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EQUILIBRATION OF PANCREATIC DIGESTIVE ENZYMES ACROSS ZYMOGEN GRANULE MEMBRANES

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SUMMARY

Zymogen granules isolated from the rat pancreas released digestive enzymes in response to lowering the enzyme concentration of the medium in which the granules were suspended, and reabsorbed enzyme when the enzyme concentration of the medium was increased. The evidence suggests that the distribution of digestive enzyme between zymogen granule and medium is the result of an equilibrium process. This equilibrium is apparently expressed across the granule membrane since the disruption of the membrane barrier eliminated the delay in establishing new equilibrium between the bound and free enzyme. The release of enzyme was continuous (equilibration did not occur) when the suspending medium was continuously filtered, thereby maintaining a constant state of disequilibrium between intragranular enzyme and enzyme in the medium. The data suggest the following: (1) that digestive enzyme passes through the intact granule membrane bidirectionally, and (2) that there is a free cytoplasmic pool of digestive enzyme in intact cells which is in equilibrium with the enzyme content of the zymogen granule.

INTRODUCTION

"Proteins destined for export remain within and are transferred through the cell in association with its membrane-bounded compartments. At no point in the pathway does there appear to be transport of molecules in soluble (i.e. non-membrane enclosed) form through the cell sap" [1]. If this view of digestive enzyme secretion by the pancreas is correct [2], then the limiting membrane of the enzyme-containing compartments must be an absolute barrier to protein movement. Accordingly, these membranes would mediate an infinite concentration gradient between enzyme within the specialized compartments and the cell sap. This does not seem to be the case for zymogen granules, the major intracellular "membrane-bounded" storage compartment for digestive enzyme. That is, the membranes of zymogen granules, either separated from cells by homogenization and differential centrifugation [3], or within intact cells [4], appear to be permeable to secretory proteins. This article considers further evidence that the membranes of isolated zymogen granules are permeable to secretory enzymes.

METHODS

Granule preparation. Zymogen granules were obtained from the pancreas of white male Holtzman rats (200–450 g). Animals were fasted for 18–24 h prior to sacrifice by spinal section after light etherization. Most fat and connective tissue was dissected free and the glandular tissue was blotted and weighed. In specified experiments glands from different animals were pooled. Tissue was homogenized in 10 times its weight of 0.3 molar sucrose (pH 5.5) at 4 °C with five short and two full shearing strokes using a Teflon pestle with 0.13–0.15 mm clearance in a glass mortar. Granule purification was accomplished by differential centrifugation [5, 6]. An initial spin of $760 \times g_{av}$ for 10 min was used to remove heavy fragments (i.e. whole cells, nuclei and some plasma membrane). The supernatant was then centrifuged at $1000 \times g_{av}$ for 10 min to sediment the "crude" granule pellet. This sediment was resuspended in one-fifth of the original volume of 0.3 M sucrose and the previous centrifugation steps ($760 \times g_{av}$ for 10 min and $1000 \times g_{av}$ for 10 min) were repeated to obtain the final "washed" pellet. This final sediment contains zymogen granules primarily [5, 7, 8].

Experimental procedures. "Washed" zymogen granule sediments were resuspended in varying volumes of 0.3 M sucrose and incubated at 37 °C. Sucrose solutions were set at either pH 5.5 or 7.0, as specified, by adding small quantities of 0.1 M NaOH or HCl. After incubation, particulate material was sedimented at $10\,000 \times g_{av}$ for 15 min. α-Amylase activity, chymotrypsinogen activity and protein were measured in both the supernatant and pellet. The sum of supernatant and pellet protein content and enzymatic activity remained constant regardless of the volume of suspending medium, the duration of incubation or the pH of the medium, the three variables employed. "$n = 1$" for a given condition refers to an experiment preformed on material obtained from a single animal or a group of animals on a given day.

"Reaggregated" granule-derived material was prepared in the following manner. The pH of relatively concentrated suspensions of granules (approx. 1 mg protein/ml) was raised to 8.5 with NaOH. Within 30 min the resultant suspension was clear (showed no appreciable light scattering at 540 nm) and contained no appreciable heavy sediment (sedimentable protein at $10\,000 \times g_{av}$ for 15 min). The pH was then readjusted to 5.5 with HCl. The solution became cloudy [6, 9]. After an additional 30 min, the suspension was centrifuged at $10\,000 \times g_{av}$ for 15 min to collect the sediment. This sediment will be referred to as the "reaggregated material". This material contains most (approx. 90 %) of the enzyme content of native granules in association with granule membrane [6]. The interactions between digestive enzyme and the structural moieties are specific and appear to be similar to those found within the native granule [6, 9]. However, these aggregates do not retain a membrane-bounded vesicular structure to enclose the digestive enzymes (see below, also Ermak, T. and Rothman, S. S., unpublished results). Reaggregated material was suspended in a volume of 0.3 M sucrose (pH 5.5) equivalent to the original suspension volume. A portion of the suspension was sedimented to determine the distribution of enzyme and protein between supernatant and pellet and then an additional volume of 0.3 M sucrose (pH 5.5) was added to dilute the suspension to approx. 1 mg protein/8 ml. Samples were taken at intervals to determine the time course for the redistribution of enzyme between sediment and supernatant as a result of dilution. Each sample was

centrifuged at $10\,000 \times g_{av}$ for 15 min and protein and enzymatic activity determined in supernatant and sediment.

A hollow fiber device (5000 molecular weight cut-off, Bio-Rad Beaker Dialyzer) was used to concentrate granule suspensions (see Fig. 3). No loss of enzymatic activity was found for at least 2 h of incubation under these conditions. Each sample removed from the dialyzer was centrifuged at $10\,000 \times g_{av}$ for 15 min to separate supernatant and pellet.

The rate of release of protein from granules was also studied with an ultrafiltration apparatus (Amicon) using a filter which allows passage of approx. 100 000 molecular weight molecules (type XM100a Dyaflo Filter-Amicon). Soluble proteins derived from the zymogen granules pass through this filter but the zymogen granules themselves are retained. A fluid reservoir of 0.3 M sucrose (pH 5.5) was used and filtration was carried out at 8–20 lb/inch^2 pressure.

Protein content was estimated using the Folin phenol reagent with a standard curve made to bovine serum albumin [10].

Chymotrypsinogen was activated in 1.5 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 0.2 mg enteropeptidase (formerly called enterokinase) (EC 3.3.4.8) (Calbiochem. Grade B) for 30 min at 37 °C. The mixture was then added to 3 ml of 0.08 M *N*-acetyl-L-tyrosine ethyl ester in 30 % methanol and the initial rate of hydrolysis measured at 25 °C by titrating the acid product with either 0.1 M or 0.02 M NaOH to maintain the pH constant at 7.8. From 0.01 to 0.1 mg of protein were present in these samples. Activity was approximately proportional to sample size and when sample size was plotted against activity, a straight line was obtained. This confirms other results which suggest that neither a reversible or irreversible inhibitor of chymotrypsinogen is present in any substantial quantity in these granules and that the activity of the enzyme is approximately proportional to the amount of

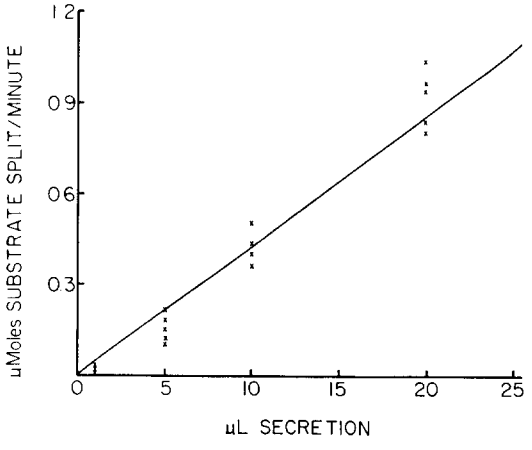

Fig. 1. Standard curve, potential chymotrypsinogen activity vs. sample size. The rate of hydrolysis of *N*-acetyl-L-tyrosine ethyl ester is plotted against sample size for pancreatic secretion diluted to 1 ml and activated in a standard manner (see Methods). Individual points were treated independently and represent a greater range of dilutions than were used in the experiments. They demonstrate an approximately linear relationship with at most only minor alinearity ($r = 0.998$). The line in the graph represents the line of assumed proportionality and the x's represent observed values.

enzyme at the concentrations used [11]. The experiments themselves also provide a number of internal checks on the linearity of the assay. These include: (1) Total measured activity did not vary as a function of dilution. While the standard deviation of the total measured chymotrypsinogen content for different dilutions had an average standard deviation of 16 % of the mean activity in nine experiments, total enzyme activity did not correlate at all with dilution (average correlation coefficient = 0.04 for the nine experiments); and (2) when aliquots of individual granule preparations were diluted (Fig. 2), the observed distribution of enzyme as a function of dilution fit the calculated regression line almost perfectly ($r = 0.99$ for pH 7.0 and 0.98 for pH 5.5). The analysis of individual granule preparations eliminated much of the variation observed previously [3] for pooled data analysis (for pooled data, $r = 0.93$ for pH 7.0 and 0.92 for pH 5.5). The non-linear relationship between chymotrypsinogen content and activity reported by others [12] does not occur to any measurable extent using the present assay technique in the range of concentrations studied.

α-Amylase (α-1,4-glucan-4-glucano hydrolase, EC 3.2.1.1) activity was estimated from the hydrolysis of amylose labeled covalently with Remazol brilliant Blue R dye (Amylose Azure, Calbiochem, Grade B). 0.5 ml of an appropriate dilution of sample in 0.1 M sodium phosphate buffer (pH 7.4) was added to 4.5 ml of a 2 % suspension of substrate in 0.02 M sodium phosphate buffer (pH 7.4) containing 0.05 M NaCl. The reaction was terminated after 15 min by the addition of 2 ml of 1 M acetic acid. Samples were removed from the water bath and centrifuged at 20 000 $\times g_{av}$ for 15 min. The absorbance of the supernatant was then read at 595 nm.

Centrifuging small aliquots of a large suspension or the whole large volume produced the same fractional separation between supernatant and sediment. Lengthening the time of centrifugation did not increase the sedimentation fraction, indicating that the effective separation was complete and the efficiency of sedimentation was not a significant problem. Furthermore, separating soluble from insoluble phase was done in the absence of centrifugation (filtration, see below) with the same experimental result.

RESULTS

The effects of volume change. In earlier studies [3] increasing the volume of fluid in which a given quantity of granules was suspended caused a major shift in the distribution of enzyme from granules (sedimentable phase) to suspending medium (non-sedimentable phase). Concentrated samples had under 10 % of the chymotrypsinogen in the soluble phase, whereas dilute suspensions were over 90 % soluble [3]. This data was derived from many individual preparations of granules diluted to varying degrees and normalized for the total chymotrypsinogen concentration of the final suspensions. The data shown in Fig. 2 are from multiple dilutions of aliquots from a single preparation of granules and show less variability than pooled data from many individual preparations [3]. The data in Fig. 2 show again [3] that enzyme is redistributed from granule to soluble phase in response to increasing volumes of dilution. The plotted least square linear regression lines (Fig. 2) have correlation coefficients of 0.99 for pH 7.0 and 0.98 for pH 5.5 and indicate that the log-log functions are linear. With S representing soluble enzyme, P enzyme sedimentable as a pellet, and V suspending volume, the line has the form;

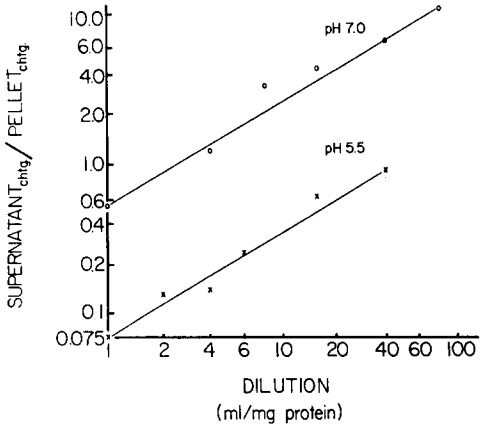

Fig. 2. The effect of dilution on enzyme distribution. The ratio of chymotryptic activity in the medium (supernatant) to that in the zymogen granules (pellet) is plotted against the granules per unit volume of medium or granule dilution (ml medium/mg zymogen granule protein) for the same group of isolated granules. (○) represents dilution in pH 7.0, 0.3 M sucrose solution and (×) represents those done at pH 5.5 in the same solution.

$$\log S/P = a + b \log V \tag{1}$$

Taking the antilog of both sides,

$$S/P = aV^b \tag{2}$$

If S/P is directly related to dilution in a linear fashion, as would be the case for certain simple equilibria, then b must equal 1. In the experiments that we previously reported [3], b approached one ($b = 0.9$) when the effects of dilution were studied at pH 7.0, but was substantially less than one ($b = 0.5$) at pH 5.5. This difference might have reflected either an intrinsic change in the association of chymotrypsinogen with the granule due to the change in pH or might have been caused simply by the fact that the distribution of enzyme between granule and medium might not have reached equilibrium after the one hour incubation period. Unlike this earlier result [3], for the data presented in Fig. 2, the slope for both pH 5.5 ($b = 0.83$) and pH 7.0 ($b = 0.80$) samples are about the same and approach 1. This is consistent with the view that the release phenomenon both at pH 5.5 and 7.0 are determined by the same kind of equilibrium in which the pH alters the affinity between enzyme and granule, but does not change the basic nature of the relationship. The Y-axis displacement of the two lines (Fig. 2) presumably indicates the pH-dependent shift in the affinity between enzyme and granule. In this experiment a change in pH from 5.5 to 7.0 produced an approx. 8-fold increase in S/P for any given dilution.

A linear relationship (b approaches 1) (i.e. $S/P = aV^1$ or $S/P = KV$) at equilibrium corresponds to $S/V = KP$, or the concentration of enzyme in the supernatant is directly proportional to the amount of enzyme in the pellet. This relationship would not hold if enzyme were stored in polymeric form within granules so that much of the enzyme would be unavailable to immediately participate in equilibrium events. That is, enzyme release from granules would be partially independent of the amount of enzyme stored within them. If the tendency for release of enzyme from the granule

were constant regardless of the total enzyme content of the granule, then enzyme release would be a function of the enzyme content of the medium, and not a function of both the medium and granule enzyme content (i.e. at equilibrium S would be directly and linearly proportional to V. b would therefore be equal to 1, when log S vs. log V is plotted). This can be written as

$$S = KV \tag{3}$$

Since the total enzyme in the system is constant and is divided between S and P, then

$$T \text{ (total enzyme)} = S+P. \tag{4}$$

A plot of log S/P vs. log V would, substituting Eqns. 3 and 4, equal a plot of

$$\log \frac{S}{T-S} \text{ vs. } \log \frac{S}{K}. \tag{5}$$

At $S \ll T$, this plot would approach

$$\log \frac{S}{T} \text{ vs. } \log \frac{S}{K} \tag{6}$$

which has a slope of 1.0 because T and K are constants.

As $S \rightarrow T$, $T-S$ becomes smaller and $S/(T-S)$ increases more rapidly than S/K, i.e. the ordinate ($S/(T-S)$ or S/P) increases more rapidly than the abscissa (S/K or V). Hence the slope must exceed 1.0. Since a slope of greater than one has not been seen, the enzyme concentration of the medium and granule apparently both determine the equilibrium distribution. This suggests that all or most of the enzyme within the granule is in direct equilibrium with enzyme in the medium.

The reversibility of the effect of volume. To determine the reversibility of the dilution-dependent release of amylase and chymotrypsinogen from zymogen granules,

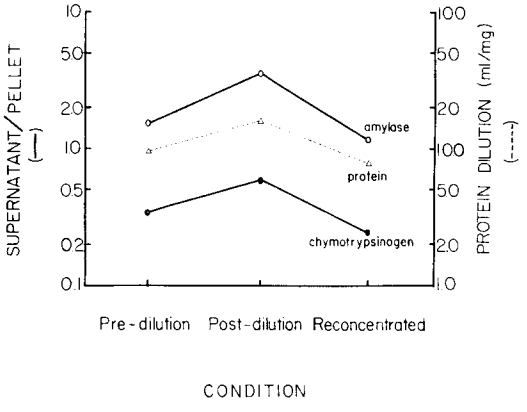

Fig. 3. Redistribution of enzyme on dilution and reconcentration. The distribution of enzyme between supernatant and pellet is shown by the solid line (—) and circles for chymotrypsinogen (●) and amylase (○) in three conditions; before diluting the granule suspension (pre-dilution), after dilution of the suspension but before reconcentration (post-dilution), and after reconcentration by dialysis (reconcentrated). The absolute degree of dilution and reconcentration is shown by the dashed line (– – –) and triangles. Its units are on the vertical axis on the right and are the inverse of protein concentration, i.e. protein dilution given in ml/mg protein. The logarithmic scale for both ordinates is equal, although the absolute values are different.

granule suspensions were first diluted and then reconcentrated in a hollow fiber dialysis chamber. Dilution, shown in Fig. 3 by the ordinate on the right labelled "protein", demonstrate the predicted release after dilution. After reconcentration a redistribution of enzyme from supernatant back into the granule (sedimentable) phase was observed. The relative effect of dilution and reconcentration on the distribution of chymotrypsinogen and amylase was roughly equivalent even though their original distributions are different (i.e. amylase started at 50 % soluble and chymotrypsinogen at 20 % for the study shown in Fig. 3). The ratio of enzyme in the supernatant to enzyme in the pellet increased 73 % for chymotrypsinogen and 107 % for amylase in response to a 60 % increase in suspending volume. These same ratios decreased 51 and 63 %, respectively, in response to a 53 % decrease in the volume of the suspending media. The similarity in these ratios is consistent with a single type of equilibrium governing the distribution of the different enzymes which only differ in their affinity constants.

The effect of time. When granule suspensions were diluted and supernatant and sediment separated after various periods of incubation, the equilibration of amylase and chymotrypsinogen between granule and medium in the new volume did not occur rapidly (within centrifugation time), but rather occurred slowly over time approaching equilibrium at 240 min (Fig. 4). More amylase was released and at a faster rate (release at 240 min = 80 %, $t_{\frac{1}{2}}$ = 30 min), than chymotrypsinogen (release at 240 min = 50 %; $t_{\frac{1}{2}}$ = 60 min). An initial delay in release was observed which may simply have been due to adding cold (4 °C) samples to a 37 °C bath causing slower initial release rates due to the initial lower temperature of the system [3]. The possibility of a long transit time, however, has not been excluded.

The effect of the granule membrane. The slow approach to a new equilibrium state may be explained by either a slow disassociation of enzyme from an intragranular aggregate (the membrane being a trivial barrier) or the restricted movement of enzyme

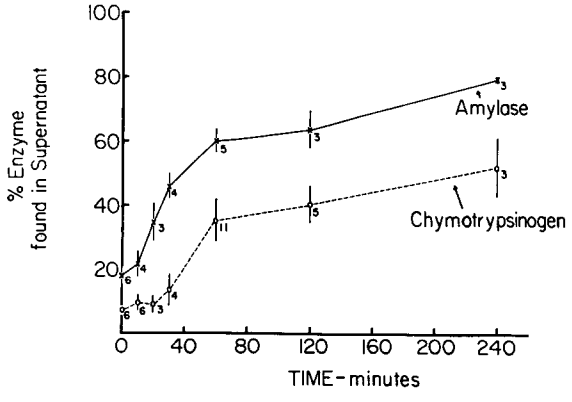

Fig. 4. The time course of enzyme redistribution after dilution. The percentage amylase (— × —) and chymotrypsinogen (---○---) activity found in the medium (supernatant) of a pH 5.5 suspension of granules at various times after dilution (time 0) of granule suspensions from approx. 1 ml/mg protein to approx. 10 ml/mg protein. Vertical bars represent the standard error of the mean and the number of experiments is noted by their side. No loss in enzyme activity was observed in samples incubated up to 120 min.

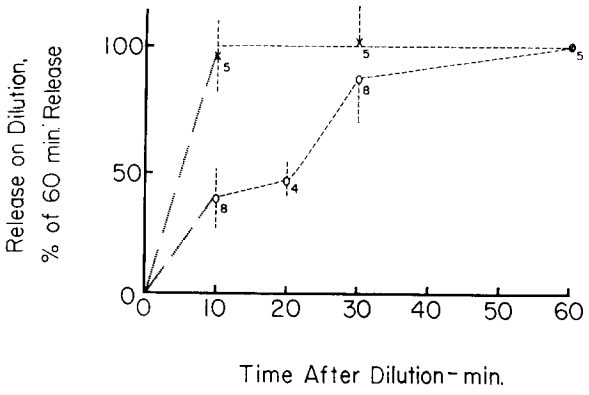

Fig. 5. The time course of enzyme redistribution after dilution for native granules and "reaggregated" granule material. The approach with time to the distribution between supernatant and pellet of chymotrypsin activity found at 60 min for whole granules and "reaggregated" granule material (with disrupted granule membranes) after an 8-fold dilution. Error bars represent standard error of the mean and the numbers represent the number of experiments. "x" is reaggregated material and "o" indicates native granules. The reaggregated material was at the new equilibrium within 10 min after dilution while whole granules continued to release enzyme with time.

across the granule membrane, or both. The contribution of the membrane to the delayed equilibration was examined by diluting reaggregated granule material which does not have vacuolar form and determining the kinetics of enzyme release from the aggregate (Fig. 5). For this material, dilution produced a new equilibrium distribution for total protein within 10 min, the first time interval measured (Fig. 5). In contrast, as discussed above, when the distribution of protein was measured for native granules as a function of time after dilution, a slow reequilibration was observed during the first hour (Fig. 5). This suggests that the impediment to equilibrium in whole granules is due to the presence of a membrane barrier rather than a slow disassociative reaction.

Release without centrifugal separation. If release were due to a disequilibrium between granule and medium (Fig. 4), then washing would remove soluble enzyme from the suspending medium and maintain the concentration gradient favoring the continuous release of enzyme. If release under these circumstances were simply due to a mechanical lysis of granules during centrifugation, then continuous release of enzyme would not be observed in such a system. To differentiate between these two possible causes for release, a filtration technique was used rather than centrifugation to separate granules from medium. On washing granules (that is, removing granule medium by filtration), release occurred continuously (Fig. 6) and did not decrease to zero as a function of time as it did in experiments where granules were suspended in a medium which was not renewed by filtration (Figs. 4 and 5). This supports the view that net release is a function of enzyme concentration in the medium and not merely a function of time.

Though granules are stable for relatively long periods of time (> 24 h) in pH 5.5 sucrose solutions [8], they might still be disrupted as a function of time in the filtration apparatus. To test for this, filtration rate was increased. If disruption were purely a function of time, then the amount of enzyme in the effluent should not change

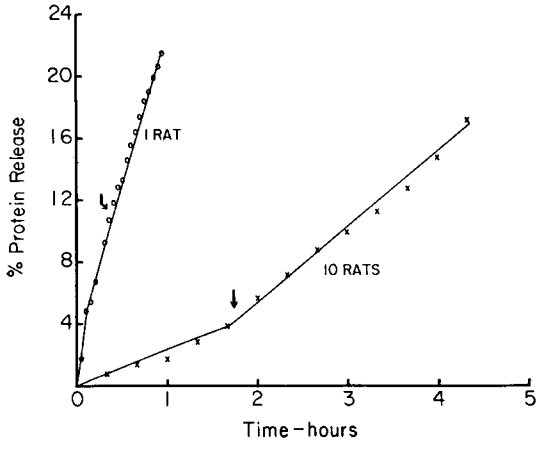

Fig. 6. Protein release from granules in response to continuous washing. Granules were placed in a mixing chamber and washed continuously with 0.3 M sucrose (pH 5.5). Granules were retained in the chamber by a 100 000 molecular weight filter through which the solvent and soluble secretory proteins passed. Protein collected in the effluent was considered "released". Two preparations are shown: one with a relatively high concentration of granules (collected from 10 rats) maintained at 25 °C (×) and one with a relatively low concentration of granules (from one rat) at 4 °C (○). The release function shown was equated for the temperature difference using the estimated Q_{10} of chymotrypsinogen release [3]. Flow rates were increased 20 % at the arrows by increasing filtration pressure from 8 to 24 lb/inch^2. The system with a low effluent protein concentration (low concentration of granules, one rat) showed no increase in the rate of enzyme released in response to increased flow rates. The system with high effluent protein concentrations showed an increased rate of enzyme released in response to increased flow ($\triangle b = 20\,\%$). This suggests that backflux of enzyme into granules is a significant factor in net enzyme release from the concentrated granule preparation at high temperatures (25 °C), but not from the low concentration of granules at low temperatures (4 °C).

but its concentration should decrease. Using granules from 10 rats filtered at 25 °C, the amount of enzyme in the effluent increased 20 % in response to a 20 % increase in the filtration rate (Fig. 6), whereas its concentration did not decrease. Thus, enzyme release in this system was a function of the suspending or filtration volume and not a function of the continuous disruption of granules with time.

The fact that the concentration of protein did not change in the effluent (75 μg/ml before and after the increase in filtration rate) although the total amount filtered did, suggests that the effluent medium is close to equilibrium with the granule content. We attempted to reduce the concentration of enzyme in the effluent by reducing the granule load in the chamber (granules derived from one rat instead of 10) and lowering the temperature of the incubation from 25 to 4 °C. These changes lowered the effluent concentration from 75 to 10 μg/ml. Such a reduction in the effluent concentration might reduce the unidirectional flux into the granule from the medium (influx) and displace the system from equilibrium. To test this the filtration rate was increased. In contrast to the result with granules from 10 rats where protein concentration in the effluent was unchanged when filtration rate was changed, with granules from one rat protein concentration decreased (10 to 6.5 μg/ml) and the rate of enzyme released was unchanged (Fig. 6). Thus the net rate of release of enzyme under these conditions appears to approach the unidirectional efflux from granules and is insensitive to changes in the concentration of enzyme in the medium.

The rate of enzyme release (before increasing perfusion pressure) was approx. 500 μg/h for the 10-rat system and 100 μg/h for the 1-rat system, but since the 10-rat system had about 10 times the load of granular protein, proportional release rates were about twice as great in the 1-rat system. Since the 1-rat system was run at 4 °C instead of 25 °C and there is a high Q_{10} for enzyme release from zymogen granules [3], the temperature-corrected proportional release rate was much greater in the 1-rat system than in the 10-rat system. The magnitude of this difference is seen in Fig. 6. This is also consistent with the view that net release from the 1-rat system is close to the unidirectional efflux from granules while net release in the 10-rat system includes a substantial influx from medium back into granules.

DISCUSSION

Evidence for equilibrium. The present observations suggest that the distribution of digestive enzyme between zymogen granule and medium is determined by equilibrium considerations: (1) Release of enzyme from granules occurs in response to an increase in the volume of the suspending medium (i.e. enzyme is redistributed in response to a displacement of an equilibrium); (2) Reassociation of enzyme with granules occurs in response to a reconcentration of the suspending medium (i.e. equilibria are reversible). This is consistent with earlier observations that bovine chymotrypsinogen associates with enzyme-depleted rat zymogen granules [3]; (3) Reconcentration after dilution restores the pre-dilution distribution of enzyme (i.e. an equilibrium is a function of state); (4) Enzyme release occurs independent of the technique used to separate granules from medium, or similarly, independent of whether released enzyme is recovered as a supernatant after centrifugation or as an ultrafiltrate (i.e. release is not a technical artifact due to granule lysis); (5) Increasing the number of granules/unit volume of suspension medium decreases the relative rate of enzyme release (Fig. 6). If lysis were an independent event the rate of enzyme release would be expected to be independent of granule concentration. Similarly, when enzyme is removed from the medium, as it is by filtration, release continues relatively unabated with time, unlike release in the absence of filtration which decreases toward zero as a function of time (Fig. 4). (i.e. net release = unidirectional release minus backflux. If backflux is decreased by the filtration of released enzyme, then a steady state is not reached).

The nature of the equilibrium. Most of the digestive enzyme within zymogen granules is probably in a bound, osmotically inactive form [8, 13]. To discuss the nature of the enzyme equilibria we should first consider some characteristics of zymogen granule structure relevant to our discussion: (1) Sufficient binding capability exists within the granules to account for virtually all of the digestive enzyme recovered in isolated zymogen granules [6, 9]; (2) Only a small fraction of the total granule mass can be accounted for by non-digestive enzyme moieties [5, 14]; (3) Granules have binding maxima for the digestive enzymes and therefore enzymes within granules do not appear to be in the form of a simple precipitate [9]; (4) The binding of one enzyme occurs independently of the presence of other enzymes [9].

Whatever structural model is proposed must be consistent with these characteristics and also with the present observation that the distribution of enzyme between granule and medium (S/P) appears to approach, at equilibrium, a direct proportionality

with the volume of the suspending medium (V), or $S/P = KV$. An earlier proposal by one of us (S.S.R.) suggested that digestive enzymes might exist as large polymers within the granules. This hypothesis was proposed in order to account for large amounts of digestive enzyme bound in the apparent absence of substantial amounts of potential binding agents [6, 9, 15]. In such a situation, the molecules at the end of the linear polymers or alternatively on the surface of the globular polymers would be released before enzyme not at the interface could be released. Hence the tendency for enzyme to be released from such an aggregate would be proportional to the number of molecules at the outer limit or interface of the polymer with its surround. Since the loss of one or several molecules would reveal others, the tendency for enzyme release would not be a function of the total amount of enzyme within the aggregate, but would only reflect the number of interface molecules at a given time. In other words, the enzyme released over a range of dilutions would not be directly proportional to the total enzyme content of the granule, or the rate of disassociation would not be k_dP but some fraction thereof. Furthermore, the rate of association is proportional to the enzyme concentration in the medium, i.e. K_a (S/V). Since the rate of association = rate of disassociation at equilibrium, $S/P = [K_d/K_a]\,V$ if all intragranular enzyme molecules are in equilibrium with the medium (individual binding sites). These two statements, however, would not be equal in the case of large polymers. Since equality was approached (i.e. S/P was related to V in a manner approaching linearity or a log-log slope approaching 1 was found (Fig. 2)), then the large polymer model must be rejected. That is, each (or more accurately, most) enzyme molecules in the granule are apparently in an independent equilibrium with enzyme in its surround.

The role of the membrane in mediating the equilibrium. The equilibrium for enzyme between the zymogen granule and its surrounding medium requires that enzyme move between these two compartments apparently across the membrane of the granule. This movement may occur either through the "intact" membrane or through defects in the membrane which might have been mechanically produced during separation. If the latter were true, then the attainment of equilibrium would only be delayed by the time required for the chemical disassociation of enzyme from individual sites in the granule aggregate. While the slow observed approach to equilibrium (e.g. $t_{\frac{1}{2}}$ for chymotrypsinogen of about 60 min) would be very unusual for such a simple non-covalent chemical equilibration between a protein and its biological ligands, we tested for this possibility directly by measuring the release of enzyme as a function of time from "reaggregated" granule material which does not have a membrane-bounded vesicular form. The delay to equilibrium was eliminated along with the elimination of the membrane boundary (100% equilibration within 10 min incubation time as compared to 40% for whole granules under similar circumstances). The movement of enzyme across the membrane of isolated zymogen granules in two directions requires that the individual enzyme molecules pass through the membrane without the deletion or consumption of granule membrane. This is so because the release of enzyme is reversible and because enzyme release in small vesicles requires much more membrane than the granule apparently contains ($[r_{\text{zymogen granule}} - r_{\text{small vesicle}}]/r_{\text{small vesicle}}$ is the proportionate increase in membrane required for small vesicles).

The observations that zymogen granule membrane is permeable to secretory enzymes in vitro (granules isolated from cells) has an in vivo (in whole cells) counterpart. That is exogenous enzyme (bovine chymotrypsinogen) enters the acinar cell and

equilibrates with the enzyme content of the zymogen granule within the intact cell when it is added to the medium-bathing tissue slices [4]. Therefore, zymogen granule appears to be permeable to secretory proteins. While both the membranes of isolated granules and those in situ appear to be permeable to digestive enzymes, the quantitative relationship in the behavior of granules in these two different environments cannot be characterized at present. Recent observations of Dandrifosse and Simar [16] using a different experimental approach suggest an in situ permeability also. While the simplest hypothesis is that the observations of zymogen granule permeability seen in situ and in isolated granules represent two aspects of a single phenomena, this cannot be rigorously established at present.

Earlier studies with isolated zymogen granules. In the experiments by Hokin [5] in which he first isolated zymogen granules from pancreatic tissue, granules were observed to lose much of their protein content at neutral pH (7–7.5), the probable pH of the cytosol. In addition, zymogen granules lose much of their content in isotonic KCl [13] a medium presumably similar to the ionic environment inside the cell. If granule membranes are unstable ("lyzed") under these conditions, then how could they exist in the intracellular milieu? If enzyme release occurs as the result of equilibrium events across intact granule membrane rather than by the lysis of whole granules, then this question will become moot. The distribution of enzyme between cytoplasm and zymogen granule would be a function of pH, ionic environment, and suspending volume, among other things. While the apparent ionic environment and pH of the cell would shift the equilibrium toward the cytoplasm, the relatively small suspending volume (i.e. the cytoplasm of single cells) would tend to shift it into the granules. This prediction is based upon the extrapolating in vitro data to a cellular system and while such extrapolation seems reasonable, it must be confirmed in situ.

Digestive enzyme secretion. There is now evidence that enzyme in the cytoplasm acts as a pre-secretory pool [11, 17, 18]. A stimulus-induced redistribution of enzyme between granule and cytoplasm may function to augment secretion via the cytoplasm. Indeed, we must consider the possibility that zymogen granules may serve as storage vesicles which release their content wholly or partially, in this manner.

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