the first 10 minutes was present in the microsomal portion of the cell. Fifteen minutes after injection, however, as well as in the later periods, the supernatant fraction contained a greater proportion of the radioactivity than any other cell fraction. The nuclei and zymogen granule fractions increased slowly in total radioactivity during the first 30 minutes.

The amount of L-valine-C\(^{14}\) which was present in the ribonuclease of each cell fraction after different intervals was determined after chromatography on XE-64 resin. Acid-treated extracts of mouse pancreas exhibit two peaks of ribonuclease activity which have been designated as Peak II and Peak III in their order of elution from the column (4). Since Peak II has been shown to be nonhomogeneous (4), it has been ignored in this work. Specific activity of Peak III has been calculated as the ratio of valine-C\(^{14}\) (c.p.m.) per unit of ribonuclease activity. A standard deviation has been calculated for each specific activity by individual analysis of each eluate tube containing sufficient enzymatic activity to give an \(A_{260}\) value of 0.1 or higher (26).

The most striking feature of the data included in Fig. 4 is the rapid labeling of the microsomal ribonuclease. The radioactivity found in this fraction was already appreciable 5 minutes after injection of the label. At that time the ribonuclease of the other cell fractions contained considerably less C\(^{14}\). The specific activity of microsomal ribonuclease attained a maximum at 15 minutes and decreased thereafter. These data have led to the conclusion that the microsomal portion of the acinar cell is the site of synthesis of pancreatic ribonuclease in the living mouse.

It can be seen that the ribonuclease of the Supernatant I fraction showed the second most rapid rate of labeled amino acid incorporation. Fifteen minutes after injection, the specific activity of the ribonuclease in that fraction is significantly greater than the ribonuclease of either the nuclei or zymogen granule fractions. However, after 30 minutes the specific activity of ribonuclease in the zymogen granule fraction was elevated sufficiently to exceed that of the enzyme of the Supernatant I fraction by a significant margin.

Three possible interpretations present themselves to account
for the distribution of radioactivity in the ribonuclease of the supernatant and zymogen granule fractions. (a) The occurrence of a precursor-product relationship between the enzymes of the two fractions. Thus, the ribonuclease of the supernatant solution may be an intermediate form in the transport of the enzyme from the site of synthesis in the microsomes to the site of storage in the zymogen granules. (b) A mixed situation. The intracellular distribution of pancreatic ribonuclease has been shown to be more diffuse than that of well recognized secretory enzymes in the mouse (3, 4), guinea pig (27), and the cow (28). The possibility that a significant proportion of the labeled ribonuclease remains in the supernatant solution, with presumably a different function from that which enters the zymogen granules must therefore be considered. (c) Contamination by small particles. If a clean separation of the soluble and microsomal portions of the homogenate was not attained by the centrifugal forces which were applied, there might be a sufficient number of microsomal particles remaining in the soluble fraction to account for the observed data.

In order to test the third possibility a further fractionation of the soluble portion of the cell was carried out. Prolonged centrifugation of a supernatant solution (15 minutes after isotope injection) yielded two "postmicrosomal" fractions and a high speed supernatant fraction (Supernatant2). The data of Table I indicate that the supernatant1 fraction was not grossly contaminated with highly labeled microsomal ribonuclease, and do not support the third possibility. Further work will be necessary, however, to determine the validity of the first two hypotheses.

**DISCUSSION**

The present data strongly implicate the microsomal (endoplasmic reticulum) portion of the acinar cells as the site of synthesis of mouse pancreatic ribonuclease. This conclusion is based on the rate of incorporation of valine-C14 into ribonuclease Peak III of the various cell fractions under conditions of net protein synthesis. Other workers have shown that these particles, derived from pigeon and guinea pig pancreas, rapidly incorporate C14-amino acids into trichloroacetic acid-precipitable proteins (29, 30). Siekevitz and Palade (31) have recently stated that incorporation of leucine-C14 into chymotrypsinogen of guinea pig pancreas was greatest in the microsome Fraction 3 minutes after injection.

On the other hand, the steps between synthesis of the enzyme and its occurrence in the zymogen granules are yet unclear. Ribonuclease which is synthesized in the microsomal portion of the acinar cells of mouse pancreas may be transferred to the soluble portion of the cell before reaching the zymogen granules. Laird and Barton (19) have reached a similar conclusion based on a kinetic study of the pilocarpine-induced depletion of amylase from the various portions of the mouse pancreas cell. These workers have proposed that the soluble enzyme is condensed into the zymogen granules. Sjostrand and Hanzon (32) examined the ultrastructure of the exocrine cells of mouse pancreas by electron microscopy and have commented on the intimate relationship between the Golgi apparatus and the zymogen granules of the acinar cells. Further, they have described granules in the Golgi zone which appear to represent a whole series of stages from the most minute granules to the well defined zymogen granules. Challice and Lacy (33) have reported similar observations in the Golgi zone of the acinar cells. Recently Palade (34) has proposed that the zymogen granules are formed

**TABLE III**

<table>
<thead>
<tr>
<th>Cell fraction</th>
<th>Ribonuclease specific activity*</th>
<th>Ribonuclease activity of fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant1</td>
<td>2.53 ± 0.24†</td>
<td>202</td>
</tr>
<tr>
<td>Postmicrosomal1</td>
<td>2.32 ± 0.28</td>
<td>99</td>
</tr>
<tr>
<td>Postmicrosomal2</td>
<td>1.25 ± 0.23</td>
<td>24</td>
</tr>
<tr>
<td>Supernatant2</td>
<td>2.37 ± 0.24</td>
<td>37</td>
</tr>
</tbody>
</table>

* Defined in "Experimental" section of the text.
† This result was obtained from a different sample.
within the endoplasmic reticulum (microsomal fraction). In such a case no soluble intermediate fraction would be expected.

An estimation of the time required for a protein molecule to be assembled from precursors can be made from the data included in Figs. 3 and 4. Since a considerable amount of labeled ribonuclease is present in the microsomal fraction 5 minutes after intraperitoneal injection of the labeled amino acid, the time required for the formation of labeled enzyme is necessarily less than 5 minutes. The time periods required for the homogenate, cytoplasmic, and zymogen granules, and nuclei. It is concluded that pancreatic ribonuclease is synthesized in the microsomes.

From the rates of incorporation of valine-C\textsuperscript{14} into microsomes and into microsomal ribonuclease, it can be calculated that approximately 3 minutes are required for synthesis of the enzyme.

REFERENCES


SUMMARY

The rate of L-valine-C\textsuperscript{14} incorporation into mouse pancreas ribonuclease has been studied in vivo under conditions of net protein synthesis. Ribonuclease of the microsomes became labeled most rapidly, followed by the supernatant fraction, zymogen

Fig. 5. Estimation of time of synthesis of ribonuclease. O—O, C\textsuperscript{14} content of microsomal fraction; △—△, C\textsuperscript{14} content of homogenate; ●—●, specific activity of microsomal ribonuclease. Each measurement is plotted as a percentage of the maximal value as a function of time after valine-C\textsuperscript{14} injection.
Biosynthesis of Ribonuclease in Mouse Pancreas
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