A Cytochemical Study on the Pancreas of the Guinea Pig

I. Isolation and Enzymatic Activities of Cell Fractions

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ABSTRACT

Pancreatic tissue, (guinea pig) homogenized in 0.88 M sucrose, was fractionated by differential centrifugation into a nuclear, zymogen, mitochondrial, microsomal, and final supernatant fraction.

The components of the particulate fractions were identified with well known intracellular structures by electron microscopy.

The fractions were analyzed for protein-N and RNA, and were assayed for RNase and trypsin-activatable proteolytic (TAPase) activity.

The zymogen fraction accounted for 30 to 40 per cent of the total TAPase and RNase activities, and its specific enzymatic activities were 4 to 10 times higher than those of any other cell fraction. The zymogen fraction was cytologically heterogeneous; zymogen granules and mitochondria represented its main components.

More homogeneous zymogen fractions, obtained by successive washing or by separation in a discontinuous density-gradient, had specific activities 2 to 4 times greater than the crude zymogen fractions.

Chymotrypsinogen was isolated by column chromatography from pancreas homogenates and derived cell fractions. The largest amount was recovered in the zymogen fraction.

The final supernatant had properties similar to those of the trypsin inhibitor described by Kunitz and Northrop.

INTRODUCTION

Microscopical studies of the mammalian pancreas have long recognized the presence of characteristic granules in the apical region of the exocrine or acinar cells (1–5) and have shown, in addition, that the granular population varies during the secretory cycle of the organ, reaching a minimum after feeding[1] when the digestive enzymes produced by the gland appear in its ducts and finally in the intestine (2, 3). Early biochemical studies have indicated that within the pancreas some of the important digestive enzymes, namely the proteases, occur in the form of inactive precursors (*cf.* 10). For these various reasons it was assumed that the apical granules of the exocrine cells consist of stored enzymes, primarily inactive proteases (3), and consequently the term "zymogen granules" was proposed for their designation. Further biochemical work resulted in the isolation of trypsinogen and chymotrypsinogen (10) from pancreatic extracts and in their identification as the inactive precursors of the proteases trypsin and chymotrypsin, respectively. More recently, the view that the zymogen granules consist of stored digestive enzymes received additional support from investigations showing that the amounts of protein (11) and proteases (11, 12) in the pan-

[1] A marked decrease in the intracellular population of apical granules was also observed after vagal excitation (6, 7) and after the introduction of lipides (6) or peptones (8) in the duodenum. In some of these cases, discharged material was detected in the lumina of the glandular ducts; in others, digestive activity was demonstrated in the intestine. A general survey concerning other agents (pilocarpine, secretin, pancreozymin, etc.) capable of inducing glandular secretion with simultaneous reduction in the number of apical granules, can be found in reference 9.

creatic tissue vary with the secretory cycle, the maxima corresponding to the highest granular accumulations in the apical regions of the acinar cells.

For a long time, however, the hypothesis concerning the enzymatic nature of zymogen granules rested entirely on indirect evidence. Only recently Marshall (13), using as specific histochemical reagents fluorescein-labelled antibodies to various pancreatic enzymes, found that chymotrypsinogen and procarboxypeptidase are localized in the zymogen granules[2] of bovine pancreas, while deoxyribonuclease and ribonuclease are more diffusely distributed, being present in all parts of the cell except nuclei and mitochondria. Hokin (14), applying the technique of differential centrifugation to homogenates of dog pancreas, succeeded in isolating a fraction which, in the light microscope, appeared to consist primarily of zymogen granules. In comparison with the whole homogenate, he found a marked concentration of proteases in this fraction with a much less marked, but still distinct, concentration of amylase and lipase activities.

In the work described in the present paper, a number of cell fractions were isolated by differential centrifugation from homogenates of guinea pig pancreas. The morphological components of these fractions were identified with known intracellular structures by electron microscopy. The protein and ribonucleic acid (RNA) contents, and the ribonuclease (RNase) and trypsin activatable protease[3] (TAPase) activities of the cell fractions were measured. In addition, a digestive pancreatic enzyme, chymotrypsinogen, was isolated by ion exchange chromatography, and the actual amounts of this protein in the various pancreatic cell fractions determined. The results confirm the classical hypothesis mentioned, and complement and extend the findings of Marshall (13) and Hokin (14). TAPase activity and chymotrypsinogen were found to be primarily concentrated in the fraction containing zymogen granules, whereas RNase appeared to be more diffusely distributed with higher concentrations in both the zymogen and the supernatant fraction of the homogenate. Finally, it was found that the supernatant fraction has properties similar to those of the trypsin inhibitor described by Kunitz and Northrop (15).

[2] This technique does not indicate an exclusive localization of chymotrypsinogen and procarboxypeptidase in the zymogen granules; according to Marshall (13), the clear cytoplasmic matrix around the granules is also intensely stained by fluorescent antibodies.

[3] The following enzyme precursors are known to be activated by trypsin: chymotrypsinogen, trypsinogen, and procarboxypeptidase. TAPase activity represents, therefore, the summated activity of the corresponding enzymes.

Experimental

Homogenization

The pancreatic glands of adult (~500 gm. weight) guinea pigs, starved for 24 to 40 hours, were removed under ether anesthesia after being separated by dissection from most of the surrounding adipose and connective tissue. The glandular tissue of 2 to 4 animals was pooled, minced, and homogenized at 0° to 4°C. either in 0.88 M sucrose, or in 1.42 M sucrose solution, or in the polyvinylpyrrolidone-containing mixtures used by Greenfield and Price (16, see also 17), or by Novikoff (18) for liver homogenization. The polyvinylpyrrolidone-containing media induced extensive agglutination of pancreatic particles and were therefore abandoned. There was no agglutination in the sucrose solutions. The preservation of zymogen granules was satisfactory at both concentrations, whereas the mitochondria appeared better preserved in the 1.42 M sucrose solution. The fractionation of pancreas homogenized in the higher concentration proved, however, to be a longer and more laborious procedure. For this reason, a 0.88 M sucrose solution was finally selected as a routine suspension medium. The final dilution of the homogenate was regularly adjusted to 1:10 (tissue weight:final homogenate volume).

Fractionation

An attempt was made to separate from pancreas homogenates a distinct zymogen fraction in addition to the usual nuclear, mitochondrial, microsomal, and supernatant fractions of current fractionation schemes. Out of a number of procedures and schemes tried for this purpose, two were finally selected.

A. Differential Centrifugation.—The differential centrifugation procedure of Hogeboom *et al.* (19) was adapted to yield the following fractions by successive centrifugations:

1. Nuclear fraction, by centrifuging the original homogenate for 10 minutes at 760 *g*.

2. Zymogen fraction, by centrifuging the supernatant of *1* (cytoplasmic fraction) for 20 minutes at 4300 *g*.

3. Mitochondrial fraction, by centrifuging the supernatant of *2* for 30 minutes at 20,000 *g*.

4. Microsomal fraction, by centrifuging the supernatant of *3* for 60 minutes at 105,000 *g*.

5. Final supernatant fraction represented by the supernatant of *4*.

This procedure was found advantageous for recovery studies, but unsatisfactory for determining the

specific activities of various cell components, because of the pronounced heterogeneity of the heavy fractions (*i.e.*, the nuclear, zymogen, and mitochondrial fractions) thereby obtained. To obviate this disadvantage, these fractions were washed as follows: The pellet of the nuclear fraction was rehomogenized in some cases in 0.88 M sucrose, and the suspension thus obtained was centrifuged for 10 minutes at 760 *g*. The resulting pellet constituted the washed nuclear fraction. The supernatant fluid was added to the cytoplasmic fraction (supernatant of *1* in the preceding scheme).

Two different washing procedures were used for the zymogen fraction. In the first procedure, the fraction was resuspended in 0.88 M sucrose and the suspension centrifuged for 20 minutes at 4300 *g*. The pellet obtained was designated zymogen-fraction-washed-once. The repetition of the washing procedure yielded a zymogen-fraction-washed-twice. A washing of this kind is expected to remove contaminants smaller or lighter than the zymogen granules and thus cut off the light "tail" of the fraction. For the other washing procedure, the resuspended zymogen fraction was centrifuged first for 10 minutes at 760 *g* and the resulting sediment was removed, thus eliminating the heavy "head" of the fraction, which contains mostly contaminants larger and heavier than the zymogen granules. The supernatant was collected and recentrifuged for 20 minutes at 4300 *g*. The new supernatant, containing light or small contaminants, was removed or discarded, whereas the corresponding pellet was saved and considered a zymogen fraction partially purified by both "head- and tail-washing." A similar combination of either successive "tail-washings" or combined "head- and tail-washings" was used for the mitochondrial fraction.

Macroscopically the zymogen pellets isolated by differential centrifugation appeared opaque and of light tan color, the mitochondrial pellets were tan and opaque, while the microsomal pellets were red and translucent. A relatively thick, opaque, white pellicle of fat droplets was found on top of the supernatants after centrifuging down the nuclear and zymogen fractions. A thinner, opaque, red pellicle formed on top of the supernatant during the sedimentation of the microsomes.

B. Differential Centrifugation in a Discontinuous Density-Gradient.—For reasons to be discussed later, the washing procedures described did not yield entirely satisfactory zymogen fractions. Consequently, a different separatory approach was tried, based this time on the procedure of Kuff and Schneider (20, 21; see also 22) and involving centrifugation in a discontinuous density-gradient. After experimenting with the number of layers, sucrose concentrations, and centrifugal fields, the following procedure was adopted: 15 ml. of a pancreatic cytoplasmic fraction were deposited on top of the following succession of layers of aqueous sucrose solutions: (from top to bottom) 1.02 M, 7 ml.; 1.60 M, 7 ml.; 1.84 M, 5 ml. The small, 50 ml. bottles containing these layers were centrifuged for 4 hours at 2000 *g* in the cold room using an International centrifuge model provided with a horizontal yoke. At the end of the centrifugal run, the top layer appeared less opaque and more reddish than the original cytoplasmic fraction; the 1.02 M sucrose layer was opaque and tan in color and contained heavy concentrations of material at the lower interface; the 1.60 M sucrose layer was milky with white flocculi at the lower interface; the bottom layer was clear and colorless, but a thin pellicle of red translucent material was found coating the bottom of the bottles. The successive layers were separated by using transfer pipettes with a long-drawn, horizontally bent tip.

Electron Microscopy

The nuclear, zymogen, mitochondrial, and microsomal pellets obtained by usual differential centrifugation were fixed *in toto* and processed as indicated in reference 23. Aliquots of the various layers in the discontinuous density gradient system were mixed with equal amounts of 2 per cent OsO_4 in 0.88 M sucrose. After 1 to 2 hours fixation at 0°C., the mixtures were centrifuged for 30 minutes at 105,000 *g*, and the tight pellets thus obtained were cut in orientable strips (*cf.* 23), and thereafter dehydrated and embedded as usually done with small blocks of tissue.

Chemistry

General.—The general procedures described in reference 23 were used for the chemical determinations carried out on pancreatic fractions. Protein nitrogen (N) was measured by a nesslerization method (24), and the RNA by the orcinol method (25).

Proteolytic Activity.—The proteolytic activity was determined by the method of Kunitz, as given in Northrop *et al.* (10). The assay system was prepared as follows: (*a*) an aliquot of the fraction to be tested was diluted to 1.0 ml. with 0.88 M sucrose solution and activated thereafter by incubation (48 hours in the cold room at 4°C.) with trypsin (2.0 ml. of a freshly prepared, 0.2 mg. per cent trypsin solution in 0.1 M phosphate buffer at pH 7.6). The activated aliquot was then provided with casein as substrate (1.0 ml. of a 1 per cent casein solution, freshly prepared by heating the casein in 0.1 M phosphate buffer pH 7.6 for 15 minutes at 100°C.), and the mixture was incubated for 30 minutes at 35°C. The enzymatic activity was stopped by the addition of 2 ml. of 10 per cent trichloroacetic acid (TCA). The incubated preparations were kept in the cold for 1 hour, and then cleared by centrifugation. The total proteolytic activity of the aliquot was determined by measuring the absorption of the supernatant at 280 mμ in a Beckman dU spectrophotometer. Two tissue concentrations were used in each experiment to make sure that the values recorded were on the straight part of the activity versus concentration curve. The controls for these determinations were: (*b*) the

same system, except that activation was carried out in phosphate buffer alone in the absence of trypsin; (*c*) the assay system to which TCA was added at zero time; (*d*) the assay system without any tissue fraction.

The amount of proteolytic activity dependent on trypsin activation (TAPase activity) was given by the difference between *a* and *b*. The amount of proteolytic activity originally present, or "spontaneously" activated during preparation procedures, corresponded to the difference between *b* and *c*. The catalytic amounts of trypsin added (4 μg.) gave a negligible hydrolysis of casein under the assay conditions (control *d*).

Ribonuclease Activity.—RNase activity was determined by a modification of the method of de Duve *et al.* (26). Preliminary assays were made to find the range of dilutions at which the enzyme activity will give a straight line when plotted against time or tissue concentration. The dilution usually required for this purpose was 1 gm. tissue equivalent in 100 ml. water. The assay system consisted of a suitably diluted aliquot of the fraction to be tested, 0.5 ml. of 0.5 M "tris" (tri- (hydroxymethyl) aminoethane) buffer pH 7.8, 1.0 ml. of RNA containing approximately 3 mg. of purified ribonucleic acid, and water to a final volume of 3.0 ml. The RNA was added last and quickly mixed with the rest of the system. The reaction was allowed to proceed for exactly 10 minutes at room temperature (~25°C.), and then stopped by the addition of 2.0 ml of 1.5 N perchloric acid (PCA) containing 0.25 per cent uranyl acetate. After sitting overnight in the cold room, the suspension was cleared by centrifugation, and the absorption of the supernatant fluid read at 260 mμ. Since it was found that commercial samples of yeast RNA gave a large, acid-soluble blank, they were solubilized as the sodium salt and purified before use by two successive precipitations with glacial acetic acid, followed by dialysis for 48 hours at 4°C. against 0.1 M acetate buffer, pH 5.0, with one change of buffer.

Chymotrypsinogen Isolation.—The preparation of acid extracts of the pancreas and the fractionation of these extracts on ion-exchange IRC-50 columns were performed by the method of Hirs (27), except that manually operated rather than automatic fractionation was employed. Pancreas tissue was homogenized in sucrose, and a part of the homogenate fractionated as described. To the whole homogenate and to the various fractions derived therefrom, sulfuric acid was added to a final concentration of 0.25 N. These suspensions were kept in the cold for 2 hours and then centrifuged. Each pellet was washed by resuspending in 0.25 N H_2SO_4, followed by recentrifugation. The washings were added to the corresponding supernatant fluid, which was subsequently dialyzed in the cold for 48 hours against frequently changed and continuously stirred 0.25 N H_2SO_4. The dialysate was brought to pH 5, the insoluble material was separated by centrifugation and discarded, while the supernatant was placed on a 0.9 square cm. x 30 cm. IRC-50 column, the resin being prepared as described in reference 28. The proteins in the extracted material were displaced from the column by washing it with consecutive 0.5 ml. aliquots of 0.2 M phosphate buffer, pH 6.0. The proteolytic activity of the eluates were determined as previously described.

Materials

The guinea pigs were obtained from the albino colony of this Institute. Crystalline chymotrypsinogen, trypsin, and RNase were obtained from the Worthington Biochemical Sales Company, Freehold, New Jersey.

RESULTS

Morphology

The isolated fractions were examined in the light microscope, under phase contrast optics, and in the electron microscope. In this section only the morphology of the fractions separated by usual differential centrifugation will be described. The morphology of the fractions obtained by centrifugation in a discontinuous density-gradient system, and changes following the washing of the zymogen and mitochondrial fractions will be covered later in conjunction with the biochemical findings.

The nuclear fraction was found to consist of isolated nuclei, many of them damaged, mixed with tissue debris (*e.g.* fragmented blood vessels and secretory ducts, collagen fibers) and, to a lesser extent, with whole or fragmented acinar cells. The fraction was also noticeably contaminated by zymogen granules and mitochondria. Washing removed part of the smaller contaminants, but the fraction, as a whole, remained highly heterogeneous.

The zymogen fraction.—Under light optics, the zymogen fraction appeared to consist of a rather homogeneous population of spherical, highly refringent granules 0.5 to 1.5 μ in diameter, thus confirming the results of Hokin (14). The electron microscope, however, showed that the fraction was disturbingly heterogeneous, with two major components in its particle population (Fig. 1). The first component was represented by spherical bodies, 0.5 to 1.5 μ in diameter, of high density and fine texture that were satisfactorily identified as zymogen granules because they had the same size, shape, density and texture as the zymogen granules found *in situ* in the apical region of the exocrine cells (compare Figs. 2 and 3). Like their intracellular counterparts, many of the isolated

granules possessed a limiting membrane. Most zymogen granules appeared morphologically "intact" after tissue homogenization and fractionation, and only occasionally damaged or disintegrating granules were encountered. The damaged granules showed irregular profiles and frequently occurred in small clusters. At their periphery, these clusters appeared to fray away into small particles and filaments (~400 A in diameter) which had the same density as the content of the zymogen granules. Occasionally, bundles of filaments of the type described were encountered "free" in the zymogen pellets. It is assumed that all these elements represent disintegration products of the zymogen granules.

The second important component was represented by mitochondria identified as such on account of their characteristic structure. Unlike the zymogen granules, most of the mitochondria appeared swollen and showed more or less extensive damage comparable to that described in liver mitochondria treated with distilled water or low concentrations of deoxycholate (29). Nuclei and microsomes were also present as minor components of the zymogen fraction. The zymogen pellets showed a certain amount of stratification, with zymogen granules predominant in the deeper layers and mitochondrial contamination particularly evident in the upper ones.

The mitochondrial fraction consisted of mitochondria contaminated by microsomes and, to a lesser extent, by zymogen granules. Most of the mitochondria isolated in 0.88 M sucrose were swollen and damaged (Fig. 4), an indication that the organelles are more fragile in the pancreas than in the liver. In 1.42 M sucrose, the mitochondria were better preserved. Although swollen and damaged specimens were still encountered, many isolated mitochondria showed clearly the two membranes, the cristae, the dense matrix, and the dense intramitochondrial granules that characterize the organelles *in situ* (Fig. 5).

The microsomal fraction has been described in a previous paper (30), in which the microsomes were recognized as being mostly fragments of the rough surfaced part of the endoplasmic reticulum of the acinar cells.

It is evident that the fractions isolated by differential centrifugation from pancreas homogenates are cytologically heterogeneous at the resolution level of the electron microscope, and that the heterogeneity is more pronounced in the "heavy" fractions (nuclei, zymogen, mitochondria). It is also apparent, however, that in each fraction the major constituent is the cell component indicated by the name. For this reason, the fractions thus obtained were considered acceptable as a starting material for biochemical work.

Biochemistry

General:

Table I shows, for a representative experiment, the amounts of protein-N and RNA found in a pancreas homogenate and in the fractions separated therefrom by usual differential centrifugation. The values per gram wet weight pancreas are not considered representative for the glandular tissue proper, because of the unknown amounts of protein and RNA contributed by the blood vessels and the connective and adipose tissue that surround and infiltrate the gland. Macroscopically the contamination was variable, but usually small. The relative values for the fractions are thought to be more significant, since in all experiments the amount of protein-N decreased in the same order, namely from the final supernatant to the nuclear, zymogen, microsomal, and mitochondrial fraction. Only 6 to 12 per cent of the protein contained in the original homogenates were recovered in this last fraction, a proportion appreciably smaller than that found in hepatic mitochondrial fractions which contain 20 to 25 per cent of the total cellular proteins (31). The low protein values found in pancreatic mitochondrial fractions can be explained in part by protein leakage during isolation (most isolated mitochondria are swollen and appear "extracted"), and by substantial losses to the zymogen fractions which appear heavily contaminated by mitochondria. It should be pointed out, however, that in the case of the pancreas, lower values than those found for liver are to be expected, since cytological observations clearly indicate that mitochondria are less numerous in the exocrine cell of the pancreas than in the parenchymal cell of the liver.

Most of the RNA of the homogenates was recovered in the microsomal fraction (~37 per cent) and in the final supernatant (~23 per cent). The relatively high RNA content of the latter is due to the presence of small ribonucleoprotein particles that can be isolated by further centrifugation as postmicrosomal fractions (30). For reasons to

TABLE I

Distribution of Protein-N, RNA, and Proteolytic Activity in Pancreas Homogenate and Fractions (Guinea Pig)

Methods are given in the Experimental section. The homogenate or fractions from one gram wet weight pancreas were diluted to 100 ml. with 0.88 M sucrose solution, and appropriate aliquots of these suspensions were used in the assays for proteolytic activity.

Preparation	Protein-N/gm.*	RNA/gm.*	RNA/mg. protein-N	Proteolytic activity			
				A‡	B§ (TAPase activity)		
				ΔE_{280}/gm.*	ΔE_{280}/gm.*	ΔE_{280}/mg. protein-N	Per cent**
	mg.	*mg.*	*mg.*				
Homogenate	24.10	7.24‖	0.30‖	34.5	113.9	4.7	(15)
Nuclear fraction	5.94	0.74‖	0.12‖	3.3	196.5	33.1	26
Zymogen fraction	3.58	0.66¶	0.18¶	0	273.5	76.5	36
Mitochondrial fraction	2.82	0.57	0.20	0	135.0	47.9	18
Microsomal fraction	3.00	2.71	0.90	2.2	86.5	28.8	12
Supernatant fraction	8.00	1.63	0.20	1.0	63.5	7.9	8
Total recovery	23.34	6.31	—	6.5	755.0	—	100

* Per gram wet weight pancreas pulp. This notation is used in all subsequent Tables.

‡ A, activity following preincubation without trypsin.

§ B, (total proteolytic activity following preincubation with trypsin) minus (A).

‖ Values corrected for amount of DNA which reacts and gives some color in orcinol reaction.

¶ Apparent RNA (*cf.* Results section).

** For calculating the figures in this column, the total additive recovery (755) is equated with 100.

be explained later, the apparent presence of RNA in the zymogen fraction is considered an artifact. In these experiments, the RNA/protein-N ratios for pancreatic microsomes were higher than those previously reported (30) and reached values comparable to those found for hepatic microsomes (23).

Phospholipides were present in small amounts in pancreatic homogenates and fractions derived therefrom and, at variance with the situation encountered in the liver, were not found to be particularly concentrated in the microsomal fraction (30). The phospholipide content per gram wet weight pancreas did not amount to more than 10 per cent of the corresponding value for liver. Low concentrations of phospholipides in pancreatic cell fractions (dog) have already been reported by Hokin (14). In his case, however, the phospholipides were more concentrated in the microsomes than in any other cell fraction.

Proteolytic Activity:

Table I shows also the proteolytic activity exhibited by the whole homogenate and by the various cell fractions after activation by trypsin. In all experiments except one, there was little activity in the whole homogenate and very little or no proteolytic activity in the fractions without activation by trypsin. Even when the tissue fractions were incubated in buffer for as long as 72 hours at 4°C., or for 3 hours at 35°C., there was little apparent proteolytic activity. The addition of catalytic amounts of trypsin caused nearly complete activation after 48 hours at 4°C., and these were the conditions used for preincubation. These results are in disagreement with those reported by Hokin (14), who found that preincubation with trypsin was not needed for obtaining full activity in pancreas homogenates and cell fractions.[4]

In all experiments performed, the amount and concentration of trypsin-dependent proteolytic (TAPase) activity was found to be highest in the zymogen fraction (Table I). It is assumed that most, if not all, of the activity found in the nuclear and mitochondrial fractions is explained by their contamination with zymogen granules. The TAPase activity of the microsomal fraction varied from one experiment to another, and amounted to 5 to 30 per cent of the activity of the zymogen fraction. Since the microsomes were not contaminated by zymogen granules, it is assumed that these findings reflect either the situation *in vivo* or are the result of enzyme adsorption on microsomal membranes. The last alternative presupposes that a number of zymogen granules disintegrate during fractionation and liberate proteolytic enzymes, which might be subsequently adsorbed on other cytoplasmic structures or remain "solubilized" in the final supernatant.

It is evident from the data in Table I and the morphological evidence in Fig. 1 that the pattern described for the intracellular distribution of proteolytic activity can be questioned on two main grounds, namely: (*a*) apparent shortcomings in the assay system, and (*b*) the heterogeneity of the

[4] In a single experiment, the homogenate and corresponding fractions showed high proteolytic activity with or without activation by trypsin. In this case, the refrigeration apparatus of the cold room broke down during the homogenization and further manipulation of the tissue, and the temperature eventually reached 15°C. Even in this case, the zymogen fraction had the highest specific activity among all cell fractions.

fractions, particularly of the zymogen fraction. These objections will be considered in turn and, to a certain extent, answered.

The reliability of the assay system can be questioned because the figure obtained by summing the activities of each and all fractions is considerably greater than the figure found for the activity of the whole homogenate. Hokin made the same observation and explained it by assuming the presence of a trypsin inhibitor in the homogenate (14). A similar explanation may be advanced for the 7-fold overrecovery obtained in these experiments. If it is assumed that a trypsin inhibitor is present in the original homogenate and that its distribution pattern among cell fractions is different from that of the zymogen, then overrecovery is to be expected, especially if most of the inhibitor separates in one fraction and most of the zymogen in another. Wherever present, such an inhibitor could inactivate totally or partially the trypsin used in preincubation, and thus block the activation of the zymogens of the preparations.

To test the hypothesis that a trypsin inhibitor is present in the homogenate and is unequally distributed among its fractions, the experiments recorded in Tables II and III were performed. It can be seen (Table II) that when the various cell fractions were combined two by two, there was a marked deviation from the calculated activity only when the final supernatant was one of the combined fractions. As a control, it was noted that the small amounts of proteolytic activity detected after preincubation without trypsin were additive upon fraction recombination, even when the final supernatant was one of the fractions involved. These observations indicate that the final supernatant contains an inhibitor which affects apparently the activation process by trypsin.

Another type of experiment demonstrating the presence of an inhibitor in the final supernatant is illustrated by Table III. In this experiment, the homogenate was divided into two aliquots; the first one was fractionated according to the usual scheme, whereas the second was centrifuged for 60 minutes at 105,000 *g*, to sediment all particulate components in a common pellet and thus separate them from the final supernatant. The original homogenate, its individual and recombined fractions, as well as the common pellet of the particulate components (taken to represent the homogenate minus the final supernatant) were tested for TAPase activity. When the final supernatant was excluded, satisfactory agreement was found between the determined and calculated TAPase activity of the remaining fractions. The table shows, for instance, that the activity of the recombined particulate fractions, and that of the common particulate pellet, come close to the sum of the activities of the same fractions tested individually. As expected, there is no agreement between determined and calculated activities when the final supernatant is included in the assayed preparations.

The data in Tables II and III indicate clearly that the factor disturbing our recovery experiments is located in the final supernatant and suggest that this factor may be a trypsin inhibitor capable of forming an inactive complex (15) with the trypsin added during preincubation for the purpose of zymogen activation.

TABLE II

The Effect of the Recombination of Pancreatic Cellular Fractions upon TAPase Activity

The methods for determining TAPase activity are given in the Experimental section. In previous experiments with each fraction, the concentration range was determined within which there was a satisfactory proportionality between activity and concentration. Concentrations within this range were then used in the recombinations shown in the table. The calculated value was obtained by adding the values given by each fraction when incubated separately.

Nuclear fraction	+	+	+	+						
Zymogen fraction	+				+	+	+			
Mitochondrial fraction		+			+			+	+	
Microsomal fraction			+			+		+		+
Supernatant fraction				+			+		+	+
Actual reading ΔE_{280}	0.790	0.620	0.720	0.190	0.910	0.880	0.265	0.715	0.145	0.135
Calculated value ΔE_{280}	0.750	0.615	0.700	0.320	0.765	0.845	0.465	0.705	0.320	0.440
Actual calculated × 100	105	101	103	59	119	104	57	101	45	31

TABLE III

Comparison of Found-with Calculated Proteolytic Activity in Pancreas Homogenate and Fractions (Guinea Pig), Incubated either Separately or Together

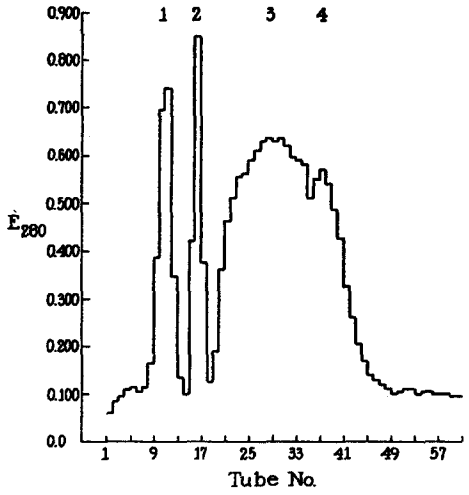
Data in this Table and in Table V are from the same experiment. Methods are given in the Experimental section and in Table V. A and B have the same connotation as in Table I.

Preparation	Proteolytic activity		
	A	B	
	ΔE_{280}/gm.	ΔE_{280}/gm.	Per cent
Whole homogenate	80.0	368.0	(27)
All derived fractions, incubated together	80.0	360.0	26
All derived fractions, incubated separately, additive recovery	72.5	1371.5	100
All derived fractions except supernatant, incubated together	53.8	1042.0	76
All derived fractions except supernatant, incubated separately, additive recovery	42.5	1199.0	87
The common pellet of the particulate fractions*	31.3	1000.0	73
Supernatant	30.0	172.5	13
Supernatant, and the common pellet of the particulate fractions,* incubated separately, additive recovery	95.0	1214.0	89

* This pellet was obtained by centrifuging the whole homogenate at 105,000 *g* for 60 minutes. It represents the homogenate minus the final supernatant.

Chymotrypsinogen Distribution among Cell Fractions:

In addition to the difficulties introduced by the presence of an inhibitor, the assay procedure thus far used has the basic disadvantage of measuring the cumulative activities of all pancreatic proteases, not the activity of a single enzyme. To obviate these shortcomings, an attempt was made to isolate a single enzyme, actually the protease precursor chymotrypsinogen, from the pancreas homogenate and its various fractions by ion exchange chromatography on columns. Preliminary experiments using crystalline bovine chymotrypsinogen gave results similar to those reported by Hirs (27), in that the major trypsin-activatable, proteolytic component came off the



TEXT-FIG. 1. Chromatography of 0.25 N sulfuric acid extracts of guinea pig pancreas homogenate on an IRC-50 column. Conditions are given in the Experimental section. The curve was obtained with an extract from 1.4 gm. wet weight pancreas. Each tube contained 0.5 ml. eluant.

column at approximately 23 ml., with a much smaller component coming off at about 10 ml. Extracts of guinea pig pancreas gave a picture (Text-fig. 1) similar to that of extracts of bovine pancreas (27) with a few differences: namely, (*a*) the major component (Text-fig. 1, peak 3) came out about 5 ml. earlier; (*b*) in some experiments there was evidence of a second component immediately following (the hump at tubes 36 to 38 in Text-fig. 1); (*c*) the major peak was preceded by two other smaller components (peaks 1 and 2 in Text-fig. 1). None of these components were spontaneously proteolytic, but all of them showed proteolytic activity upon the addition of catalytic amounts of trypsin. Peak 1 might be chymotrypsinogen B (27), since in some cases it began to come off the column as soon as elution was started. Peak 2 is similar in placement to a peak of unknown identity found in bovine pancreas (27). Peak 3 corresponds to chymotrypsinogen A, and peak 4 is as yet unidentified. These four peaks were found in all experiments with extracts of guinea pig pancreas, with peaks 2, 3, and 4 coming off at approximately the same place each time. When the homogenate was fractionated and extracts made of the cellular fractions, these three peaks were present in various amounts in all fractions. The data on their distribution are given in Table IV. Measurements for peak 1 were not considered reliable enough

TABLE IV

Amounts of Chymotrypsinogen Isolated by Column Chromatography from Pancreas Homogenate and Cell Fractions

Methods are given in the Experimental section. The figures represent chymotrypsinogen equivalents; that is, that amount of crystalline chymotrypsinogen which, after activation, would give proteolytic values equal to those found in this experiment. These amounts were taken from a curve prepared with crystalline bovine chymotrypsinogen tested under assay conditions identical to those used for the determinations in this table. The figures represent the amount found in one gram wet weight pancreas pulp. For the calculation of percentage figures, the total additive recovery (368 and 1330) was equated to 100.

Preparation	Peak 2		Peaks 3 and 4	
	Chymotrypsinogen Equivalent	Per cent	Chymotrypsinogen Equivalent	Per cent
	μg.		μg.	
Homogenate	265	(72)	902	(68)
Nuclear fraction	85	23	370	28
Zymogen fraction	234	64	785	59
Mitochondrial fraction	5	1	47	4
Microsomal fraction	8	2	80	6
Supernatant fraction	35	10	48	3
Total recovery	368	100	1330	100

for tabulation, since in some cases the corresponding component began to come off immediately after the elution was started. The reason for the greater than 100 per cent recovery, when the activity of all the fractions were summated is not known. It may be explained by assuming that the extraction was adequate for the separated fractions, but not entirely satisfactory for the whole homogenate. The table shows clearly that the major portions, *i.e.*, ~60 per cent, of peaks 2 and peaks 3 and 4 are found in the zymogen fraction. It follows that the results obtained by isolating chymotrypsinogen from the various cell fractions are substantially in agreement with the measurements of total proteolytic activity in the same fractions (Table I). The proteolytic components separated by column chromatography from the nuclear fraction can again be explained by contamination with zymogen granules either free or included in cell fragments. A similar explanation may be advanced for the chymotrypsinogen present in the mitochondrial fraction. The amount of material recoverable in peaks 3 and 4 represents about 4 per cent of the calculated original dry weight of the pancreas, a figure higher than that reported by Hirs (27) for chymotrypsinogen in bovine pancreas, but close to the value reported by Daly and Mirsky (12) for the same zymogen in mouse pancreas. The small amount of chymotrypsinogen found in the final supernatant vindicates the results of the enzymatic assays shown in Table I and shows that, irrespective of the inhibiting effect described, this fraction has a low proteolytic activity, at least as far as chymotrypsinogen is concerned.

Solubilization of Zymogen Granules:

As it is known (*cf.* 32), erratic results in enzymatic assays can also be caused by structural "barriers" which prevent the ready access of substrate to enzyme or *vice versa*. In the case of the zymogen granules, the possible effect of such barriers must be considered, since these granules are bounded by a membrane and their dense content is tightly packed (30). When pellets of the zymogen fraction were resuspended into the assay medium (which has a pH of 7.6 and in which the final sucrose concentration is below 0.03 M), and were examined in the light microscope under phase contrast optics, it was found that the zymogen granules lost their high refringence and disintegrated. It appears, then, that under the conditions of the assay, lysis of the granules occurs, and that contact of substrate with enzyme could be achieved without structural limitations.[5] The preceding observation is in agreement with the findings of Hokin (14), who reported that zymogen granules isolated in 0.25 M sucrose disintegrate when resuspended in distilled water, or when the pH of the original suspension (0.25 M sucrose) is brought up to 7.2. Earlier findings by Claude (33) also indicate that zymogen granules retain their morphology when isolated in isotonic saline solution, but swell and break when isolated in distilled water.

Attempts to Purify the Zymogen Fraction:

Washing.—From the preceding experiments it can be concluded that the assay procedure used

[5] Notwithstanding these observations, attempts were made to increase enzymatic activity by procedures expected to disrupt the zymogen granules. When 0.3 per cent deoxycholate, pH 7.5 to 7.7, a detergent known to disrupt microsomes (23, 30) and mitochondria (29) was tried, it was found that upon its addition (during the trypsin activation carried at 4°C. for 48 hours), the zymogen fractions gelled. Incubation of cell fractions for 3 hours at 35°C., instead of the usual 48 hours at 4°C., produced a uniform 80 to 90 per cent decrease in activity in all the fractions, but did not affect the crystalline chymotrypsinogen controls.

is reliable, and that a large part of the TAPase activity of the cell and of its chymotrypsinogen content is recovered in the zymogen fraction. This fraction, however, is noticeably heterogeneous, and for this reason it was deemed necessary to eliminate its various contaminants (nuclei, mitochondria) before specifically localizing the enzymes under study in the zymogen granules. To this intent washing procedures were initially used. The zymogen fraction washed once showed no striking modification in the morphology of its components, except that the zymogen granules appeared frequently clumped. After two tail-washings the granules were definitely agglutinated (Fig. 6), and the periphery of their clumped masses frequently showed signs of disintegration in the form of fraying filaments (Fig. 7) or rods. Bundles of similar filaments were encountered in profusion throughout the pellets (Fig. 8). Bundles and masses of similar density and comparable texture were frequently encountered *in situ*, in the lumina of pancreatic acini and ducts (Fig. 9), during zymogen discharge. The mitochondrial contamination seemed to be reduced after washing, and all remaining mitochondria appeared swollen and extensively extracted. Head- and tail-washing of the zymogen fraction induced less agglutination and disruption of zymogen granules, but left the fraction still noticeably contaminated.

The washing of the mitochondrial fraction reduced the microsomal contamination and caused further extraction, swelling, and bursting of the mitochondria. Chemical determinations and enzymatic assays performed on these washed fractions are given in Table V. It is clear that both head- and tail-washing produced a marked increase in the specific TAPase activity of the zymogen fractions, supposedly by eliminating part of the nuclear and mitochondrial contamination of the original preparations. By contrast, the washing of mitochondrial fractions did not lower their specific TAPase activity. It reduced, however, their

TABLE V

Effects of Washing on Properties of Zymogen and Mitochondrial Fractions

Chemical and assay methods are given in the Experimental section. The zymogen (Z) and mitochondrial (Mt) fractions were prepared by the differential centrifugation procedure. For washing, the zymogen pellet was resuspended in 0.88 M sucrose solution. Spinning at 760 *g* for 5 minutes produced a pellet and supernatant, the latter being the "head-washed" zymogen fraction. Another spinning at 4,300 *g* for 20 minutes produced a pellet and supernatant, the pellet being the "tail-washed" fraction. Combining the two procedures produced a "middle" zymogen fraction with "head" and "tail" sections removed. Similarly, the mitochondria fraction was resuspended in 0.88 M sucrose solution. Spinning at 4,300 *g* for 10 minutes produced a pellet and supernatant, the latter being the head-washed mitochondrial fraction. Another spinning at 20,000 *g* for 30 minutes produced a pellet and supernatant, the pellet being the tail-washed fraction.

Experiment No.	Fraction	Protein-N/gm.	RNA/gm.	RNA/mg. protein-N	TAPase Act.		RNase Act.	
					$\Delta E_{280/gm.}$	$\Delta E_{280/mg. protein-N}$	$\Delta E_{260/mg.}$	$\Delta E_{260/mg. protein-N}$
		mg.	*mg.*	*mg.*				
1	Z-unwashed	3.77	0.72*	0.19*	228	61	1660	441
	" -tail-washed	1.67	0.34*	0.20*	158	94	930	556
	" -head-washed	2.47	0.56*	0.22*	227	92	1550	628
	" -head- and tail-washed	0.92	0.16*	0.17*	115	125	740	805
2	Z-unwashed	2.80	0.45*	0.16*	196	70	1620	579
	" -tail-washed once	1.62	0.22*	0.14*	191	118	1370	845
	" -tail-washed twice	1.27	0.16*	0.12*	158	124	1150	897
1	Mt-unwashed	1.85	0.56	0.30	66	36	750	405
	" -head-washed	1.53	0.53	0.35	55	36	620	400
2	Mt-unwashed	1.57	0.36	0.23	60	38	550	350
	" -tail-washed once	0.77	0.10	0.13	27	35	240	312
	" tail-washed twice	0.52	0.05	0.10	18	34	140	259

* Apparent RNA (*cf.* Results section).

RNA content and lowered their RNA/protein-N ratios, a finding which could be satisfactorily correlated with the partial elimination of microsomal contaminants. It should be pointed out that the washing of zymogen fractions caused little change in the same ratios. This situation will be discussed later.

When recovery experiments of the type shown in Table I were repeated with washed zymogen and mitochondrial fractions, the data in Table VI were obtained. A comparison of the two tables makes it clear that head- and tail-washing of the zymogen fraction raised appreciably the specific activity: the washed fractions were twice as active as the unwashed parent preparations, and 4 to 15 times more active than any other cell fraction. At the same time, however, the total amount of proteolytic activity in the zymogen fractions decreased, while that in the other fractions increased, indicating that, within the frame of a recovery experiment, the washing produced a transfer of activity from the zymogen fraction into the adjacent fractions. These results indicate that a partial purification of the zymogen fraction can be obtained by washing, at the expense, however, of heavy losses in protein (~75 per cent) and in total TAPase activity (~50 per cent). Repeated manipulations result, in addition, in clumping and disintegration of zymogen granules in a still heterogeneous fraction, a situation which evidently precludes further purification. It follows that if a more homogeneous zymogen fraction is desired, procedures other than usual differential centrifugation followed by washing should be tried.

TABLE VI

Distribution of Protein-N, RNA, and Proteolytic Activity in Pancreas Homogenate and Washed Fractions (Guinea Pig)

Methods are given in the Experimental section and in Table V. The zymogen fraction was "head-" and "tail-washed," the head-wash being put with the nuclear fraction and the tail-wash with the mitochondrial fraction. The mitochondrial fraction was head-washed, the head-wash being put with the zymogen fraction. A and B have the same connotation as in Table I.

Preparation	Protein-N/gm.	RNA/gm.	RNA/mg. protein-N	Proteolytic activity			
				A	B (TAPase activity)		
				ΔE_{280}/gm.	ΔE_{280}/gm.	ΔE_{280}/mg. protein-N	Per cent‡
	mg.	*mg.*	*mg.*				
Homogenate	18.57	7.75	0.42	80.0	368.0	19.7	(27)
Nuclear fraction	5.52	1.82	0.33	12.5	409.5	74.1	30
Zymogen fraction	0.69	0.11*	0.15*	1.2	294.0	427.0	21
Mitochondrial fraction	3.50	0.94	0.27	7.5	229.0	65.4	17
Microsomal fraction	2.67	3.16	1.18	21.3	266.5	99.5	19
Supernatant fraction	6.05	1.93	0.32	30.0	172.5	28.6	13
Total additive, recovery	18.43	7.96	—	72.5	1371.5	—	100

* Apparent RNA (*cf.* Results section).

‡ For calculating the figures in this column the total additive recovery (1371.5) was equated to 100.

Isolation of a Zymogen Fraction by Centrifugation in a Discontinuous Density-Gradient:

For the reasons mentioned in the preceding paragraph, pancreas homogenates were differentially centrifuged in the discontinuous density-gradient described in detail on page 205, where the macroscopic results of this type of fractionation have already been presented. In the light microscope, under phase contrast optics, the milky, 1.60 M sucrose layer appeared to contain only zymogen granules; the 1.02 M sucrose layer contained mitochondria with a few zymogen granules; whereas the 0.88 M sucrose layer retained only microsomes. When pellets obtained from these layers were examined in the electron microscope, they were found to have the following composition:

1.60 M sucrose layer: numerous, intact, easily recognizable zymogen granules,[6] and a relatively small number of damaged mitochondria. The clumps of disintegrating zymogen granules and the bundles of filamentous material were noticeably less numerous than in the fractions obtained by usual differential centrifugation (Fig. 10).

1.02 M sucrose layer: numerous mitochondria (most of them damaged), a few zymogen granules, and a few microsomes.

0.88 M sucrose layer: microsomes, small particles, lipide droplets, and unidentified membranous material.

No attempt was made to recover quantitatively each of these fractions, because of the interface

[6] In more recent experiments in which a much stronger centrifugal field was used (53,000 *g* x 2 hours; Spinco model L centrifuge provided with a swinging bucket rotor SW 25.1), the zymogen granules were again recovered in the 1.60 M sucrose layer. Assuming that under these conditions equilibrium is reached, it follows that the specific gravity of the zymogen granules is around 1.26. This value is close to the one presented in the literature for the density of crystals of various proteins.

TABLE VII

Comparison of Enzymatic Activities of Zymogen Fractions Obtained by Usual Differential Centrifugation and by Centrifugation in a Discontinuous Density-Gradient

Methods are given in the Experimental and Results sections. The fractions compared were obtained from the same homogenate prepared in 0.88 M sucrose solution. In the differential centrifugation technique the zymogen fraction was "head-" and "tail-washed" as described in Table V. The zymogen fraction obtained by centrifugation in a discontinuous density-gradient was not washed.

Technique	RNA/mg. protein-N*	TAPase activity	RNase activity
		ΔE_{280}/mg. protein-N	ΔE_{280}/mg. protein-N
	mg.		
Differential centrifugation	0.19	147.2	124.8
Discontinuous density-gradient	1.00	202.1	203.0

* Apparent RNA (*cf.* Results section).

disturbances produced when the layers were pipetted off. Representative samples were obtained, however, from the various layers, and the ones taken from the 1.60 M sucrose layer proved to have high TAPase activity. Table VII presents the results of an experiment in which the specific TAPase activity of a zymogen fraction obtained by usual differential centrifugation followed by head- and tail-washing was compared with the specific TAPase activity of a zymogen fraction (1.60 *M* sucrose layer) obtained by centrifugation in a discontinuous density-gradient. Both fractions were separated from aliquots of the same homogenate. It is evident that the fraction isolated by the second procedure is ~60 per cent more active than the best zymogen fraction obtained by the previous technique. The result parallels the decrease in contaminating elements, primarily mitochondria, in the corresponding pellets. A comparison with data in Table VI shows that the specific activity of the new zymogen fraction is six to twenty times higher than that of any other cell fraction.

Distribution of Ribonuclease Activity:

Data concerning the RNase activity of the various pancreas fractions are given in Tables V, VII, and VIII. No difficulties were encountered in the assay system for this enzyme.[7] The activity of the original homogenate was satisfactorily recovered in the fractions isolated therefrom and tested either individually or upon recombination (Table VIII). RNase activity, like proteolytic activity, was found concentrated in the zymogen fraction. Its concentration was, however, less pronounced, as indicated by the fact that the specific activity of the zymogen fraction for RNase was only 4 to 9 times higher than that of any other fraction. Of the total activity of the homogenate, 30 to 40 per cent (a figure comparable to that found for TAPase activity) was recovered

TABLE VIII

The Distribution of Ribonuclease Activity in Pancreas Homogenate and Fractions (Guinea Pig)

Enzymatic assay as given in the Experimental section. In Experiment 1 the fractions (as in Table I) were not washed. In Experiment 2 the zymogen and mitochondrial fractions were washed (as in Table VI).

Preparation	Experiment 1			Experiment 2		
	ΔE_{280}/gm.	Per cent	ΔE_{280}/mg. protein-N	ΔE_{280}/gm.	Per cent	ΔE_{280}/mg. protein-N
Homogenate	3350	100	139	4200	100	284
Nuclear fraction	250	8	42	685	16	155
Zymogen fraction	1180	35	321	490	12	892
Mitochondrial fraction	550	16	195	550	13	196
Microsomal fraction	630	19	210	830	20	388
Supernatant fraction	1010	30	126	1450	34	300
Total recovery	3620	108	150	4005	96	270
All fractions incubated together	2830	85	121	4080	97	275

[7] As in the case of the assays for proteolytic activity, attempts were made to eliminate structural barriers by using procedures expected to break up the particulates and thus activate latent enzyme activity. When the preparations were preincubated in the assay medium for 60 minutes at 35°C., and then tested for RNase activity, no increase in activity was found in any of the fractions nor in crystalline RNase controls. When the fractions were tested in the regular way, but in the presence of 0.3 per cent DOC, pH 7.5 to 7.7, there was a *general and proportional* increase (of the order of 50 per cent) for all the fractions and the homogenate. Since the crystalline RNase controls were not affected by the presence of 0.3 per cent DOC, no reason for the increase in the RNase activity of the fractions can be given at present. No indication was found of the presence of an RNase inhibitor, particularly in the supernatant fraction, as had been found for various tissues of the rat (34, 35).

in the zymogen fraction, but more than 30 per cent remained in the final supernatant in all experiments. The specific activity of the zymogen fractions for RNase was increased in approximately the same proportion as their TAPase activity by washing (~80 per cent) (Tables V and VIII), and by separation in a discontinuous density-gradient (~80 per cent) (Table VII). Again, as in the case of the TAPase activity, a severe loss in total activity (~70 per cent) occurred during washing, apparently by transfer to other fractions (Table VIII).

The Orcinol Reaction in Extracts of the Zymogen Fraction:

In our experiments a positive orcinol reaction was found in the hot TCA extracts of all zymogen fractions. The color was initially attributed to RNA, which appeared to be present in amounts large enough to give a RNA/protein-N ratio of 0.15 to 0.22 in all experiments. The finding was in disagreement with the data of Hokin (14), who reported only traces of RNA (3 μg. RNA-P/mg. N) in his zymogen fractions, as well as with classical histochemical observations,[8] but could be tentatively ascribed to the heavy contamination of the fraction. The washing of the zymogen fractions was not followed, however, by a lowering of the ratio, although the contamination was reduced (Tables V, VI, and VII). Because of these unexpected results, the absorption spectrum of the pale green color developed by the orcinol reaction (25) on the hot TCA extract of the zymogen proteins was recorded. In addition to the small peak at 660 mμ, another peak of equal size was found at 420 mμ. This second peak indicates the presence of hexoses (25). A further advance in this direction was made possible by the isolation of zymogen fractions in a discontinuous density-gradient. Extracts of this fraction gave with the orcinol reagent a distinctly brown color with a large peak at 420 mμ, and with a smaller but distinguishable peak at 660 mμ, this second peak being high enough to bring the "RNA/protein-N ratio" of the preparation to the seemingly high value shown in Table VII. The absorption curve of the brown compound was very similar to that produced by fructose, in both cases the extinction at 420 mμ being four times greater than at 660 mμ. The compound obtained with mannose yielded a similar curve, except that the peak at 420 mμ was only twice as high as that at 660 mμ. The hot TCA extract of the zymogen fraction isolated in a discontinuous density-gradient gave a strong positive anthrone reaction (36) with a time curve of color production similar to that given by mannose and clearly different from that yielded by fructose (*cf.* 36). However, tests for the presence of reducing sugars (37) were negative for both unhydrolyzed and hydrolyzed (in 0.1 N HCl at 100°C. for 60 minutes and in 6 N HCl at 100° for 15 minutes) extracts. These preliminary results are compatible with the hypothesis that the zymogen granules contain a glycoprotein with a non-reducing sugar moiety. It is assumed that the glycoprotein comes down with the TCA-precipitated material of the zymogen fraction, is not washed out by cold TCA, warm alcohol, and alcohol-ether, but is extracted (or at least the glucosidic part of the compound is extracted) by hot 5 per cent TCA. The reason for the difference between the reactions given by the extracts of the two types of zymogen fractions described in this paper is not known. It may be assumed that the compound responsible for the color is more easily extractable in 0.88 M than in 1.60 M sucrose. The presence of this compound renders uncertain the results of RNA determinations in zymogen fractions. Absorption at 660 mμ may be due to the hexose of a glycoprotein and not, as initially assumed, to the ribose of RNA; consequently the zymogen granules may contain very little, if any, RNA.[9] The type of orcinol reaction described for the extracts of the zymogen fraction has not been encountered in the extracts of the other cell fractions.

DISCUSSION

The experiments reported indicate that a zymogen fraction, consisting primarily of easily recognizable, apparently intact zymogen granules and of damaged mitochondria, can be isolated from homogenates of guinea pig pancreas by differential centrifugation. In the usual fractionation scheme, this fraction occupies an intermediary position between the nuclear and mitochondrial fractions. The zymogen fraction thus obtained accounts for approximately 40 per cent of the TAPase of the pancreatic homogenates, and its specific TAPase activity is 5 to 10 times higher than that of the other cell fractions. Systematic examination of the various pellets in the electron microscope showed that the zymogen fraction did not contain all the zymogen granules of the homogenate; in smaller numbers, such granules were found as contaminants in the adjacent nuclear and mitochondrial fractions. Their presence therein could explain the relatively high total and specific activity of these fractions. By repeated washings

[8] The cytoplasm of the apical region of the acinar cells, in which the zymogen granules are located, is distinctly and characteristically acidophilic.

[9] Further work is proposed to verify this point by measuring nucleic acid phosphorus, and by determining the identity of the carbohydrate seemingly contained in the zymogen granules.

the specific activity of the zymogen fraction was almost doubled. The centrifugation of pancreas homogenates in a discontinuous density-gradient yielded a morphologically less heterogeneous and biochemically more active zymogen fraction. It is admitted that a zymogen fraction entirely free of mitochondrial contamination could not be obtained, but it is evident that the specific activity of this fraction was increased by the progressive reduction of mitochondrial contamination, and it is also clear that the mitochondrial fractions themselves were not particularly active.

In view of these results, it can be concluded that a large part of the trypsin-activatable proteolytic enzymes (*i.e.* mainly trypsinogen and chymotrypsinogen) produced by the pancreatic exocrine cells is localized in the zymogen granules. This conclusion appears to be well founded for chymotrypsinogen which was isolated in a purified state from zymogen fractions by ion exchange chromatography and applies also, in all probability, for trypsinogen. Our findings thus confirm and extend the results obtained by Hokin, who isolated only a zymogen fraction and compared its proteolytic activity to that of the whole homogenate (14).

In our experiments, the zymogen fraction also contained about one third of the RNase activity of the whole homogenate and had a higher specific activity than all the other cell fractions. An equal amount of activity was present, however, in the final supernatant, which is supposed to correspond primarily to the cell sap. It is not known whether the presence of RNase in the final supernatant is a true representation of the situation *in situ* or whether it is due to leakage from the zymogen granules during tissue homogenization and differential centrifugation. In this respect it should be pointed out that Marshall (13), by using labelled antibodies, arrived also at the conclusion that the intracellular localization of RNase is more diffuse than that of chymotrypsinogen.

Hokin found that, in addition to proteolytic activity, the zymogen granules show lipase and amylase[10] activity at concentrations higher than those of the whole homogenate (14). It appears, then, from our results, from those of Hokin, and from the observations of Marshall, the five hydrolytic enzymes or enzyme precursors, namely chymotrypsinogen, trypsinogen, procarboxypeptidase, amylase, and lipase are concentrated to an appreciable extent in the zymogen granules. Since they represent the main pancreatic enzymes known to be secreted for digestive purposes, it can be assumed, as already implied by Schucher and Hokin (39), that that part of the pancreatic RNase which is located in the zymogen granules is also a digestive enzyme produced for excretion in the intestine.

The segregation of all these hydrolytic enzymes into compact, membrane-bound zymogen granules could represent a protective device, since these enzymes could autolyze the cell if free to move throughout it. In the case of the proteolytic enzymes, a further degree of protection is afforded by the presence of a trypsin inhibitor in the supernatant fraction (cell sap ?) and by the fact that these enzymes are elaborated and stored intracellularly in the form of inactive precursors. It is probable that mechanisms which prevent the intracellular activity of ribonuclease, amylase, and lipase are also present within the pancreatic exocrine cell, but thus far they have escaped detection.

The distribution of RNase activity among pancreatic cell fractions is noticeably different from that revealed by similar methods in the case of the liver. Schneider and Hogeboom (40) found, for instance, that the RNase activity of mouse liver homogenates was almost entirely recovered in the mitochondrial fraction, while the microsomes and the final supernatant showed respectively little and negligible activity. They tentatively ascribed microsomal activity to adsorbed enzyme. By using a more critical cell fractionation, de Duve *et al.* arrived at the conclusion that in the liver cells of the rat the RNase is located (together with a whole series of other hydrolytic enzymes) in granules smaller than, and distinct from, mitochondria for which the name "lysosomes" was proposed (26). The morphological identification of the lysosomes is still uncertain, but the structure of the putative bodies (41) is noticeably different from that of the zymogen granules. In the kidney, Strauss (42) found the same series of hydrolytic enzymes, RNase included, concentrated in dense "droplets" (0.1 to 5 μ) apparently distinct from mitochondria. Finally, an inhibitor of alkaline RNase has been found (34) in the final supernatant of the rat liver, and other rat tissues (35), but thus far a similar inhibitor has not been detected in the pancreas.

[10] According to Laird (38), most of the amylase activity is recovered in the microsome fraction. In her experiments, however, a distinct zymogen fraction was not isolated.

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Explanation of Plates

Plate 104

Fig. 1. A relatively large, representative field in the sectioned pellet of a crude zymogen fraction isolated by differential centrifugation from pancreatic tissue (guinea pig), homogenized in 0.88 M sucrose.

The dense profiles marked *z* represent zymogen granules. Their size variation is due, at least in part, to sectioning: the smaller profiles usually correspond to lateral sections, whereas the larger ones represent medial or median sections through the granules. The oval form of the profiles is due to deformation during sectioning, as indicated by the fact that all long axes are oriented in the same direction. In less deformed, thick sections, the profiles are circular. Close examination reveals numerous cutting faults, such as roughness of the cut surfaces (see profile marked *ir*) and masses of dense material piled up where the knife left the granules (arrows). These faults indicate that the zymogen granules are difficult to embed and section.

Clusters of agglutinated granules with irregular outline appear at *c* and ill defined masses of dense material at *x*. Both these appearances presumably represent disintegrating zymogen granules.

Numerous profiles of damaged mitochondria (m_1, m_2) are also present in the pellet. Their general appearance indicates that they swelled and were extensively extracted during the homogenization and fractionation of the tissue. Remnants of characteristic structural details, *e.g.*, cristae, can be seen at m_1; they are easily recogniazble at higher magnifications (see Figs. 4 and 5).

In conclusion, the analysis of the micrograph shows that the zymogen fraction consists of zymogen granules, most of them intact, and of extensively damaged mitochondria.

Magnification: 10,000.

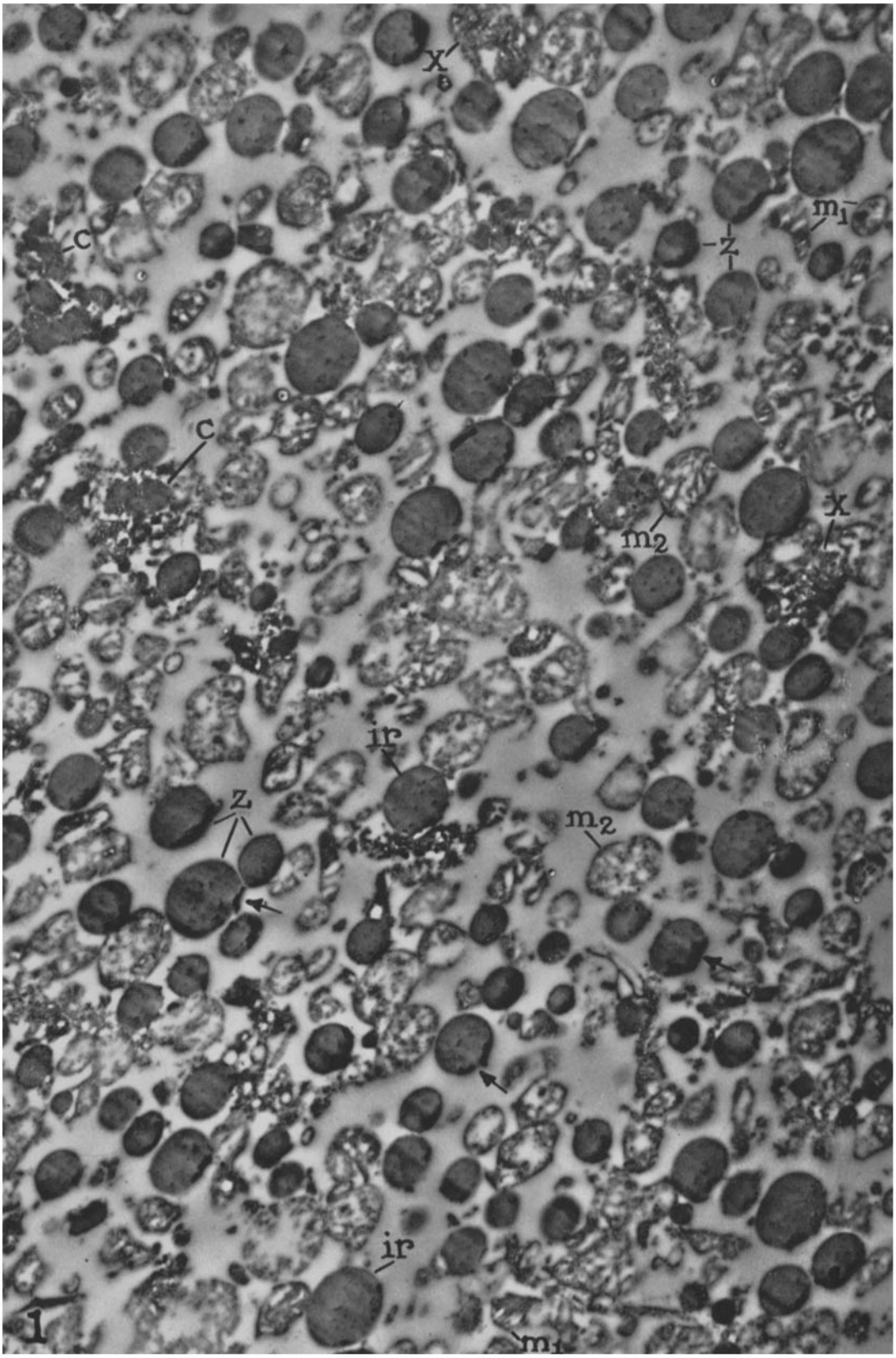

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PLATE 105

FIGS. 2 and 3 compare zymogen granules sectioned *in situ* (Fig. 3) and in the pellet of a zymogen fraction (Fig. 2).

It can be seen that in both cases the profiles of the granules (*z*) are similar in size and shape and characterized by high density and fine, apparently amorphous texture. Similar cutting artifacts are visible in both situations. They include ovalization of the granules (note that the direction of cutting was different in the two preparations), roughness of cut surfaces (white arrows), and terminal piling up of dense material (black arrows). Some profiles are bound by a fine, dense line, which is interpreted as a limiting membrane (*mz*). This membrane is expected to appear clearly outlined when normally sectioned (*mz* in both figures), and to be blurred to a varied extent when sectioned obliquely (as for the granules marked *z* in both figures).

FIG. 2. The appearance designated *dz* is probably a disintegrating zymogen granule; mitochondrial "ghosts" are marked *m*.

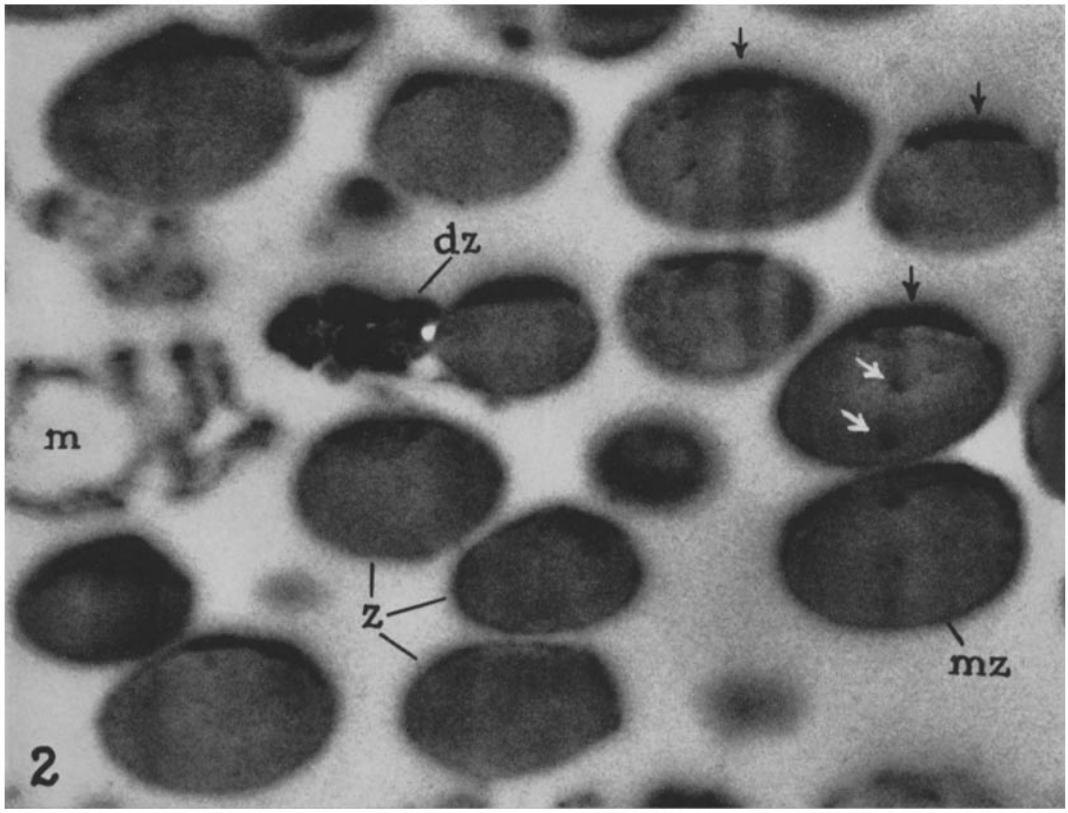
FIG. 3. An acinar lumen filled with amorphous material approaching the density of zymogen granules can be seen along the upper margin of the micrograph. It is bordered by a centro-acinar cell (upper right) and two exocrine cells (middle and upper left). Note the "microvilli" (*v*) that protrude in the acinar lumen and appear in both longitudinal and transverse sections.

In the centro-acinar cell can be seen a mitochondrion (*m*), smooth surfaced elements of the endoplasmic reticulum, and clusters of small, dense particles.

In the acinar cells, the cytoplasm in between the zymogen granules contains circular, oval, and irregular profiles of the endoplasmic reticulum (*rs*). Most of them bear attached particles.

Part of the centrosphere region (Golgi zone), with its clusters of smooth surfaced vesicles, appears at *cs*.

Magnification for both figures: 30,000.



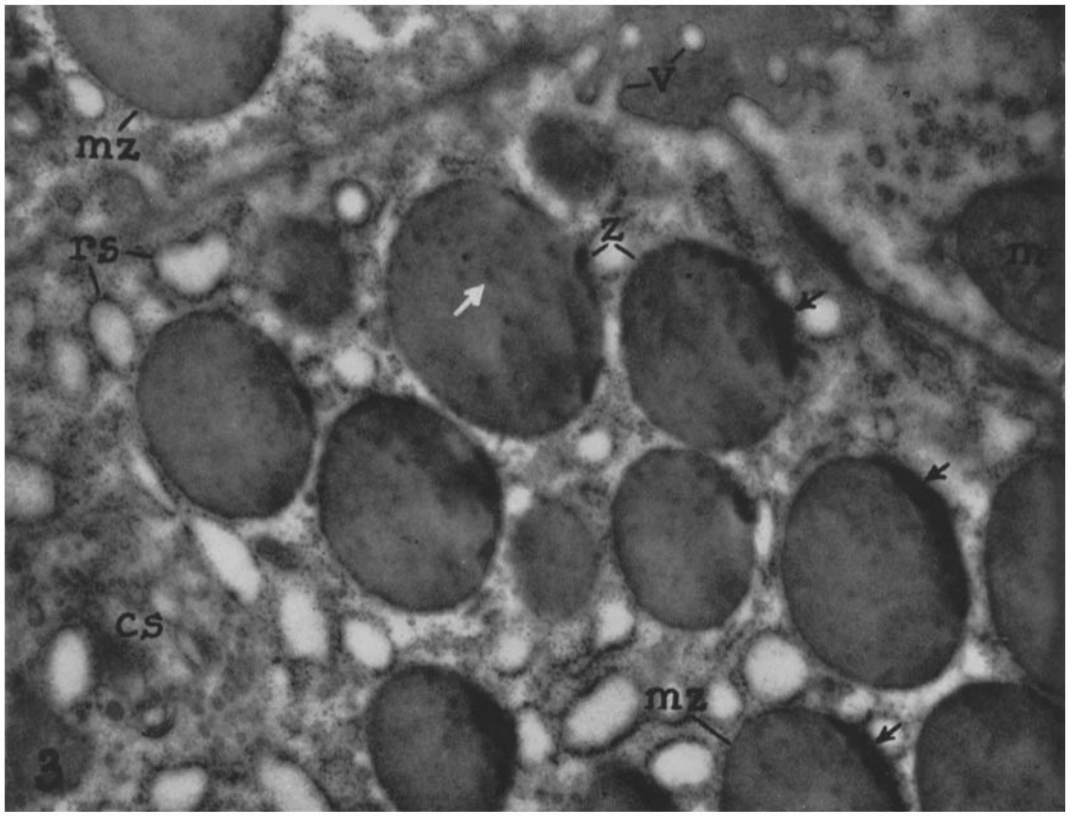


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PLATE 106

FIGS. 4 and 5. Mitochondrial fractions, sectioned pellets.

FIG. 4. Mitochondrial fraction isolated from a pancreas homogenate prepared in 0.88 M sucrose.

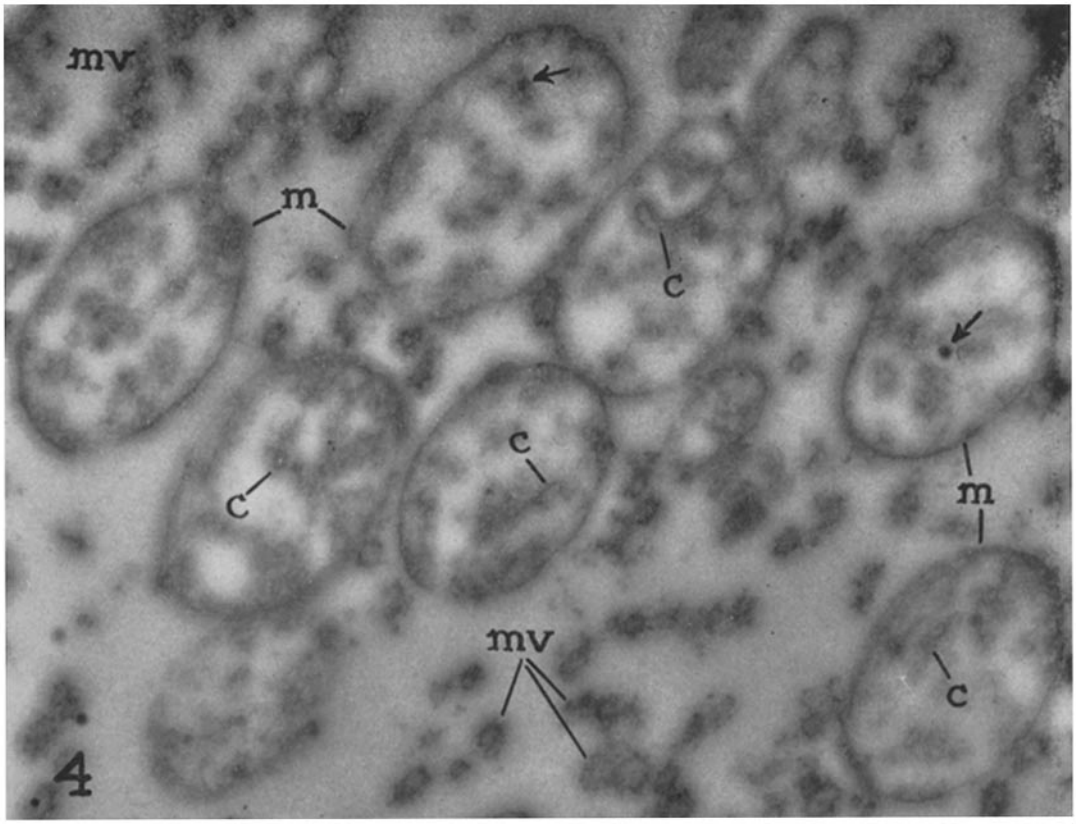
The relative large, oval structures marked *m* are identified as mitochondria by the remnants of their cristae (*c*). A comparison with Fig. 5 indicates that in 0.88 M sucrose pancreatic mitochondria swell into relatively large spheres (the oval shape of the profiles is due to deformation during cutting); their matrix is extensively extracted; few intramitochondrial granules persist (arrows); the internal membrane and the cristae break down into small vesicles; only the external membrane shows relatively little damage.

Microsomes (*mv*) and clusters of small, dense particles contaminate the fraction.

FIG. 5. Fraction isolated from a pancreas homogenate prepared in 1.42 M sucrose. Mitochondria (m_1 to m_5) appear in longitudinal (m_1) or oblique (m_2 to m_5) sections. The structural details are relatively well preserved, so that the outer mitochondrial membrane (long arrow), the inner membrane (short arrow), the cristae (*c*), the relatively dense matrix, and the dense intramitochondrial granules (*mg*) can be easily recognized. Note, however, the great variation in the quality of preservation: in some cases (m_1, m_2) only the outer membrane appears partially disrupted; in others, the pattern of the cristae is partially (m_3) or totally (m_4) changed so that the matrix seems fragmented in discontinuous masses; finally, in other cases, the matrix has been extensively extracted, leaving behind a damaged membranous ghost (m_5).

Microsomal vesicles (*mv*) and chains of small dense particles (*p*) contaminate the fraction.

Magnification for both figures: 45,000.



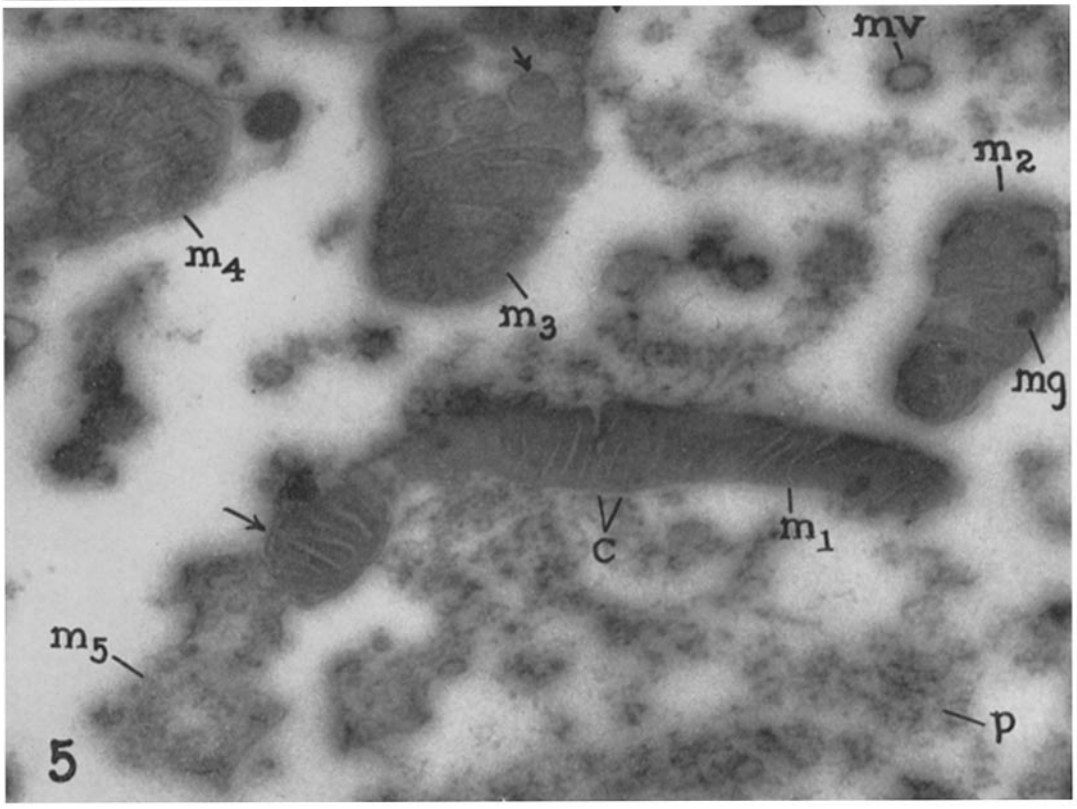


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PLATE 107

FIGS. 6 to 8. Sections through the pellet of a zymogen fraction, isolated by differential centrifugation, from a pancreas homogenate prepared in 0.88 M sucrose. The fraction was washed twice with a sucrose solution of the same concentration.

FIG. 6. The micrograph shows relatively large masses (ms_1, ms_2) of dense, apparently homogeneous material found at the bottom of the pellets of repeatedly washed fractions. Some of these dense masses (ms_2) are formed by clumped, though still distinct, round bodies of 0.5 to 1 μ diameter. This feature and the density of the material suggest that the large, irregular masses are produced by an extensive agglutination of zymogen granules. Magnification: 15,000.

FIG. 7. The dense bodies marked z_1 and z_2 probably represent disintegrating zymogen granules, judging by their form (z_1) and density (z_1 and z_2). They are surrounded by, and continuous with, masses of filamentous material (f) of similar density. It is assumed that these filaments derive from the content of the zymogen granules. Magnification: 28,000.

FIG. 8. The irregular dense masses and the bundles of dense filamentous material (f) presumably represent forms taken by the content of disintegrated zymogen granules. A few more or less intact zymogen granules are marked z. Magnification: 37,000.

FIG. 9. Disintegrating zymogen in the lumen of a pancreatic duct. Parts of three cells of the duct epithelium appear in the upper right and lower left corners. Their relatively dense cytoplasm contains mitochondria (m) and vesicular elements of the endoplasmic reticulum. The junction line between two cells can be seen at *cj*, and obliquely sectioned adhesion plates at *ap*. The free surface of the epithelial cell bears a few microvilli (v).

The lumen is occupied by a network of relatively large irregular trabeculae of dense material (dz) that represents the discharged content of the zymogen granules of acinar cells. The light matrix surrounding the zymogen in the lumen is the embedding plastic replacing an aqueous phase, presumably present *in vivo*. The zymogen in the duct lumen bears some resemblance to the disintegrating zymogen in Figs. 7 and 8. In other cases the intraluminal zymogen is more clearly disposed in bundles of filaments or fibrils.

Magnification: 25,000.

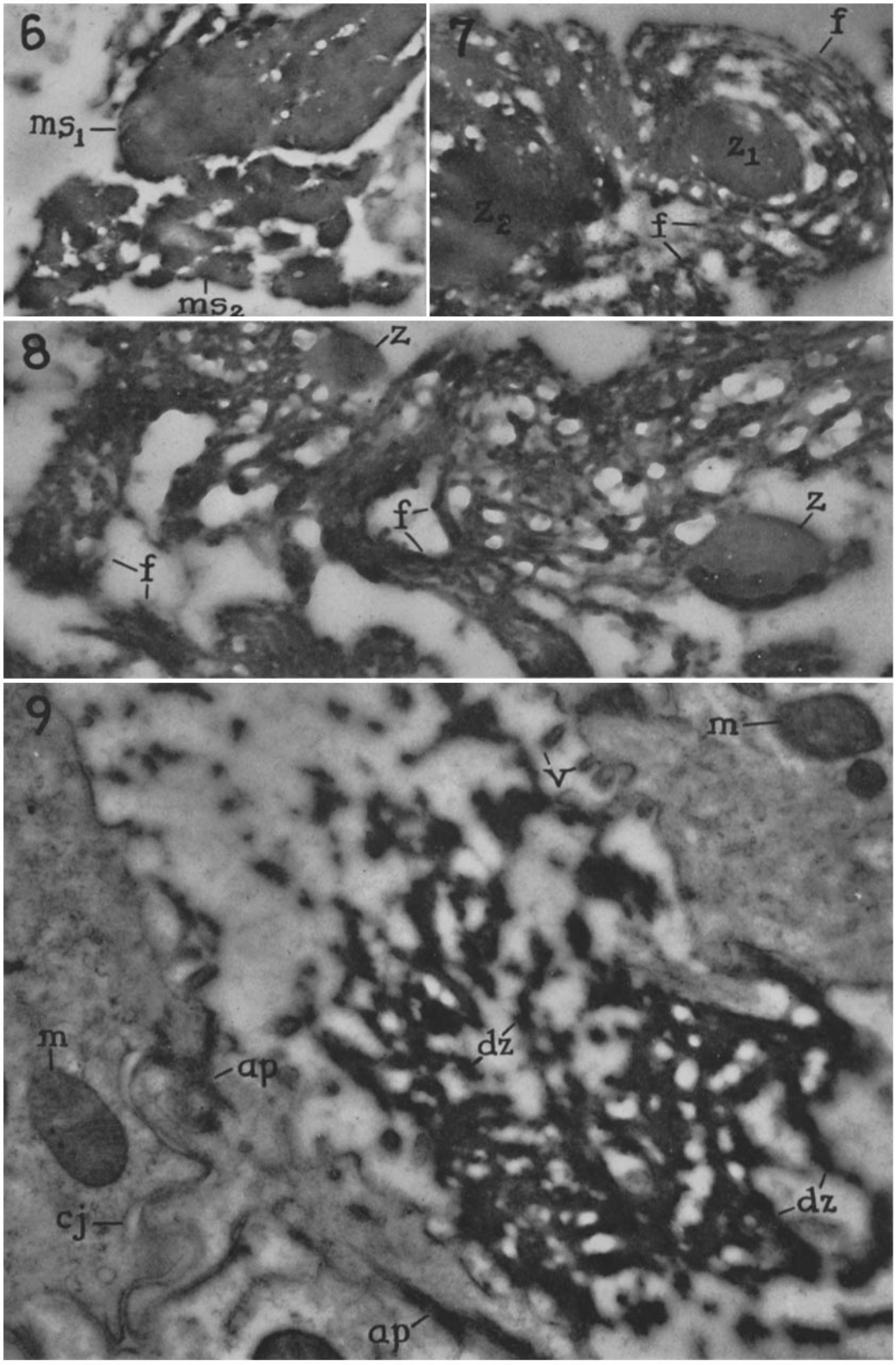

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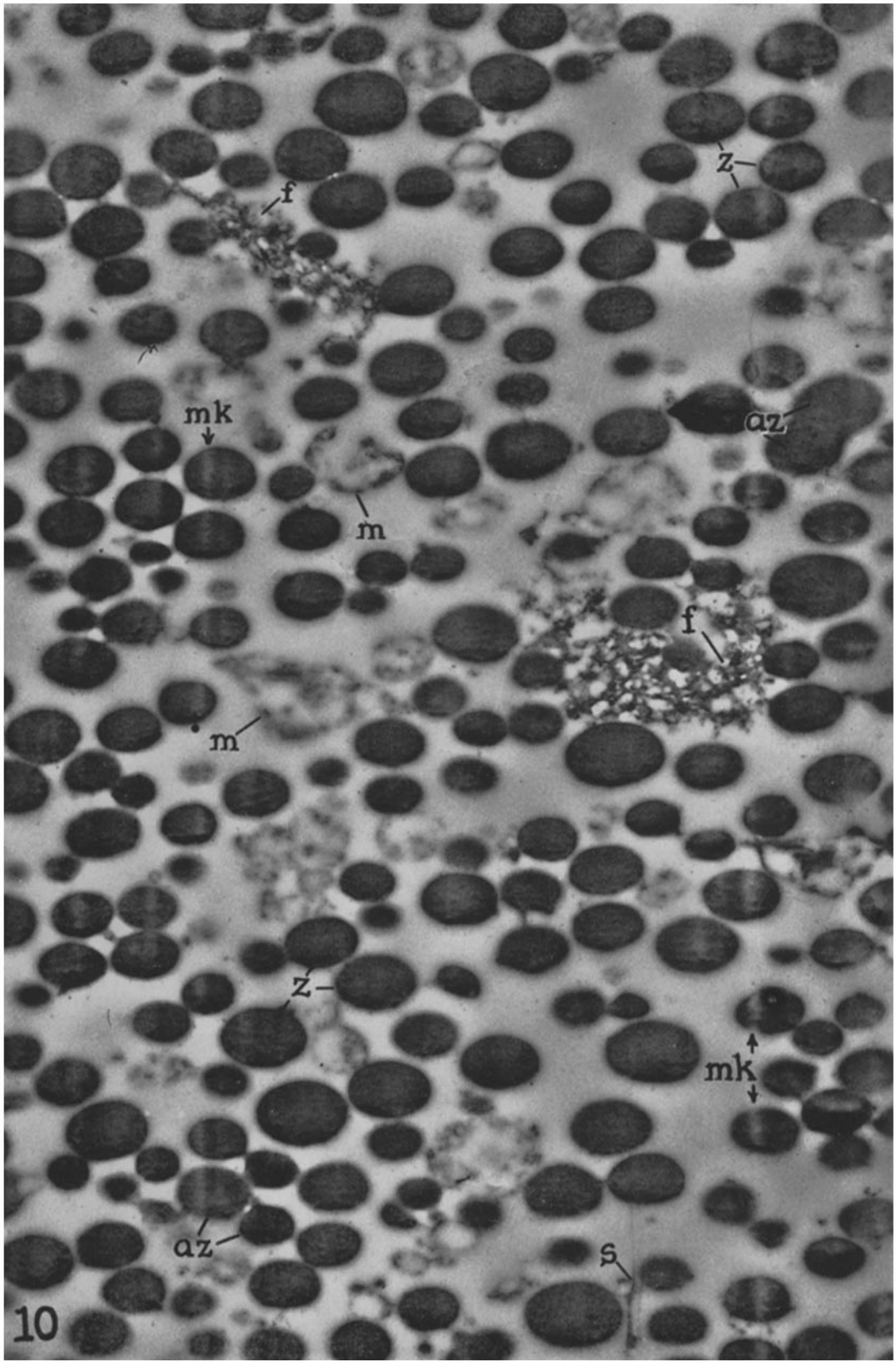
PLATE 108

FIG. 10. Zymogen fraction isolated by centrifugation in a discontinuous density-gradient (1.60 M sucrose layer). Representative field in the bottom layer of the pellet. The dense, oval profiles marked *z* represent spherical zymogen granules deformed during sectioning. Note that all the long axes of the oval profiles are oriented in the same direction, which is perpendicular to the direction of cutting. The latter is indicated by a few knife marks (*mk*) and streaks (*s*). Minor cutting artifacts, *e.g.* roughness of the cut surfaces, knife vibrations, and terminal piling up of dense material, are also visible. The majority of the granules are apparently intact, and only a few appear agglutinated (*az*). Fibrous material (*f*), presumably derived from disintegrating zymogen, is scarce. Damaged mitochondria (*m*) occur in relatively small numbers.

A comparison with Fig. 1 shows that this fraction isolated in a discontinuous density-gradient is much less heterogeneous and that its zymogen granules are better preserved.

Magnification: 12,000.



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