A Cytochemical Study on the Pancreas of the Guinea Pig

V. *In vivo* Incorporation of Leucine-1-C^{14} into the Chymotrypsinogen of Various Cell Fractions*

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ABSTRACT

Chymotrypsinogen synthesis in the exocrine cell of the guinea pig pancreas was studied under the following conditions: Animals fed after a fast of ~48 hours received ~1 hour after feeding an intravenous injection of DL-leucine-1-C^{14}. At various time intervals (1 to 45 minutes) after the injection, the glands were removed and fractionated into a series of cell fractions of known cytological significance. Ten to twelve animals were used for each time point. From each cell fraction, the chymotrypsinogen was isolated by acid extraction and purified by $(NH_4)_2SO_4$ fractionation, isoelectric precipitation, and chromatography. Because of the minuteness of the quantities involved, chymotrypsinogen amounts were calculated from enzymatic activity figures, and a carrier method was used to precipitate and count the enzyme. The chymotrypsinogen isolated from the attached ribonucleoprotein particles of the microsomal fraction had the highest specific radioactivity at the early time points (1 to 3 minutes). After long intervals (at 15 to 45 minutes), the specific radioactivity of the enzyme increased in the microsomal contents and finally in the zymogen granules. The results are compatible with the view that the chymotrypsinogen is synthesized in or on the attached RNP particles and subsequently transported to other cell compartments.

INTRODUCTION

Based on results obtained in our previous work on the pancreas of the guinea pig (2–6), we have formulated the following hypothesis about the role played in the secretory process by the various structures which compose the pancreatic exocrine cell: The digestive enzymes produced by the cell are synthesized in or on the ribonucleoprotein particles attached to the membrane of the endoplasmic reticulum. The newly synthesized enzymes are subsequently transferred across the limiting membrane of the network and segregated into its cavities, sometimes in the form of visible intracisternal granules. As granules or in solution, the product of secretion moves through the channels of the reticulum to the centrosphere (Golgi) zone, where it is quantized and where each quantum acquires a membranous envelope within which it is condensed, thereby becoming a zymogen granule. These latter granules are stored in the apical region of the cell until the next food intake, when their membranes fuse with the cell membrane and, through the openings thus created, their contents are discharged into the glandular lumina (7, see also 8 and 9).

The basic premise that the microsomes, more specifically their attached RNP particles, are intracellular sites for protein synthesis is derived from work carried out with radioactive tracers in many laboratories. Because of technical difficulties, past work in this field has usually involved the isolation, washing, and counting of mixtures of acid-precipitated proteins (*cf.* reviews 10, 11). Since a clearer picture can emerge from an inquiry re-

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stricted to a single protein of known physiological significance, we have tried to isolate, in the present work, a well defined protein—α-chymotrypsinogen—from the various pancreatic cell fractions after *in vivo* labeling with DL-leucine-1-C^{14}. The enzymatic activity of this protein,[1] known to be synthesized for secretion, was used to quantitate our findings. The results indicate that:

(*a*) the attached ribonucleoprotein (RNP)[2] particles are the intracellular sites of the earliest radioactive labeling of α-chymotrypsinogen;

(*b*) the specific radioactivity of the enzyme increases subsequently in the microsomal contents and finally in the zymogen granules.

These findings are clearly compatible with our hypothesis. Admittedly, they do not constitute final proof thereof, but they represent the strongest supporting evidence we have thus far obtained in the matter.

EXPERIMENTAL

Methods and Materials

Preparation of Animals and in vivo Labeling.—Six guinea pigs were used in each experiment in which the microsomes were not subfractionated. The number of animals was raised to 10 or 12 when microsomal subfractions were investigated. Each experiment covered a single time point.

The guinea pigs, both males and females, weighing 300 to 400 gm., were fed a meal of cabbage after a fast of 48 hours. One hour to 1 hour and a half after the beginning of the meal, each animal received intravenously, under light ether anesthesia, 1 ml. of a 0.15 M NaCl solution containing 2 mg. DL-leucine-1-C^{14} with a specific activity of 4.66 μc./μM. The interval of ~1 hour between feeding and injection was chosen because at this time the rate of protein synthesis apparently increases (5) and the endoplasmic reticulum of many exocrine cells contains intracisternal granules in its cavities (4). Within each group of animals, feeding and injections were staggered so as to allow exact timing for the *in vivo* labeling. At the required interval after injection, the circulation was interrupted by severing, under ether anesthesia, the aorta and cava inferior below the diaphragm. Immediately afterwards, the pancreas was removed, minced, and homogenized in cold 0.88 M sucrose. The final concentration of the homogenate was 1/10 (weight/volume). In each experiment the individual homogenates were pooled before cell fractionation. Accordingly, each time point in our tables refers to an experiment carried out on pancreatic tissue pooled from either 6, or 10 to 12 animals. By careful timing, the interval during which the labeled leucine had circulated in the blood was almost exactly the same for all animals within each pool.

Cell Fractionation.—The homogenate was fractionated by a modification of the procedure mentioned in reference 3. After the nuclear fraction was removed by centrifugation for 15 minutes at ~760 g, its supernatant, or cytoplasmic fraction, was spun for 20 minutes at 20,000 *g* to sediment zymogen granules and mitochondria in a common pellet. The supernatant of this sediment was further centrifuged for 60 minutes at 105,000 *g* to separate the main microsomal fraction.

The common pellet of zymogen granules and mitochondria was resuspended by gentle homogenization in 0.88 M sucrose and made up to the original volume of the homogenate. Ten ml. aliquots of this suspension were layered on a series of discontinuous density-gradients, each built up from bottom to top with 7 ml. layers of 1.90 M, 1.52 M, and 1.40 M, sucrose solutions (*i.e.*, 65, 52, and 48 per cent). The tubes containing the density-gradient systems were centrifuged for 60 minutes at 53,500 *g* using a swinging-bucket rotor. At the end of the run, a thin pellicle containing nuclei and red blood cells was found at the bottom of each tube; a thick band of white opaque material (zymogen fraction) accumulated above the first interface from the bottom; a yellowish, broader, and much less opaque band (mitochondrial fraction) formed at the following interface and spread well into the 1.52 M sucrose layer. The top layer remained red and slightly cloudy, although reddish opaque material (microsomes) piled up in a band at the top of the upper interface of the system. The layers were separated by cutting each tube immediately below the corresponding interfaces with a Spinco Co. tube-slicer. The 0.88 M sucrose layers were discarded. The mitochondrial layers were pooled in a common fraction, diluted with water to a sucrose concentration of ~0.88 M, and then centrifuged for 60 minutes at 105,000 *g* to obtain its components in well packed pellets. The layers of the zymogen fraction were similarly processed.

In experiments in which the microsomes were subfractionated (*cf.* 4), the microsomal pellets were resuspended in 0.88 M sucrose and enough of the latter and of a 3 per cent solution of Na deoxycholate (DOC) (pH 7.6–7.8) were added to bring up the suspension to the original volume of the homogenate and to obtain a final DOC concentration of 0.1 per cent. The cleared suspension was spun at 105,000 *g* for 30 minutes to isolate a heavy microsomal subfraction (MS_{30}). The corresponding supernatant was further centrifuged for

[1] Chymotrypsinogen is actually an enzyme precursor or zymogen, which is converted into an active enzyme (chymotrypsin) by the action of trypsin or erepsin. It is for convenience only that we refer to it as an enzyme in this paper.

[2] Abbreviations used are: RNP, ribonucleoprotein; RNA, ribonucleic acid; RNase, ribonuclease; DOC, deoxycholate.

120 minutes at 105,000 *g* to obtain a sediment, the intermediary subfraction (MS_{120}), and a new supernatant, the light or DOC-soluble subfraction (SS_{120}).

In some experiments, the original microsomal supernatant was further fractionated (by centrifugation at 105,000 *g* first for 3, and then for 15 hours) into two postmicrosomal fractions (PM_1 and PM_2) and a final supernatant (FS) (*cf.* 2, 6). In other experiments, the postmicrosomal fractions were separated together in a common pellet.

An International centrifuge model SBV, running in the cold room at ~4°C. was used for the isolation of the nuclear fraction. The rest of the fractionation was carried out by using, simultaneously, two or three Spinco model L centrifuges provided, as required, either with No. 40 angle head rotors or with No. SW 25.1 swinging-bucket rotors. When no postmicrosomal fractions were isolated, the entire fractionation procedure took ~5 hours.

Electron Microscopy, Methods and Results.—In pilot experiments, the morphological composition of the various fractions, isolated as indicated above, was checked by electron microscopy using procedures previously devised to survey the whole depth of the pellets (12).

The zymogen fraction was found to consist primarily of zymogen granules with an admixture of mitochondria.

The mitochondrial fraction contained, in addition to mitochondria, zymogen granules and microsomes.

The microsomal fraction, the most homogeneous of these preparations, was made up of "healed" fragments of the rough surfaced part of the endoplasmic reticulum (2). The composition of the red layer above the upper interface in the discontinuous density-gradient was similar to that of the usual microsomal fraction except for a noticeable contamination by mitochondria. The heavy microsomal subfraction (MS_{30}) was a concentrate of intracisternal granules (4) with an admixture of various microsomal debris (membranes and particles). The intermediary subfraction (MS_{120}) consisted almost entirely of small (~150 A), dense RNP particles.[3] The contamination by membranous material was negligible (4). The light subfraction (SS_{120}) (not examined in the electron microscope) is supposed to represent the fluid phase of the microsomal content but it also comprises whatever materials are solubilized from the other microsomal components (membrane, attached particles, intracisternal granules) by DOC treatment (4).

The two postmicrosomal fractions consisted of free RNP particles of similar morphology, but dissimilar RNA content (2, 6).

The final supernatant did not contain structured elements. It assumedly represents the soluble components of the cytoplasmic matrix, but inevitably comprises all materials extracted from the other cell components during the preparation and fractionation of the tissue brei.

We were obliged to modify our previous procedure (3) for fractionation in a discontinuous density-gradient because, in experiments with fed animals, it yielded mitochondrial fractions heavily contaminated by microsomes, and microsomal pellets smaller and more loosely packed than those we had obtained in the past by conventional differential centrifugation (2). We assume that this unfavorable result is due to the frequency of microsomes loaded with intracisternal granules which, because of their density, sediment in concentrations of sucrose higher than 0.88 M. The modification described above combines the advantages of conventional differential centrifugation, which yields sizable, well packed, microsomal pellets (2), with those offered by fractionation in a discontinuous density-gradient by which less heterogeneous zymogen and mitochondrial fractions can be obtained. Even with this modification, these latter fractions are not entirely satisfactory; the mitochondrial fraction for instance, although improved, remains noticeably contaminated by zymogen granules and microsomes. The microsomal contamination is, however, smaller than in earlier preparations (3).

Chymotrypsinogen Isolation (*cf. 13, 3*).—Each fraction obtained in pellets was resuspended in 7.5 ml. of cold, distilled water, and 2.5 ml. of cold 1 N H_2SO_4 were added to bring the final concentration of the acid to 0.25 N. To the liquid fractions (SS_{120} and FS), enough 10 N H_2SO_4 was added to reach the same final concentration of acid (0.25 N). The sulfuric acid extracts were kept at ~4°C. for ~1 hour and then cleared by centrifugation. The sediment was resuspended in 0.25 N H_2SO_4, left again at ~4°C. for ~1 hour, then centrifuged and the ensuing supernatant added to the first 0.25 N H_2SO_4 extract.

To the combined acid extracts, enough $(NH_4)_2SO_4$ was added to reach a final concentration of 35 per cent (*w/v*). The preparation was left at ~4°C. for 1 to 2 hours and the protein thereby precipitated was isolated by centrifugation. The precipitate was redissolved in 0.25 N H_2SO_4 and the $(NH_4)_2SO_4$ fractionation repeated once. The new precipitate was redissolved in 0.25 N H_2SO_4 and the solution dialyzed at ~4°C. for 40 to 48 hours against stirred and once changed 0.25 N H_2SO_4. At the end of the dialysis, the content of the sac was brought to pH 5.8 to 6.0 and the resulting precipitate was spun down and discarded. The pH 6 extract of each fraction was finally placed on a previously prepared and washed 30 × 0.9 cm. IRC-50 ion-exchange column and eluted therefrom with 0.2

[3] About 80 to 85 per cent of this material gave a sedimentation coefficient of ~85 S (*cf.* 28). We wish to thank Dr. D. A. Yphantis of The Rockefeller Institute for performing the analytical ultracentrifugation experiments.

M, pH 6.0, phosphate buffer with no detergent added (13, 14, 3). The elution was run at 1.5 to 2.0 ml. per hour, about 0.5 ml. (13 drops) being collected in each tube. The contents of tubes 21 to 51, which corresponded to eluant volumes ~12 ml. to ~29 ml. and which contained the α-chymotrypsinogen, were pooled (together with 2 to 3 ml. of buffer used to rinse the tubes) and the volume measured. This volume ranged from 15 to 18 ml. among the fractions.

Enzymatic Assays.—For each cell fraction, aliquots of the pooled eluates were activated by trace amounts of trypsin and then assayed by Kunitz's procedure (15, 13, 3), which measures the amount of acid-soluble products liberated during casein digestion. Enzyme activity figures were converted into amounts of enzyme by using an activity/weight curve obtained for crystalline bovine chymotrypsinogen and by assuming that the same relationships obtain for the guinea pig enzyme.

Counting of α-Chymotrypsinogen Radioactivity.—After trying several methods for counting liquid eluate samples, the following carrier-method was chosen: an amount of bovine crystalline chymotrypsinogen, ranging from 9 to 12 mg. and weighed to a precision of 0.1 mg., was dissolved in the eluate from the column, after aliquots had been taken for enzymatic assays. Trichloroacetic acid was then added to a final concentration of 10 per cent and the precipitated protein was collected, washed, and counted as previously described (6). The counted sample was then weighed to a precision of 0.1 mg. The amounts of radioactivity were accurately measurable in all fractions. For instance, the total counts in the α-chymotrypsinogen extracted from the attached RNP particles ranged from 100 to over 500 C.P.M. The enzyme extracted from the other fractions gave many more counts, running into thousands in the case of the whole microsomal fraction. From the counting rate, the total radioactivity in the whole eluate was calculated, by correcting the figure for the aliquots removed for enzymatic assays and for the amount of carrier not recovered in the precipitate. In each experiment the precipitated protein represented only 70 to 90 per cent of the amount of added carrier, due mostly to losses incurred during the washings of the protein. The specific radioactivity of the α-chymotrypsinogen in the fraction was expressed as counts per minute divided by the weight (in mg.) equivalent to the enzymatic activity of the corresponding eluate (*cf. Enzymatic Assays*).

Materials

The animals were supplied by the colony of albino guinea pigs of this Institute. The chemicals were obtained from the following sources: labeled leucine: Isotopes Specialities Co., Burbank, California; salt-free bovine crystalline chymotrypsinogen and RNase: Worthington Chemicals Co., Freehold, New Jersey; Na deoxycholate: Wilson Laboratories, Chicago.

Rationale and Evaluation of Experimental Procedures

Enzymatic Assays and Enzyme Stability.—Since in some cell fractions there is not enough α-chymotrypsinogen for gravimetric determinations, we were obliged to calculate enzyme amounts from enzyme activity figures using crystalline bovine chymotrypsinogen as a standard. We assumed, therefore, that activity and amounts are directly and proportionally related and that the guinea pig enzyme has the same specific enzymatic activity as its bovine counterpart. By using this procedure we determined that the amount of α-chymotrypsinogen finally isolated from the attached RNP particles varied from 25 to 50 μg. in the various experiments, while the amount obtained from the intracisternal granules was about twice as much. The other fractions yielded higher amounts of enzyme: up to 500 and 800 μg. for the zymogen and the whole microsomal fractions respectively. Enzymatic activity has been repeatedly used in the past to demonstrate an increase *in vitro* in the amount of such pancreatic enzymes as lipase and RNase (16–18), amylase (19–22), and esterase (23).

Because the quantitation of our results was based on enzymatic activity, and because of the long duration of the experiments, we were obliged to ascertain that there was no significant change in chymotrypsinogen activity during extraction and storage. We determined first that two successive 0.25 N H_2SO_4 extractions removed 95 per cent of the chymotrypsinogen of the sample (the amount obtained by four successive extractions when a plateau was reached being considered 100 per cent). Storage of the acid extract at ~4°C. for periods up to 24 hours caused no change in activity. We found, however, that after $(NH_4)_2SO_4$ precipitation and dialysis, the dialyzed extract lost ~75 per cent of its activity when stored for 6 days at ~4°C. in 0.25 N H_2SO_4. This loss could be prevented by bringing the pH up to ~6.0 at the termination of the dialysis. Hence in the experimental procedure finally adopted, all the steps, *i.e.* double extraction with 0.25 N H_2SO_4, treatment with $(NH_4)_2SO_4$, dialysis and pH adjustment to 6.0, were performed successively (immediately upon the termination of the cell fractionation procedure) for all cell fractions involved, and the extracts were stored at pH 6.0 until placed on the respective columns. We also ascertained that the enzymatic activity of the eluate from the first column did not change over the period of 6 days necessary for the chromatography of all extracts.[4] Hence all eluates were stored at 4°C. and assayed at the same time after all extracts had been chromatographed. The influence

[4] Since the extract of one fraction was chromatographed at a time, it usually took 5 to 6 days to chromatograph the extracts of all the cell fractions of a single time point experiment.

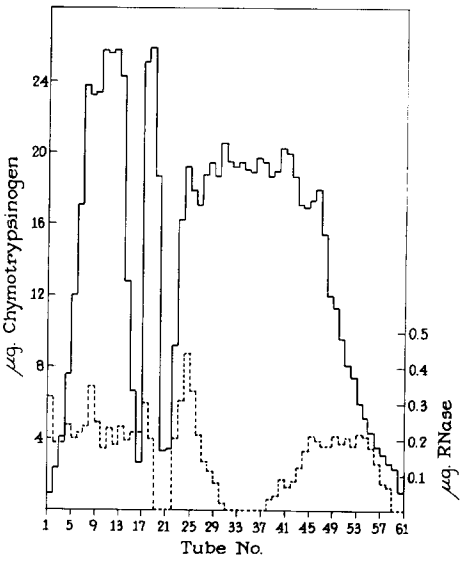

FIG. 1. Elution pattern of α-chymotrypsinogen and RNase on IRC-50 columns after two $(NH_4)_2SO_4$ fractionations. The experimental procedures are given in the text. Proteolytic activity was measured by the procedure of Kunitz (13, 15, 3). Solid line is trypsin-activatable proteolytic activity. The peak from tube 22 onwards is α-chymotrypsinogen; the dotted line is RNase. The amount of enzyme was calculated from calibrated curves obtained with bovine crystalline enzymes.

of the pH of the eluant (6.0) upon the enzymatic assay carried out at its apparent pH optimum of 7.6 was checked and found to be negligible under our experimental conditions.[5]

Chymotrypsinogen Purification by $(NH_4)_2SO_4$ Fractionation.—Hirs has shown that bovine α-chymotrypsinogen can be satisfactorily separated from RNase by IRC-50 ion exchange chromatography (13, 14). In the case of the guinea pig, however, the separation is less satisfactory: in fact two major RNase peaks come off together with the α-chymotrypsinogen peak, presumably because the nuclease is less firmly held by the column (Fig. 1). Using bovine crystalline enzymes as standards, we calculated that 10 per cent of the proteins of the eluate in tubes 21 to 51 is RNase, the rest being α-chymotrypsinogen. To eliminate this contamination we decided to take advantage of the difference in solubility between the two enzymes in $(NH_4)_2SO_4$ solutions. Bovine RNase, for instance, is still soluble (24) whereas chymotrypsinogen precipitates in 43 per cent (*w/v*) $(NH_4)_2SO_4$. At the same concentration, however, approximately half of the guinea pig RNase is precipitated and thus another difference is revealed between this enzyme and its bovine counterpart. At 40 per cent $(NH_4)_2SO_4$, all guinea pig chymotrypsinogen is salted out but 10 per cent of the RNase is also precipitated. By two separate precipitations at 35 per cent $(NH_4)_2SO_4$, the chymotrypsinogen is recovered with only small losses and the RNase contamination is reduced to less than 1 per cent. This figure can be reduced to less than 0.5 per cent by four successive $(NH_4)_2SO_4$ fractionations, but about half of the chymotrypsinogen is also lost in the process. For these reasons, we decided to subject all acid extracts to only two successive fractionations with 35 per cent $(NH_4)_2SO_4$, a contamination by less than 1 per cent RNase being considered tolerable. Since RNase contains less than 2 per cent leucine (25), whereas the corresponding figure for chymotrypsinogen is about 8 per cent (15, 26), contamination by radioactive RNase can not exceed 0.2 to 0.3 per cent. Fig. 1 shows chromatographically the results obtained after two $(NH_4)_2SO_4$ fractionations. In this typical case it can be calculated, by using crystalline bovine enzymes as standards, that the area under the curve from tubes 22 to 55 represents 535 μg. α-chymotrypsinogen and 5 μg. RNase.

Contamination of α-Chymotrypsinogen by Radioactive Leucine.—Since leucine is held by IRC-50 columns and eluted by pH 6.0 buffer, contamination of α-chymotrypsinogen by radioactive leucine has to be taken into consideration. Our pilot experiments (Fig. 2) showed that after *in vivo* labeling most of the leucine counts in the acid extract of a whole homogenate came out before tube 21, the counts in tubes 21 to 50 representing mostly α-chymotrypsinogen. Contamination was possible only at the beginning of the α-chymotrypsinogen peak. Indeed after the addition of 10^6 C.P.M. leucine to an extract, chromatographed thereafter without previous dialysis (Fig. 2), most of the leucine counts were eluted before tube 21 but the immediately following tubes still contained 123,000 C.P.M. Most of this contamination could be removed however by dialysis. For instance out of 200,000 C.P.M. added as leucine to an unlabeled pancreatic acid extract, 7,575 C.P.M. were eluted in tubes 21 to 50 when the extract was chromatographed without previous dialysis; but, after an overnight dialysis without change of the dialysate, only 112 counts came out in the same tubes. Although highly efficient, removal by dialysis was not good enough in our case because of the high specific radioactivity of the leucine and of the small number of counts in the α-chymotrypsinogen isolated from certain fractions. It was found, however, that trichloroacetic acid precipitation followed by washing of the precipitated protein (which, together with dialysis, is part of our purification procedure) reduced the contamination with radioactive leucine to practically nil. Out of the 300,000 C.P.M. added as leucine to a solution of crystal-

[5] An inhibition of 20 to 30 per cent was recorded only when equivalent amounts of the two buffers were used in the assay system, a situation never encountered in our determinations.

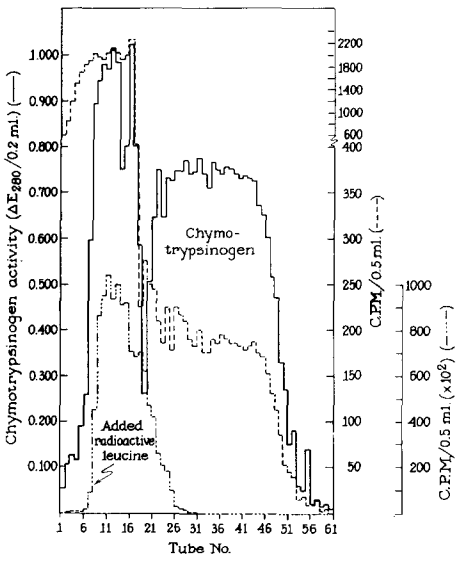

FIG. 2. Elution patterns of radioactive α-chymotrypsinogen and radioactive leucine on IRC-50 columns. The experimental procedures for α-chymotrypsinogen are given in the text. The radioactive enzyme was obtained from whole pancreas tissue after injecting radioactive leucine into a guinea pig, and after treating the pancreas tissue as described. Radioactive leucine brought to pH 6.0 was added directly to an identical column. In the graph the curves obtained in the two experiments are superimposed.

line bovine chymotrypsinogen, only 7 C.P.M. were recovered in the protein precipitated by, and washed twice with, 10 per cent trichloroacetic acid as done in our counting procedure. It appears, therefore, that the contamination of α-chymotrypsinogen by radioactive leucine can be eliminated to the extent of 98 per cent by dialysis, and the remainder washed out almost entirely during the precipitation and washing of the protein preparatory to counting.

Reproducibility of the Carrier Method.—Because in the eluates of most fractions the α-chymotrypsinogen was present in too small amounts for direct precipitation, we were obliged to use the carrier method already described. Its reproducibility was tested by the following experiments carried out after *in vivo* labeling with leucine-1-C^{14}.

(*a*) The pooled eluate of the extract of the microsomal fraction was divided into two aliquots and weighed amounts of crystalline bovine chymotrypsinogen were added to each. The chymotrypsinogen was then precipitated by trichloroacetic acid, and the precipitate washed, counted, and weighed as described above. The eluate of the zymogen fraction extract was similarly processed. The recovery of the carrier varied from 80 to 90 per cent. After correction for 100 per cent recovery, the counting rate of the duplicate samples agreed within 10 per cent over a wide range (570 and 519 C.P.M. for the microsomal α-chymotrypsinogen, and 55 and 49 for the α-chymotrypsinogen of the zymogen granules).

(*b*) The α-chymotrypsinogen peak was divided into two halves, the first comprising tubes 21 to 35 and the second, tubes 36 to 50. Each half was processed as usual and the specific radioactivity of the two samples compared. The two values (27,900 and 29,600 C.P.M./mg. enzyme) agreed within 10 per cent.

(*c*) The eluate of a whole homogenate was divided into two aliquots and one of them chromatographed again on an IRC-50 column. Only 20 per cent of the enzymatic activity of the first eluate was recovered upon rechromatography. Weighed amounts of chymotrypsinogen were added separately to the once and twice chromatographed eluates which were then processed as usual. After corrections for carrier losses, the specific radioactivities of the two aliquots were found to be 21,760 and 19,690 C.P.M./mg. enzyme respectively. The values were again in agreement within 10 per cent.

The results indicate that we can recover a representative chymotrypsinogen sample by the carrier method, although recovery is incomplete. To avoid heavy α-chymotrypsinogen losses, we decided to chromatograph the extracts just once.

Chymotrypsinogen Isolation on IRC-50 Columns; Estimation of Purity.—The effectiveness of IRC-50 columns for the isolation and purification of bovine α-chymotrypsinogen has been discussed in detail by Hirs (13). In previous experiments (3) we have shown that guinea pig α-chymotrypsinogen behaves chromatographically almost exactly like its bovine counterpart and in the present work we ascertained that all the α-chymotrypsinogen activity of pancreatic homogenates is retained by the columns. The enzyme isolated from the various cell fractions has the same chromatographic profile (3); accordingly, it can be reasonably assumed that we are dealing with the same enzyme in all cell fractions tested.

The purity of the chromatographed enzyme was tested as follows:

(*a*) Ninhydrin reactive material and proteolytic activity were determined in each tube of the α-chymotrypsinogen peak. The corresponding curves were found in close parallelism to one another.

(*b*) Specific assays[6] showed considerable chymotrypsin activity and very little trypsin activity in the same peak. The extent of trypsinogen contamination cannot be accurately determined because the assay system is not sensitive enough for the quantities

[6] Trypsin was tested against benzoyl-L-arginine ethyl ester, and chymotrypsin against acetyl-L-tyrosine ethyl ester by Mr. L. Greene, The Rockefeller Institute, to whom we are grateful.

involved and because small amounts of trypsin are necessarily added to the eluate to activate the zymogen. It can be said, however, that trypsinogen contamination, if present, can not exceed 5 per cent.

(*c*) The addition of increasing amounts of trypsin to the eluate did not elicit more chymotrypsin activity indicating the absence of appreciable amounts of trypsin inhibitor.

Since RNase contamination has already been shown to amount to ~1 per cent, and since RNase, trypsin inhibitor, trypsinogen, and α-chymotrypsinogen are the only known cationic enzymatic proteins expected to be held by the IRC-50 column (*cf.* 27), it would follow that the main enzymatic component of the peak is α-chymotrypsinogen. Contamination by other cationic, non-enzymatic proteins is, however, not excluded. Furthermore, it should be realized that any contaminant, if endowed with high specific radioactivity, could easily mislead our interpretations.

To test for such possible contaminants, the eluate of the α-chymotrypsinogen peak was dialyzed against 0.001 N HCl and then lyophilized. The ensuing powder was taken up in ~0.1 ml. H_2O and small samples (10 to 20 lambda) of the solution were placed on strips of Whatman 3 MM filter paper and subjected to electrophoresis[7] at 150 volts for 20 hours in pH 8.6 veronal buffer of 0.1 ionic strength (*cf.* 28, 29). After electrophoresis, the proteins were stained with 0.5 per cent bromphenol blue and the excess color removed with 2 per cent acetic acid. Crystalline bovine chymotrypsinogen, treated with the phosphate-buffer wash of the column, then dialyzed against 0.001 N HCl, and finally lyophilized, served as control. The protein of the eluate and the known chymotrypsinogen each moved as a single spot to exactly the same position. In both cases, however, a small amount of protein remained at the origin and the moving spot left behind a fine tail. Since crystalline bovine chymotrypsinogen, subjected directly to electrophoresis, left no residue at the origin (it also showed slightly greater mobility), it can be assumed that a certain amount of enzyme is denatured in the presence of the wash of the column either during dialysis or lyophilization. Discounting the amount of protein left at the origin, it can be estimated that 90 per cent or more of the material in the peak is α-chymotrypsinogen. A contaminant amounting to 10 per cent of the material would have been detected by electrophoresis.

To rule out the presence of a highly radioactive contaminant, the eluate of the acid extract of a pancreas, labeled *in vivo* with DL leucine-1-C^{14}, was subjected to electrophoresis as described above. Autoradiography of the paper strip showed that most of the radioactivity moved with the chymotrypsinogen spot, little trailing behind or being left at the origin. In this case again, a contaminant with 10 per cent of the radioactivity of the chymotrypsinogen, but with a different electrophoretic mobility, would have been detected. We should add that the rate of amino acid incorporation of the hypothetical, highly radioactive contaminant we have considered would have to be 10 times higher than that of the total proteins in the α-chymotrypsinogen peak and 50 to 100 times higher than that of the mixed pancreatic proteins. It is clear that the existence of such a protein is highly improbable.

From these experiments and considerations, it can be concluded that at least 90 per cent of the protein in the peak examined is α-chymotrypsinogen, and that at least 90 per cent of the radioactivity in the peak is incorporated in this zymogen.

RESULTS

Distribution of Radioactivity among Protein Fractions.—To evaluate the relative importance of α-chymotrypsinogen production within the general process of protein synthesis carried out by the pancreas, we followed the distribution of radioactivity in the various protein fractions obtained during the extraction and purification of the enzyme. We started with a pancreatic homogenate prepared 45 minutes after the injection of 2 mg. leucine-1-C^{14}. The combined 0.25 N H_2SO_4 extracts gave 215,800 C.P.M., whereas the corresponding acid precipitate contained 211,500 C.P.M. The H_2SO_4 extract is known to comprise most of the digestive enzymes produced by the pancreas, namely α-chymotrypsinogen, chymotrypsinogen B, trypsinogen, RNase, and deoxyribonuclease (15, 27). Since these proteins are released in the pancreatic juice (27) it can be assumed that proteins synthesized for secretion predominate in the H_2SO_4 extract, while proteins produced for intracellular use prevail in the precipitate. For convenience, and on this basis only, the two fractions obtained by H_2SO_4 treatment will be referred to as "exportable" and "non-exportable" proteins in the rest of this paper. It follows from the data, that the "exportable" proteins account for more than 50 per cent of the counts incorporated over 45 minutes into the proteins of the gland. From the radioactivity of the combined acid extracts, 5,700 C.P.M. were soluble, and 192,800 precipitated, in 35 per cent $(NH_4)_2SO_4$, giving a recovery of ~92 per cent at this step in the procedure. The results suggests that RNase, the main enzyme soluble in 35 per cent $(NH_4)_2SO_4$, is less heavily labeled by leucine than the other

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hydrolytic enzymes. The $(NH_4)_2SO_4$ precipitate was redissolved in 0.25 N H_2SO_4, dialyzed, and the pH of the dialysate adjusted to ~6.0. The precipitate formed at this step contained 149,000 C.P.M. whereas the corresponding supernatant, which comprised the α-chymotrypsinogen, gave 52,000 C.P.M. or 25 per cent of the 192,000 C.P.M. in the original $(NH_4)_2SO_4$ precipitate. At this step the recovery amounted to ~100 per cent indicating the absence of detectable amounts of free radioactive leucine in the original extract. The situation is not surprising since at the time the pancreas had been removed (45 minutes after the injection of the tracer) there were very few counts left in the acid-soluble fraction of pancreas homogenates (5). Of the 52,000 C.P.M. of the pH 6.0 supernatant put on columns, 7,720 C.P.M. (corrected to 100 per cent carrier recovery), or 14.5 per cent, were recovered in the eluate pooled from tubes 21 to 50. This figure represents only 3.6 per cent of the radioactivity in the original H_2SO_4 extract. The reasons for the low recovery at this step are still obscure: it can be due to adsorption of a certain amount of α-chymotrypsinogen on the glass surfaces of the collecting tubes (26), or to the presence of other radioactive proteins of different chromatographic behavior in the pH 6.0 supernatant put on columns. We already know that the major α-chymotrypsinogen peak is preceded by other proteolytic peaks in the chromatogram (13, 3). If instead of radioactivity, trypsin-activatable proteolytic activity is followed, 43 per cent of the activity of the pH 6.0 supernatant is found in the major α-chymotrypsinogen peak and in this case the incomplete recovery can be easily explained by the known presence of other proteases in the starting preparation. It follows that only a relatively small fraction of the incorporated leucine is used for α-chymotrypsinogen synthesis; that the extent of labeling is not necessarily the same for all enzymes produced by the gland (*cf.* 30); and finally that substantial losses of α-chymotrypsinogen are probably incurred during chromatography.

Labeling of "Non-Exportable" Proteins.—The figures in Table I show how the specific radioactivity of the "non-exportable" proteins varied with time in the various cell fractions. At the early time points (1 to 3 minutes) the specific activity of these proteins in the attached RNP particles was higher than in any other cell fraction studied. At the late time points (15 and 45 minutes), comparable values were obtained for the proteins of the microsomal, mitochondrial, and zymogen granule fractions. The course of labeling and the differences in specific activity among various cell fractions were generally comparable to those found in previous experiments (5) in which the labeling of the total proteins precipitated by trichloroacetic acid from similar cell fractions was studied. A marked difference appeared, however, at 45 minutes in the case of the zymogen fraction: the specific activity of its total proteins was higher than that of any other cell fraction, whereas the corresponding figure for "non-exportable" proteins was comparable to that of the other cell fractions. The difference is probably due to the accumulation of highly labeled "exportable" proteins in the zymogen granules at the late time points mentioned.

TABLE I

Specific Radioactivity of Proteins Precipitated by 0.25 N H_2SO_4 from Various Pancreatic Cell Fractions after in vivo Labeling with DL-*Leucine-1-*C^{14}

The H_2SO_4-precipitable proteins were obtained, washed, and counted as described in the text. The figures are C.P.M./mg. protein.

Cell Fraction	Time after injection					
	1 min.	2.5 min.	3 min.*	3 min.	15 min.	45 min.
Zymogen granule fraction	—	—	440	1250	1590	3750
Mitochondrial fraction	—	—	955	3125	2645	3290
Microsomal fraction (whole microsomes)	—	—	1695	—	—	4000
MS_{120} (attached particles)	4175	6820	—	8675	2650	—
MS_{30} (intracisternal granules)	1350	1480	—	3130	2800	—
SS_{120} (microsomal contents)	—	2900	—	2725	3400	—
First postmicrosomal fraction (PM_1) (free particles)	1375	2030	—	2600	—	—
Second postmicrosomal fraction (PM_2) (free particles)	580	1295	—	2085	—	—
PM_1 and PM_2 in a common pellet	—	—	—	—	—	2130
Final supernatant	—	845	—	1115	—	2810

* In this experiment only 1 mg. of 4.66 μc./μM DL-Leucine-1-C^{14} was injected to each animal. In all the other experiments the amount injected was 2 mg.

Labeling of α-Chymotrypsinogen.—Table II gives the specific activity of the α-chymotrypsinogen isolated from various cell fractions after 1 to 45 minutes labeling *in vivo*. At the early time points, the attached RNP particles yielded α-chymotrypsinogen with a specific activity higher

TABLE II

Specific Radioactivity of α-Chymotrypsinogen Isolated from Various Pancreatic Cell Fractions after in vivo Labeling with DL-*Leucine-1-*C^{14}

The details of the experiments are given in the text. The figures are C.P.M./mg. enzyme.

Cell fraction	Time after injection					
	1 min.	2.5 min.	3* min.	3 min.	15 min.	45 min.
Zymogen granule fraction	—	—	215	1,770	10,300	58,500
Mitochondrial fraction	—	—	2,240	4,100	13,950	51,250
Microsomal fraction (whole microsomes)	—	—	3,140	—	—	27,700
MS_{120} (attached particles)	22,100	13,780	—	10,000	15,480	—
MS_{30} (intracisternal granules)	7,970	2,920	—	2,770	18,300	—
SS_{120} (microsomal contents)	—	8,160	—	5,740	14,950	—
First postmicrosomal fraction (PM_1) (free particles)	2,840	9,280	—	2,970	—	—
Second postmicrosomal fraction (PM_2) (free particles)	3,900	9,150	—	8,370	—	—
PM_1 and PM_2 in a common pellet	—	—	—	—	—	27,500
Final supernatant	—	6,220	—	3,930	—	24,450

* In this experiment only 1 mg. of 4.66 μc./μM DL-Leucine-1-C^{14} was injected to each animal. In all the other experiments the amount injected was 2 mg.

than in any other fraction; the same protein isolated at 3 minutes from the intracisternal and the zymogen granules was less active by 3.5 and 7 times respectively. At 15 minutes the specific radioactivity of the enzymes showed little variation from fraction to fraction, and at 45 minutes the α-chymotrypsinogen in the zymogen granules was twice as radioactive as the microsomal enzyme. α-Chymotrypsinogen labeling in the cell fractions studied followed a time course closely parallel to that of the total proteins of the same fractions (5). This situation suggests that the pattern encountered in the case of α-chymotrypsinogen applies to other "exportable" proteins as well, and that the labeling of the total proteins of the gland reflects primarily the labeling of the digestive enzymes it produces. The first inference is supported by the presence of other enzymes, *e.g.* trypsinogen, RNase, and amylase (31) in the attached RNP particles, and by the more or less parallel variations in the amount of proteases and RNase associated with the microsomes during the secretory cycle (4). The second inference is validated by a comparison of the specific activities of the α-chymotrypsinogen (Table II) and "non-exportable" proteins (Table I) isolated from the same cell fractions. The comparison demonstrates convincingly that the "exportable" protein is labeled at a much higher rate in all cell fractions, especially in the attached RNP particles, and that it accumulates progressively in the microsomal and zymogen fractions.

DISCUSSION

The findings presented are entirely compatible with the hypothesis that the α-chymotrypsinogen is synthesized in or on the attached RNP particles, to be subsequently transferred to the cavities of the endoplasmic reticulum and finally concentrated and stored in the zymogen granules. This sequence of events is not, however, the only possible explanation of our results, for they could be accounted for not only by synthesis at one site (the RNP particles) followed by transport to other intracellular sites (the intracisternal and zymogen granules), but also by synthesis carried on at different rates at all the sites mentioned. We prefer the first alternative because it seems to us more logical in view of our morphological and cytochemical findings, but we realize that final proof remains to be obtained by studying *in vitro* amino acid incorporation into a digestive enzyme by isolated cell fractions.

Our findings show that the attached RNP particles are an important, possibly unique, site for α-chymotrypsinogen synthesis. They cannot tell by themselves whether complete synthesis occurs at this site, or whether the leucine is incorporated by peptide bond formation into a precursor at some other intracellular site, the possible role of the RNP particles being the conversion of this precursor into α-chymotrypsinogen. But all previous work on the *in vitro* incorporation of amino acids into the proteins of microsomes (*cf.* 10) and isolated RNP particles (32) shows that these particles can form the peptide bonds required for protein synthesis. We favor, therefore, the first alternative, *i.e.* the complete synthesis of the enzyme by the RNP particles.

The results presented raise and leave without clear answer many questions. They indicate, for instance, the existence of different rates of synthesis for "exportable" and "non-exportable" proteins and suggest that the latter, like the former, are synthesized—at least in part—by the attached RNP particles because at this site they have the

highest specific radioactivity at the early time points. The results also suggest that the "non-exportable" proteins are less rapidly removed from these particles than α-chymotrypsinogen, for while the specific activity of α-chymotrypsinogen decreases with time (Table II) that of "non-exportable" proteins continues to increase (Table I) at least during the first 3 minutes. The specific nature and the intracellular role of the "non-exportable" proteins remains, however, unknown.

The free RNP particles of the cytoplasm (PM_1 and PM_2) yield a certain amount of α-chymotrypsinogen whose specific radioactivity is definitely lower than that of the enzyme extracted from the attached RNP particles, but higher than that of the enzyme obtained form most other cell fractions at the early time points (Table II). The significance of this finding is obscure. It may reflect relocation by adsorption of a mixture of "hot" and "cold" α-chymotrypsinogen liberated from other cell components during tissue homogenization and fractionation. Or it may have functional significance, these particles partaking in the process of α-chymotrypsinogen synthesis *in situ*. In this case their relationship to the attached RNP particles becomes a matter of considerable interest. The view that free and attached RNP particles represent physiologically distinct cell components is rendered unlikely. The free particles appear to be mixtures which contain either detached particles or particles that would become attached in due time to the membrane of the endoplasmic reticulum. Their content of "cold" α-chymotrypsinogen or their apparently lower rate of synthesis might be connected to their inability to discharge the synthesized enzyme as long as they are not in contact with the membrane of the endoplasmic reticulum (*cf.* 30).

The presence of labeled α-chymotrypsinogen and labeled "non-exportable" proteins in the mitochondrial fraction is due—at least in part—to contamination by microsomes and especially by zymogen granules. This view is in agreement with the morphological findings and with the fact that the α-chymotrypsinogen isolated from this fraction has a specific radioactivity intermediary between that of the microsomal and zymogen enzyme, but closer to the latter. Finally, results obtained in our pilot experiments showed that the specific activity of the α-chymotrypsinogen extracted from various mitochondrial fractions decreased in parallel with the reduction of the microsomal contamination in these fractions. Ullmann and Straub (33) have assumed that amylase is formed in the mitochondria from a precursor synthesized in the microsomes. In the case of α-chymotrypsinogen synthesis, we are inclined to consider the mitochondrial involvement only apparent because: (*a*) the amount of enzyme in the fraction is small; (*b*) its specific activity at the early time points is considerably lower than in the whole microsomes or attached RNP particles; and (*c*) the contamination of the fraction by zymogen granules and microsomes is well established by the electron microscopy of the corresponding pellets. The significance of the labeled "non-exportable" proteins of the fraction is more difficult to assess. Their presence may again reflect the contamination already mentioned, but can also be explained through the synthesis of mitochondrial proteins by mitochondria, a process comparable to the synthesis of cytochrome *c* by liver and heart mitochondria (34, 35).

The recent biochemical literature includes a large number of reports on topics related to ours. Although the corresponding results are only in part comparable, they are generally in agreement with our findings and interpretations. Incorporation of radioactive amino acids *in vivo* has been repeatedly used to demonstrate the synthesis of a number of specific proteins such as muscle proteins (36) and lung antibodies (37) isolated from tissue extracts, as well as radioactive RNase (30, 38), trypsinogen, and α-chymotrypsinogen (28, 30) obtained from pancreatic extracts (38) and juice (28, 30). Pancreatic slices labeled *in vitro* have yielded radioactive amylase (39, 40), RNase (41), and insulin (41, 42). Under similar conditions, radioactive albumin was obtained from liver slices (43–46).

The isolation of specific proteins from various cell fractions after *in vivo* or *in vitro* labeling has also been used in several studies concerned with the intracellular location of sites for protein synthesis. For instance, radioactive albumin has been produced by hepatic microsomes after *in vitro* labeling (46, 47); *in vivo* labeled RNase has been extracted from pancreatic microsomes (48); and both labeling and net synthesis of cytochrome *c* has been achieved *in vitro* with heart mitochondria (34, 35). Hemoglobin synthesis in reticulocytes—a particularly favorable material for this type of work—has been studied extensively at both the cellular (49–53) and subcellular levels (54, 55). In this case, the synthesis of a "hemoglobin-like protein"

by "microsomal particles" has been demonstrated *in vitro* (54, 55). Although the morphology of the active cell fraction was not examined, the organization of the intact reticulocyte suggests that this fraction consists mainly of free RNP particles. A number of recent reports describe the *in vitro* incorporation of radioactive amino acids into the mixed proteins of preparations known, or assumed, to consist of RNP particles, isolated from a variety of sources including mammalian liver (32, 56), pea seedlings (57–59), yeast (57), and ascites tumor cells (60). All these observations suggest, in agreement with ours, that the RNP particles constitute an important, but not necessarily unique, site of intracellular protein synthesis.

We have investigated a more complex system, the pancreas, in which the synthesis of a variety, of proteins is followed by intracellular segregation transport, and storage. We have included in our inquiry all cytoplasmic fractions and we have secured information about the morphological composition of these fractions. We know, for instance, that by current standards the attached RNP particles represent a homogeneous preparation (*cf.* 31). Our results strongly suggest that the protein under study—α-chymotrypsinogen—is synthesized by the attached RNP particles and that the appearance of the protein in other cell components is related to intracellular transport and storage.

In vivo chymotrypsinogen synthesis has been investigated before in mouse pancreas by Daly *et al.* (61) who found that at all points examined the enzyme, as isolated by paper electrophoresis, was more heavily labeled in the final supernatant than in any other cell fraction. The discrepancy between their results and ours could be easily explained by differences in timing and technique. In their case, 30 minutes was the erliest time point investigated, a zymogen fraction was not isolated, and a substantial amount of chymotrypsinogen was regularly found in the final supernatant.

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