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THE BEHAVIOR OF ISOLATED ZYMOGEN GRANULES: pH-DEPENDENT RELEASE AND REASSOCIATION OF PROTEIN

S. S. ROTHMAN

Department of Physiology, Harvard Medical School and Harvard School of Dental Medicine, Boston, Mass. 02115 (U.S.A.)

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SUMMARY

1. The release of chymotrypsinogen, trypsinogen, and amylase from zymogen granules which were isolated from rat pancreas was studied as a function of pH. Enzyme release was lowest at slightly acid pH and increased progressively as pH was raised above neutrality. When pH was plotted against the percentage of enzyme released from the granules different curves were formed for different enzyme species.

2. At alkaline pH's enzyme release was essentially complete within the time required to separate the granules from the suspending medium by centrifugation, but release kinetics could be followed in "unstable" acid conditions (pH 4.5). At pH 4.5 a time-dependent release of trypsinogen could be followed under conditions in which the homologous protein, chymotrypsinogen, was completely retained within the granules.

3. Granule contents which were released at alkaline pHs could be reassociated or reaggregated when the pH of the suspension was again made acidic. As much as 90% of the original protein content of the granules could be reassociated. This pH-dependent reassociation required the presence of granule membrane and perhaps a non-secreted lipoidal moiety as well.

4. These experiments support the view that the zymogen granule is either internally ordered or that different granules contain different enzymes or groups of enzymes. Furthermore, the digestive enzymes appear to associate in some manner with molecules forming part of the granule membrane. A hypothesis is proposed to explain the results in which polymers of a particular digestive enzyme form as a result of the association of a molecule of this enzyme with granule membrane.

INTRODUCTION

"Zymogen granules" found in pancreatic acinar cells are membrane-bound particles which contain of variety of digestive enzymes or their proenzymes[1-3] apparently within the granule population of single cells[4]. It is not known whether each granule contains all of the various digestive enzymes, perhaps more than 20, or whether they are partitioned either singly or in groups in different granules. Furthermore, the granules do not appear to contain any substantial quantity of organic molecules other than these digestive enzymes[1-3].

When zymogen granules were first isolated from homogenates of dog pancreas they were found to be most stable at slightly acid pH [2]. If the pH of a granule suspension was raised to between 7 and 8, the turbidity of the suspension decreased dramatically. The change in turbidity, due to a decrease in the light scattering properties of the granules, was associated with the release of exportable protein from the granules into the suspending medium[2]. This release of digestive enzyme at neutral or slightly alkaline pH has been referred to as "lysis". Even though the mechanisms involved in the phenomenon were not clear, the simplest explanation was that membrane defects resulting from the pH-dependent dissociation of membrane proteins or lipids or both made it possible for the secretory proteins to diffuse out of the granule. The fact that the enzymes released at alkaline pH's did not "reassociate" when the pH was again made acidic seemed to eliminate a molecular dissociation of intragranular protein as part of the process.

In the course of developing a system to study isolated zymogen granules, in this case extracted from rat pancreas, we made two somewhat unexpected observations regarding the pH-dependent behavior of granule suspensions; first, the release of digestive enzymes from granules at various H^+ concentrations was not identical for different molecular species, and second, unlike the earlier study using dog granules[2], pH-dependent reassociation of previously released protein was observed. These observations will be the primary focus of this paper.

METHODS

Zymogen granule fraction

Pancreatic tissue was taken from male Holtzman rats (300–450 g body wt.) killed by spinal section after light etherization. Most of the fat and connective tissue was dissected from the glandular tissue and wet weights were then determined. Glands from 6 animals were pooled (4.5–8.0 g tissue wet wt.) for homogenization and subsequent centrifugation. The tissue was homogenized by shearing in 0.3 M sucrose (1:10, w/v) (0.13–0.15 mm clearance between mortar and pestle)[5]. Heavy fragments (nuclei, plasma membrane, and whole cells) were removed by centrifugation at $760 \times g_{av}$ for 10 min. The supernatant was recentrifuged at $1000 \times g_{av}$ for 10 min to produce a "crude" granule pellet. This pellet was resuspended and further purified by one "head" and one "tail" rinse ($760 \times g_{av}$ for 10 min and $1000 \times g_{av}$ for 10 min). Granule pellets prepared in this way contained between 75 and 95 % dense profiles (zymogen granules) when thin sections were viewed with the electron microscope. Protein/nucleic acid ratios obtained on "lysed" granule suspensions were never less than 25:1 using ultraviolet absorption methods[6], and the measurement of RNA and DNA using diphenylamine[7] and orcinol reagent[8] on trichloroacetic acid extracts of zymogen fraction material[9,10] also indicated only minor nuclear and microsomal contamination (Table I). The highest specific activity of trypsinogen, chymotrypsinogen and amylase relative to other subcellular fractions was found in the zymogen granule fraction[5].

The washed zymogen pellet was usually resuspended in 8.0 ml 0.3 M sucrose (pH 5.5), divided into four samples containing approx. 1–2 mg protein apiece which were then subjected to a variety of experimental manipulations. Solutions containing salts were not used owing to the instability of zymogen granules even in solutions of relatively low ionic strength[11].

After experimental treatment particulate material was recovered by centrifugation at 10000 × g_{av} for 10 min. This spin yielded well packed pellets and apparently clear supernatants. The 10000 × g_{av} pellet was considered to contain all the material retained in the particulate phase (zymogen granules) while the 10000 × g_{av} supernatant was considered "soluble". This is an operational definition of course, and under appropriate conditions particulate material can be recovered from 10000 × g_{av} supernatants (see RESULTS).

Enzyme assays

Proenzymes were activated by incubation for 30 min at 37° in 40 mg purified enteropeptidase (formerly called enterokinase, EC 3.4.4.8) (grade B, Calbiochem)[12] per 100 ml 0.1 M sodium phosphate buffer (pH 7.4). Granule contents were completely solubilized by this treatment and maximum activation of both trypsinogen and chymotrypsinogen could be obtained. The presence of trypsinogen and chymotrypsinogen was estimated from the esterolytic activity of activated samples using *p*-toluene sulfonyl-L-arginine methyl ester·HCl as substrate for trypsin (EC 3.4.4.4) and *N*-acetyl-L-tyrosine ethyl ester·H_2O as substrate for chymotrypsin (EC 3.4.4.5)[13,14]. The measurements are, therefore, more properly of enteropeptidase-activable *p*-toluene sulfonyl-L-arginine methyl ester·HCl and *N*-acetyl-L-tyrosine ethyl ester·H_2O esterases. In rat zymogen granules these activities primarily represent trypsinogen 1 and 2 (ref. 15) and the various chymotrypsinogens, respectively. All measurements were of initial reaction velocities and were not substrate limited. The presence of trypsin and chymotrypsin inhibitors was not detectable by examining the effect of zymogen granule fractions "lysed" but not activated on the activity of standard solutions of bovine trypsin and α-chymotrypsin. It has previously been reported that rat zymogen granules do not contain pancreatic trypsin inhibitors[16]. Irreversible inhibitors were not found in any quantity and plots of activity *vs.* protein content passed through the origin.

α-Amylase (α-1,4-glucan 4-glucanohydrolase, EC 3.2.1.1) activity was measured by the release of the dye remazolbrilliant blue R (covalently bound to sugar subunits) from insoluble amylose[17]. Aliquots were diluted from 1:20 to 1:100 in 0.1 M sodium phosphate buffer (pH 7.4) before assay. The amount of dye solubilized was measured after 15 min incubation at 37°. Dye bound to sugar subunits was separated from polymer-bound dye by filtration through 1.2 μ pore diameter cellulose ester filters. Absorbance of the supernatant was measured at 595 nm. The release of dye with time was linear in the range of sample size used.

Protein content was determined after granule lysis by ultraviolet absorption[6] and by using the Folin-phenol reagent[18]. Unless otherwise specified, the total protein and enzyme yield was unchanged by treatment.

RESULTS

pH-dependent variations in the release of protein from isolated zymogen granules

Granules isolated from rat pancreas and suspended in 0.3 M sucrose solutions of varying pH behaved in essentially the same way described by HOKIN[2] for isolated dog granules (bovine material also behaves similarly[1]). That is, at slightly alkaline pH a sharp increase in the "soluble" protein content of the suspension was observed

accompanied by a decreased turbidity of the suspension (Fig. 1). Protein release was at its minimum at pH 4.5, although a rather broad pH band of granule stability was observed from 4.5 to 6.5. The data given in the insert of Fig. 1 are for the distribution observed after a 30-min incubation of granule suspensions at 24°.

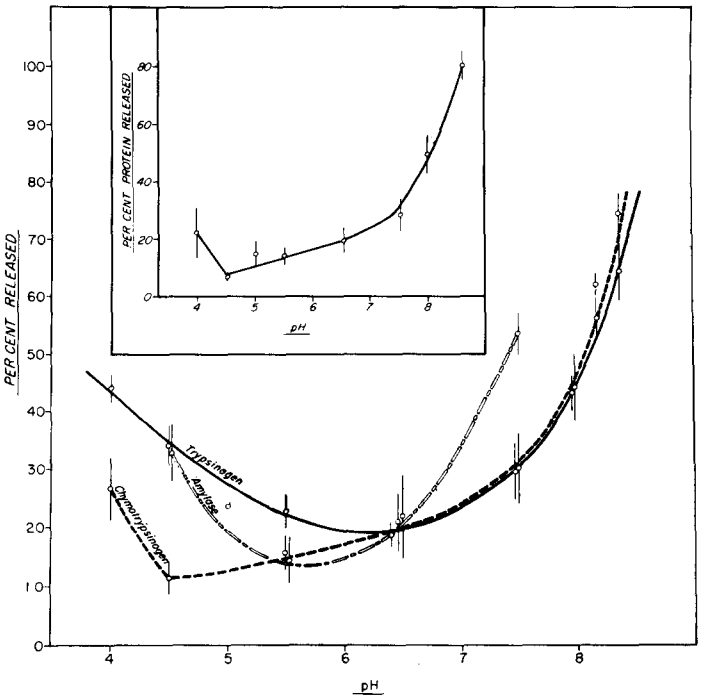

Fig. 1. The release of protein, trypsinogen, amylase and chymotrypsinogen from suspensions of zymogen granules at various pH's. The circles and vertical bars represent the mean value ± S.E.. Moving from lower to higher pH for protein, n = 4, 12, 7, 8, 5, 5, 5, and 5. Also moving from lower to higher pH for trypsinogen and chymotrypsinogen, n = 3, 14, 7, 3, 3, 7, 4, 5. n = 4 at each point for amylase. Per cent released refers to the percentage of the total enzyme activity or protein recovered in a supernatant after centrifugation at 10000 × g_{av} for 10 min. All granule pellets were suspended in 0.3 M sucrose (pH 5.5) to which small amounts of either 0.01 M HCl or NaOH were added to alter the pH. All suspensions were incubated for 30 min at 24° prior to centrifugal separation. The total yield of enzyme and protein was not altered by pH in the range presented in this figure.

TABLE I

SOME CHEMICAL CHARACTERISTICS OF THE ZYMOGEN GRANULE FRACTION

Protein (mg/g tissue)	*DNA (mg/g tissue)*	*RNA (mg/g tissue)*	*N-Acetyl-L-tyrosine ethyl ester·H_2O esterase specific activity (μmoles substrate split per min per mg protein)*	*N-Acetyl-L-tyrosine ethyl ester·H_2O esterase relative specific activity*
1.94	0.03	0.06	23	3.9

pH-dependent variations in the release of digestive enzymes from isolated zymogen granules

Release from granules of the structural homologues, trypsinogen and chymotrypsinogen, as well as release of amylase was examined over a wide pH range (from 4.0 to 8.6 for trypsinogen and chymotrypsinogen; from 4.5 to 7.5 for amylase). In general, the pattern of release was similar to that found for total protein (Fig. 1). That is, enzyme "solubilization" was greatest at acidic pH's below 4.5 and also at moderately alkaline pH's (7.5–8.6).

Although the release patterns of the enzymes were similar, each described its own unique "parabola" with different minima (pH of maximal stability), percentage of total enzyme lost at the minimum, and rate of loss from the particulate phase at both acidic and alkaline pH (Fig. 1).

At pH 4.5 (30 min incubation at 24°) only 11.5 % of the total chymotrypsinogen was recovered in the soluble phase while 35 % of the trypsinogen and amylase was found in the supernatant (chymotrypsinogen *vs.* trypsinogen or amylase, $P < 0.001$). During the alkaline swing another point of divergence was observed at pH 7.5, with over 50 % of the amylase released while chymotrypsinogen and trypsinogen release was a little less than 1/3 complete (amylase *vs.* trypsinogen or chymotrypsinogen, $P < 0.001$). At alkaline pH the release of trypsinogen and chymotrypsinogen was found to closely parallel each other (Fig. 1).

The time-course and temperature dependence of trypsinogen and chymotrypsinogen release

It was not possible to study the time-course of enzyme release at alkaline pH using the present separation techniques since release was essentially complete within centrifugation time. However, the rate at which enzyme was liberated from the granules could be studied when granules were suspended in an acidic medium in which they are not stable; for example, at pH 4.5. At pH 4.5 trypsinogen was slowly released from the particulate phase reaching the maximum amount set free after about 30 min incubation at 24° (Fig. 2). Approx. 1/3 of the total trypsinogen content of the granules was released under these conditions. In the same environment chymotrypsinogen

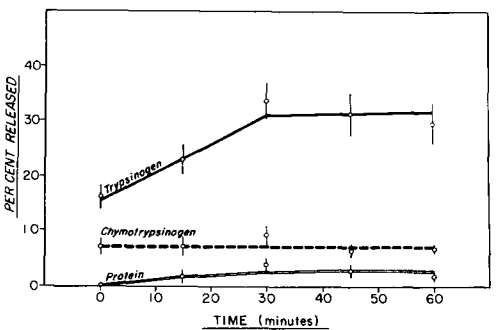

Fig. 2. The time-course of trypsinogen, chymotrypsinogen and protein release from zymogen granule suspensions at pH 4.5. Granule pellets were suspended in 0.3 M sucrose (pH 5.5) and the pH was then immediately lowered to 4.5. The circles and vertical bars represent the mean value ± S.E. for a given time interval. In time-zero studies the suspension was brought to pH 4.5 by the addition of 0.01 M HCl and then centrifuged immediately in order to separate the granules from the suspending medium. Moving out from zero time, $n = 3, 3, 12, 5, 5$ for trypsinogen, chymotrypsinogen, and protein. Per cent released refers to the percentage of the total recovered in the supernatant after centrifugation at 10000 × g_{av} for 10 min. All studies were done at 24°.

distribution was virtually unchanged with time and was almost exclusively recovered within the particulate phase (Fig. 2). Trypsinogen release appeared to be the result of an irreversible event, such as lysis, and was not due to a new steady-state distribution between phases since varying the volume of incubation medium did not produce the redistribution of enzyme between soluble and particulate phases.

The amount of trypsinogen discharged was found to be independent of temperature (comparing 4, 24, 30 and 37°). Temperature did not alter the chymotrypsinogen distribution either and it remained associated with the particulate phase throughout the same temperature range.

pH-dependent reaggregation or reassociation of zymogen granule proteins

When the pH of a suspension of zymogen granules "lysed" at alkaline pH (8.6) in 0.3 M sucrose was returned to pH 5.5 by the addition of small amounts of 0.01 M HCl, the "clarified solution" of granule contents again became cloudy. When the resultant suspension was incubated for 30 min at 24° along with a pH 8.6 control and both were then centrifuged at 10000 × g_{av} for 10 min to collect the sediment, the protein distribution was altered from approx. 90–100 % recovered in the soluble phase in the pH 8.6 control to only about 22 % in "lysed" material returned to pH 5.5. Chymotrypsinogen activity in the 10000 × g_{av} pellet was also increased by this pH change, but to a lesser degree than was total protein (Table II). The distribution of trypsinogen activity on the other hand was unaffected by treatment. The difference in the reaggregation of total protein and of chymotrypsinogen and trypsinogen indicates that other protein components of granules must have been affected to a greater extent than either of these two enzymes. Which proteins in particular are more effectively reaggregated has not yet been determined.

TABLE II

THE PERCENTAGE OF TRYPSINOGEN, CHYMOTRYPSINOGEN, AND PROTEIN FROM WHOLE ZYMOGEN GRANULE "LYSATES" (pH 8.6) REASSOCIATED AT pH 5.5 AND 4.5

pH	% *Total recovered in 10000 × g_{av} pellet in 10 min*		
	Trypsinogen	*Chymotrypsinogen*	*Protein*
8.6	31*	18	20
	39	7	4
	30	6	6
5.5	38	57	80
	33	40	78
	38	44	85
4.5	40**	44	83

* These data are from three experiments in which single granule pellets were divided into four equal aliquots after being resuspended in 0.3 M sucrose (pH 5.5). 0.01 M NaOH was added to each sample until pH 8.6 was reached. They were then incubated at 24° for 1 h at which time one sample was returned to pH 5.5 (with 0.01 M HCl), two to pH 4.5 (with 0.01 M HCl), and the fourth was left at pH 8.6. All four samples were again incubated at 24°, but this time for only 30 min, and then spun at 10000 × g_{av} for 10 min. The resultant pellet and supernatant were analyzed.

** Numbers represent the mean of six for all at pH 4.5.

Protein solubility and protein reassociation

Reassociation was found to be approximately equally effective over a wide range of initial granule concentration (from approx. 10 to 100 mg protein per 100 ml) (Fig. 3). Suspension concentrations were all well below predictable solubilities for the kinds of proteins in the mixture. To investigate this further, pure pancreatic juice was collected from the secretory ducts of anesthetized rats (for methods see ref. 19) and the pH-dependent behaviour of secretion was compared to that of material from granule suspensions. Pancreactic secretions in protein concentrations similar to that of granule suspensions did not exhibit any pH-dependent changes in turbidity. At high concentrations of protein (*i.e.*, non-diluted juice) some turbidity developed as pH was lowered and approx. 15 % of the total protein in the sample could be sedimented by centrifugation as a result of pH-dependent solubility effects (Fig. 3).

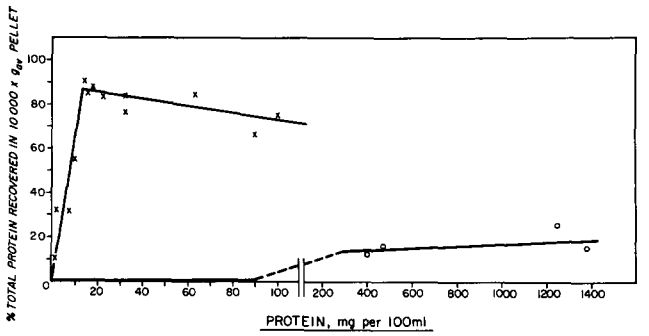

Fig. 3. A comparison of the reassociative behavior of zymogen granule "lysates" (pH 8.6) and pancreatic juice. Aliquots of zymogen granule material were resuspended in different volumes of 0.3 M sucrose (pH 5.5) so that the final protein concentration ranged from approx. 1 to 100 mg protein per 100 ml 0.3 M sucrose. The pH of each sample was raised to 8.6 with 0.01 M NaOH to "clarify" the suspension and then immediately lowered to 5.5 (by the addition of 0.01 M HCl). The resultant suspensions were incubated for 30 min at 24° and then centrifuged at 10000 × g_{av} for 10 min. The pellet was then separated from supernatant and protein measured in both. ×, per cent of the total protein recovered in the pellet. Pancreatic juice was collected directly from the duct of anesthetized rats[19]. The pH of the juice was lowered to 5.5 either after dilution in varying amounts of 0.3 M sucrose (pH 8.6) or undiluted. When undiluted juice was used some turbidity developed (○). When diluted pancreatic juice was used at protein concentration similar to that of the zymogen granule "lysates" no associative behavior was seen. This is represented by the dark line drawn along the abscissa from 0 to 90 mg protein per 100 ml.

The reassociation of zymogen granule contents in dilute suspensions at slightly acidic pH does not appear to be simply the result of pH-dependent changes in the solubility of enzymes in the mixture of proteins. Rather, it results from changes in the potential for association of these proteins with each other or some other moiety in the presence of a "structural" component of zymogen granules which is not secreted with the digestive enzymes.

The reassociative behavior of a 100000 × g_{av} sediment from zymogen granule "lysates" at pH 8.6

When thin sections of sediments recovered from high pH granule suspensions (after centrifugation at 100000 × g_{av} for 1 h) were viewed with the electron microscope membranous profiles predominated. Similar sedimentable material collected by others from high pH suspensions of bovine granules have been shown to also consist mainly of membranous profiles apparently of granule membrane[1].

When these 100000 × g_{av} pellets (pH 8.6) were resuspended in 0.3 M sucrose (pH 8.6), a reasonably "clear" mixture resulted. If, however, the pH of the sucrose solution was subsequently reduced to pH 5.5 (by the addition of 0.01 M HCl), turbidity developed and a considerable 10000 × g_{av} sediment could again be recovered (Table III). The percentage of the protein from the 100000 × g_{av} pellet which was recovered in the 10000 × g_{av} sediment was about the same as that recovered using whole granule "lysates" (pH 8.6) (compare Table III to Table II). The percentage of chymotrypsinogen reassociated was enhanced when the 100000 × g_{av} pellet (pH 8.6) was used instead of whole granule "lysates" (pH 8.6) (% recovered using 100000 × g_{av} pellet = approx. 75 %; using whole granule "lysates" = approx. 47 %). The reason for this enhancement is not clear at present.

TABLE III

pH-DEPENDENT REASSOCIATION OF PROTEIN AND CHYMOTRYPSINOGEN USING 100000 × g_{av} PELLETS OBTAINED FROM "LYSATES" OF ZYMOGEN GRANULES (pH 8.6)

	% of 100000 × g_{av} pellet recovered in 10000 × g_{av} (10 min) pellet after being resuspended in 0.3 M sucrose			
	Protein		*Chymotrypsinogen*	
	pH 8.6	*pH 5.5*	*pH 8.6*	*pH 5.5*
	9*	77	19	71
	8	87	18	68
	12	89	26	86
$\bar{x}$	10	84	21	75

* The results are from three separate experiments.

A flocculent layer and the 100000 × g_{av} supernatant (pH 8.6)

After removal from the ultracentrifuge, as the tube containing sedimented granule material (pH 8.6) warmed up slightly, a flocculent or turbid layer appeared on the surface of the supernatant. The chemical composition of this material has not been determined, but since it was observed floating on 0.3 M sucrose it probably contains lipid or lipoprotein.

To examine the possible importance of the soluble protein itself in the reassociation phenomenon, experiments were done testing for association in the usual way (pH 8.6–5.5 and then 10000 × g_{av} for 10 min) using 100000 × g_{av} supernatant from high pH granule suspensions from which the flocculent layer was initially removed. Under these circumstances reassociation was found to be minimal (Table IV). If the surface layer was removed with care immediately after the tube was removed from the centrifuge, it was possible to completely suppress any visible turbidity changes in the supernatant fraction when the pH was made acidic. When the flocculent layer was added back to the 100000 × g_{av} supernatant a small reaggregative effect was observed (Table IV). Even though about 40 % of the protein now sedimented at 10000 × g_{av} in 10 min, no increase in chymotrypsinogen sedimentation was observed (Table IV).

TABLE IV

THE pH-DEPENDENT REASSOCIATION OF 100000 × g_{av} SUPERNATANT FROM GRANULE "LYSATES" (pH 8.6) WITH AND WITHOUT FLOCCULENT LAYER

	% *Sedimented at 10000 × g_{av} (in 10 min) after the pH was changed from 8.6 to 5.5**			
	100000 × g_{av} supernatant (pH 8.6) + 0.3 M sucrose (pH 8.6)		*100000 × g_{av} supernatant (pH 8.6) + flocculent layer (pH 8.6)*	
	Protein	*Chymotrypsinogen*	*Protein*	*Chymotrypsinogen*
	17**	55	36	13
	19	6	40	13
	10	13	43	11
$\bar{x}$	15	11	40	12

* A small volume of fluid (approx. 0.1–0.5 ml; total supernatant volume approx. 8–12 ml) containing a flocculent material was taken off of the surface of the 100000 × g_{av} supernatant (pH 8.6). The remaining supernatant was then divided in half, the flocculent layer added back to one-half and an equal volume of 0.3 M sucrose to the other. The pH of both samples was lowered to 5.5, suspensions incubated for 0.5 h at 24° and then centrifuged at 10000 × g_{av} for 10 min. The resultant pellet and supernatant were analyzed for protein and chymotrypsinogen.

** Three different experiments are presented in this table.

DISCUSSION

The organization of zymogen granules

The different "pH-enzyme release" curves for amylase, chymotrypsinogen, and trypsinogen (Fig. 1) suggest that zymogen granules are organized structures and cannot be adequately described simply as membrane-bound sacs which contain randomly arranged digestive enzymes either in or out of solution. A non-ordered granule containing a mixed population of proteins would not be likely to have such different release patterns for various enzymes especially to the extent that chymotrypsinogen is almost completely retained within the granules at pH 4.5 while the chemically and structurally similar enzyme, trypsinogen, is liberated in a time-dependent manner (Fig. 2).

These different release patterns might occur because of the sequestering of digestive enzymes within granules that are homogeneous for a particular enzyme or group of enzymes. Structures such as granule partitions could also account for the distinctive behavior of different molecules (but have never been observed in numerous electron microscopic investigations of zymogen granules, their "ghosts", "immature" granules, or broken granules[1,20,21]). This kind of subgranular organization need not be anatomical, however, and exportable proteins could be bound or linked to other molecules (*e.g.*, proteins or phospholipids) within the granules by specific weak interactions in a way which does not display ultrastructural organization. What evidence there is suggests that the organic content of these cellular granules may be almost exclusively exportable protein and whatever interactions exist would be limited to those between secretory proteins and the granule membrane[1–3].

pH-dependent reassociation of zymogen granule proteins

Since the zymogen granule membrane can be estimated to account for at most 10 % of granule protein and as much as 90 % of the protein of a granule suspension can be "reaggregated", reassociation must result from interactions involving the

protein contents of the zymogen granule. And since similar pH-dependent reassociative phenomena were not observed using secretion either non-secreted intragranular molecules or granule membrane must also be involved. Since very effective reassociation occurs with 100000 $\times$ g_{av} sediments (pH 8.6) which are primarily composed of granule membrane "ghosts", a membrane-digestive enzyme interaction apparently takes place. Considerable dilution does not affect reaggregative efficiency (Fig. 3) and the idea that enzyme contents could just be trapped within granule membrane spherules formed as a result of changes in pH can be eliminated. That reassociation requires membrane can also be seen from the fact that it does not occur when the 100000 $\times$ g_{av} supernatant (pH 8.6) is used alone (at least when the "flocculent" layer is carefully removed).

Soluble granule contents do not have any independent role in reassociation, but when the "flocculent" layer is added back, some reassociation of supernatant protein occurs in a pH-dependent manner. Whether this material is non-secreted zymogen granule lipid or lipoprotein which also plays some role in the organization of the granule or is just pH-extracted parts of granule membrane has not been determined. "Flocculent" material has only been recovered from lysed granules and on this basis seems unlikely to be a contaminant from other parts of the cell.

The formation of enzyme polymers associated with granule membrane

If reassociation of zymogen granule protein results primarily from digestive enzyme–granule membrane interactions, then there probably is not enough membrane to account for reaggregation solely by monomolecular binding of enzyme to molecules on the surface of the membrane either by lipid or protein interactions or, for that matter, both. If we consider the possibility of a protein–protein interaction, and assume that membrane proteins and the digestive enzymes have similar mean molecular weights, then we can estimate that at least eight digestive enzyme molecules would have to associate with every protein molecule in the membrane. One way to explain this imbalance is by the formation of polymers of the various digestive enzymes attached by one of its member molecules to the membrane; the interaction between membrane and digestive enzyme in some way enhancing the potential for polymerization of that particular enzyme.

Whether the pH-dependent association between proteins normally contained within zymogen granules and the granule membrane has an important bearing on the way in which these elements are normally formed and associated *in situ* cannot be determined from these experiments alone. Certainly the coprecipitation and association *in vitro* of proteins originally derived from the same organelle have often been useful in attempting to understand natural functional relationships.

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REFERENCES

1 L. J. GREENE, C. H. W. HIRS AND G. E. PALADE, *J. Biol. Chem.*, 238 (1963) 2054.
2 L. E. HOKIN, *Biochim. Biophys. Acta*, 18 (1955) 379.
3 P. J. KELLER AND E. COHEN, *J. Biol. Chem.*, 236 (1961) 1407.
4 K. YASUDA AND A. H. COONS, *J. Histochem. Cytochem.*, 14 (1966) 303.
5 S. S. ROTHMAN, *Am. J. Physiol.*, 219 (1970) 1652.
6 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1941) 384.
7 Z. DISCHE, *Z. Mikrochem.*, 8 (1930) 4.
8 W. MEJBAUM, *Z. Physiol. Chem.*, 258 (1939) 117.
9 W. C. SCHNEIDER, *J. Biol. Chem.*, 161 (1945) 293.
10 W. C. SCHNEIDER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. 3, Academic Press, New York, 1957, p. 680.
11 S. J. BURWEN AND S. S. ROTHMAN, *Fed. Proc.*, 29 (1970) 445.
12 M. KUNITZ, *J. Gen. Physiol.*, 22 (1939) 447.
13 G. W. SCHWERT, H. NEURATH, S. KAUFMAN AND J. E. SNOKE, *J. Biol. Chem.*, 172 (1948) 221.
14 J. E. SNOKE AND H. NEURATH, *J. Biol. Chem.*, 182 (1950) 577.
15 A. VANDERMEERS AND J. CHRISTIAN, *Biochim. Biophys. Acta*, 188 (1969) 101.
16 B. ARNESJÖ AND A. GRUBB, *Acta Physiol. Scand.*, 75 (1969) 139.
17 H. RINDERKNECHT, P. WILDING AND B. J. HAVERBACK, *Experientia*, 23 (1967) 805.
18 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
19 S. S. ROTHMAN AND H. WELLS, *Am. J. Physiol.*, 216 (1969) 504.
20 F. S. SJÖSTRAND, *Ciba Foundation Symp. on the Exocrine Pancreas, London, 1961*, Churchill, London, 1962, p. 1.
21 G. E. PALADE, P. SIEKEVITZ AND L. G. CARO, *Ciba Foundation Symp. on the Exocrine Pancreas London, 1961*, Churchill, London, 1962, p. 23.