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Protein flux across the membrane of single secretion granules

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We have applied, for the first time to our knowledge, X-ray microscopy to measure the mass of protein contained in *single* sub-cellular membrane-bound structures and to make high resolution, time-resolved observations on them. Using this method we have been able to follow the flux of protein out of secretion (zymogen) granules isolated from the acinar cells of the exocrine pancreas. The results provide direct visual and quantitative confirmation of the hypothesis that the membrane enclosing this object is permeable to its various contained proteins, although the mechanism remains unknown.

1. Introduction

It has been about twenty years since the trans-membrane transport of macromolecules was first reported [1]. In this original study, evidence was presented that the some 20 different proteins stored in a particular microscopic membrane-bound sub-cellular structure (the zymogen granule) were released from the isolated object into the suspending medium in response to mass action. The proteins released from these granules are the digestive enzymes that the acinar cell of the exocrine pancreas manufactures and secretes into the digestive tract. The fact that they were able to cross the granule membrane suggested that protein secretion, at least in the case of the exocrine pancreas, might include the transport of individual macromolecules through granule and cell membrane [2]. Other evidence, both in situ and in vitro, supported this idea [3].

At the time, this notion clashed with the already accepted view that the secretion of large organic products, especially proteins, resulted from their gross movement by vesicle-based transfer processes. Added to this was the widely held presumption that transport of a protein molecule across a membrane would be analogous to solubilizing highly charged spheres (polar globular proteins) in a nonpolar phase (the lipid bilayer) – an energetically unfavorable event. For these reasons, the idea of an alternative, non-vesicular, modality for secretion became a subject of continuing controversy and the original observations were often discounted as artifact.

This particular secretion process aside, in the intervening years, it has become evident that the direct transmembrane transport of proteins subsequent to their synthesis is a common process; for example, into mitochondria and the nucleus. With the discovery of specialized pores through which proteins can pass, membrane porter molecules for proteins, and cytoplasmic helper molecules or co-factors, such as chaperones, notions of a priori thermodynamic limitations have been replaced by a growing realization that the complex, physically and chemically diverse structures of biomembranes and proteins are variously adapted to carry out protein transport functions.

Also, important new evidence for protein transport in zymogen granules has recently been reported [4]. Applying Debye-Mie scattering theory, the relationship between decreases in the light scattered by a suspension of zymogen granules and protein release was found to be the result of changes in the size and/or refractive index (loss of content) of intact granules. Dissolution or lysis of the granule, the artifact to which protein release was most often ascribed [5,6], could not account for the results.

Nonetheless, and in spite of these new understandings, it has remained the common view that cells that manufacture and secrete protein as a major activity, such as the acinar cell of the exocrine pancreas, do so by means of vesicle transport processes and such processes alone [7]. The details of these processes, which involve an extensive membrane recycling system and a large number of associated receptor targets [8], have

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yet to be fully established. Given current knowledge of protein transport across membranes, we have revisited the early observations on zymogen granules [1] both to further validate its occurrence in this system and to characterize the permeability properties of the membrane, with a view towards eventually discovering the intimate mechanism(s) of transport, and the respective physiological roles of membrane- and vesicle-based protein transport in the process of secretion.

2. Application of the X-ray microscope

In the current work, we sought new, and uniquely direct, evidence for the permeability of the zymogen granule membrane to its various formed proteins. An examination on a visual and quantitative level of *individual* granules as they released their contained proteins would clarify the events that occur. Direct visual proof for the mechanism of protein release from these objects has not been available, or perhaps not obtainable. The granules are just barely discernible in the ordinary visible light microscope (appearing as dark spots in a bright field) and morphologic changes, such as decreases in size, cannot be resolved. Nor can changes in the protein content of individual granules be measured by even the most sensitive chemical methods. Similarly, even though standard transmission electron microscopy provides the needed resolution, time-resolved imaging and examination of the samples in an aqueous medium (so that release could occur) is not possible.

At least in theory, the newly developed method of scanning transmission X-ray microscopy (STXM) offered the possibility of making the needed measurements. It can, (1) provide the necessary resolution, approx. 50 nm, to observe the morphologic consequences of protein release from individual granules. (2) allow the needed aqueous environment in which to suspend whole granules and (3) permit the simultaneous quantitative measure of protein release from individual granules on the order of femtograms.

Two, relatively recent technological advances, high brightness, partially coherent soft X-ray radiation sources (e.g., synchrotrons) [9–12] and diffractive X-ray lenses capable of focusing such radiation to a small spot size [13–15] have made high resolution X-ray microscopes a practical reality. Although their diffraction-limited resolution is still an order of magnitude larger than the wavelength of the illuminating radiation (3.5 nm), these microscopes are capable of 30–50 nm resolution today and will probably approach 20 nm in the near future [16]. A special feature of the method is its ability to combine high resolution microscopy and the examination of biological samples in their natural state [17]. That is, the sample can be viewed without alteration, other than separation from its source tissue or cell, in a physiological environment at ambient or body temperature. Fixation, staining and sectioning are not required, and the object can be suspended in and contain water as it normally does. In addition, and importantly for the current experiments, because the contrast mechanism is based on the differential absorption of X-rays by particular elements naturally present in the sample, quantitative estimates of elemental distribution can be made simultaneously. By tuning the incident radiation to specified energies between the oxygen and carbon k edges (the soft X-ray 'water window'), X-ray absorption becomes predominantly a measure of the carbon content of the sample, and consequently in most biological structures an estimate of its protein content.

The work that we shall discuss applies all these features simultaneously in a single dynamic-biological experiment. Examination of individual zymogen granules in the X-ray microscope show them releasing their protein contents into the suspending medium in a continuous fashion, decreasing in size and protein content over time (preliminary results presented at the Third International Symposium on X-Ray Microscopy, London, 1990, Rothman, S.S., Goncz, K.K. and Loo, B.). Simple equilibrium displacements (viz. perifusion) caused the continuous release of protein from zymogen granule suspensions and concurrent time-dependent reductions in both mass and the size of individual granules, as opposed to changes in their number (lysis). The results provide confirmation of the hypothesis that the membrane that encloses the zymogen granule is permeable to many, if not all, of its contained proteins, and releases them in response to mass action.

3. Materials and Methods

Preparation and handling of zymogen granules

Zymogen granules isolated from the rat pancreas will release up to 90% of their protein content when subjected to a variety of simple environmental manipulations in vitro, including suspending granules in media whose composition is similar to the intracellular environment in which they are normally found. For example, granule contents are lost at neutral pH, in isotonic ionic media and in the presence of physiological concentrations of certain sugars and amino acids [18–22]. Also, protein release occurs slowly from granules in what is called 'stable' suspension (slightly acid pH and non-ionic suspending media), even when no particular perturbation is introduced. Net protein efflux can be increased in a continuous fashion by mass action simply by introducing equilibrium displacements to reduce the concentration of digestive enzyme in the suspending medium [1,23,24]. And indeed, release can be brought to completion (approaching 100% of granule

content), if the gradient is renewed continuously (perifusion).

The granule sample was prepared from acinar cells of rat pancreas. Sprague-Dawley males (Taconic Farms, Germantown, NY), 200–225 g, were used. Animals were fasted overnight prior to removal of the pancreas. Granules were isolated from ground, fresh tissue by methods described in detail elsewhere [23]. The granule sediment was resuspended in 0.3 M sucrose with 5 mM $NaHPO_4$ buffer (pH 6.0) prior to viewing in the microscope.

Once resuspended, the granules were either kept on ice until they were used or immediately introduced into a specially designed sample chamber, called the Lawrence Berkeley Laboratory wet cell (LBL-WC). The sample suspension was flowed into the interior of the chamber by means of a peristaltic pump at a flow rate of about 1 μl/min. The specimen could be observed in a light microscope as it passed by the viewing window of the LBL-WC (made of 60 nm thick silicon nitride) and we were able to assess adherence of the sample to the window. With the sample in place, the LBL-WC was placed in the X-ray microscope. The introduction of experimental solutions was done using the peristaltic pump, with the LBL-WC in place in the microscope. The LBL-WC holds the sample vertically in the microscope and is thin enough to fit in the 400 μm working distance between the focusing optics and the sample stage. The wet cell device and manufacturing process is described in more detail in a paper soon to be published (Goncz, K.K. et al. (1992) J. Microsc. (London)). All experiments took place at room temperature, 25°C.

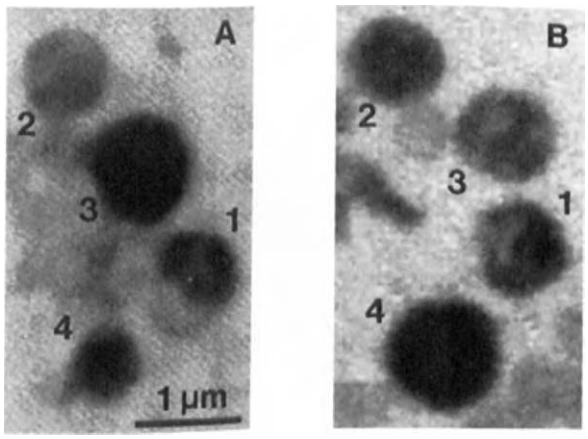

Fig. 1. Two images of zymogen granules from different preparations (suspended in 0.3 M sucrose with 5 mM $NaHPO_4$ buffer (pH 6.0)). A.1 and B.1 are examples of a dense end or 'cap' structure that is commonly seen in X-ray images. The granules in A have average protein concentrations of 172 mg/ml (A.1), 204 mg/ml (A.2), 433 mg/ml (A.3) and 488 mg/ml (A.4) and in B, 290 mg/ml (B.1), 338 mg/ml (B.2), 267 mg/ml (B.3) and 450 mg/ml (B.4). In previous reports, we have shown similar X-ray images of the structure of the zymogen granule [42,43]. All of these images characteristically show the granule population to be heterogeneous in appearance; some granules appear uniformly dense while others are partially empty with a dense region on one end or a cap. From physical and biochemical analysis it is known that the structures seen in the granule are (predominantly secretory) proteins held as insoluble complexes formed by weak (non-covalent and probably in great part polar) high-affinity (protein-specific) bonds [19,23,44]. Electron micrographs of partially depleted granules suggest an underlying structure of dense strands of variable thickness that border lucent polygonal spaces [23]. Image parameters: pixel size, 19 nm × 19 nm (A), 32 nm × 32 nm (B); dwell time, 3 ms/pixel (A), 5 ms/pixel (B); λ = 3.6 nm (A), λ = 3.4 nm (B).

Calculation of granule protein content

The X-ray microscope used to perform these experiments is located at the National Synchrotron Light Source in Brookhaven National Laboratory at the undulator beam line (X1A) [25–27]. The specimen is scanned across a focused spot of X-rays using piezoelectric motors and the transmitted radiation is detected by means of a gas flow proportional counter, and recorded digitally. The resulting image is an array of picture elements or pixels whose size was determined in multiples of 30 nm or approximately half the resolution of the microscope.

Since this digital image is a record of the photons transmitted through the sample, it can be analyzed to give quantitative information about the mass of the object. In the energy range of the X-rays that are used, essentially all of the photons interact with the sample through photon absorption (scattering effects are negligible) and a relationship between the transmitted intensity (I) and the incident intensity (I_0) of

$$I = I_0 e^{-\mu \rho z} \tag{1}$$

is given and can be solved. The variables are: μ (cm^2/g), the mass absorption coefficient which depends on the atomic composition of the sample and the energy of the radiation; ρ (g/cm^3), the density; and z (cm), the thickness of the sample. The variable I_0 is normally considered to be the intensity value of the unattenuated beam. In our calculations, we have used the value of the transmitted intensity (I) from regions in the window that are clear of granules as I_0 so that absorption due to the air path and the silicon nitride windows is normalized. As a result, Eqn. 1 becomes,

$$I = I_0 e^{-[(\mu\rho)_p - (\mu\rho)_s]a} \tag{2}$$

Absorption due to the lipid bilayer was not accounted for and space inside the granule not occupied by protein is assumed to be filled with the suspending medium, 's'. Here, 'p' refers to zymogen granule protein. Because the granule contains a quantitatively unknown combination of 20 or so digestive enzymes, we chose to use a general formula for animal protein in calculating the molecular mass m; $C_{313}H_{498}S_3O_{101}N_{85}$. The variable '$a$' is the pathlength of protein within the

granule, which we can solve for on a pixel by pixel basis.

Using a standard value for the density of protein, the protein mass can then be calculated. The protein concentration per pixel volume (voxel) can also be calculated by assuming a spherical model for the granule; the pathlength through the granule at a specific pixel location is just the chord length through a sphere and the voxel value is the chord length times the pixel area.

For the limited population of granules examined thusfar (approximately 100 objects), the range of protein concentration calculated within the granule varies from about 50 mg/ml to 700 mg/ml. Literature values for average protein concentration in populations of granules range from 150-400 mg/ml, depending upon the method of estimation [28]. Dense regions are often seen interspersed with the more lucent areas giving the granule a reticulated appearance and it is common for individual granules to have one 'end' more dense than other regions (Fig. 1).

4. Results and Observations

Changes in the protein concentration of granules over time: control experiments

In our first experiments, we observed that granules decreased in protein concentration (protein mass/granule volume) after their resuspension from the final sediment in the absence of any other particular treatment, and over time approached new steady-state values (Fig. 2). The initial granule resuspension was introduced into the LBL-WC and the granules imaged over time without the introduction of other solutions. De-

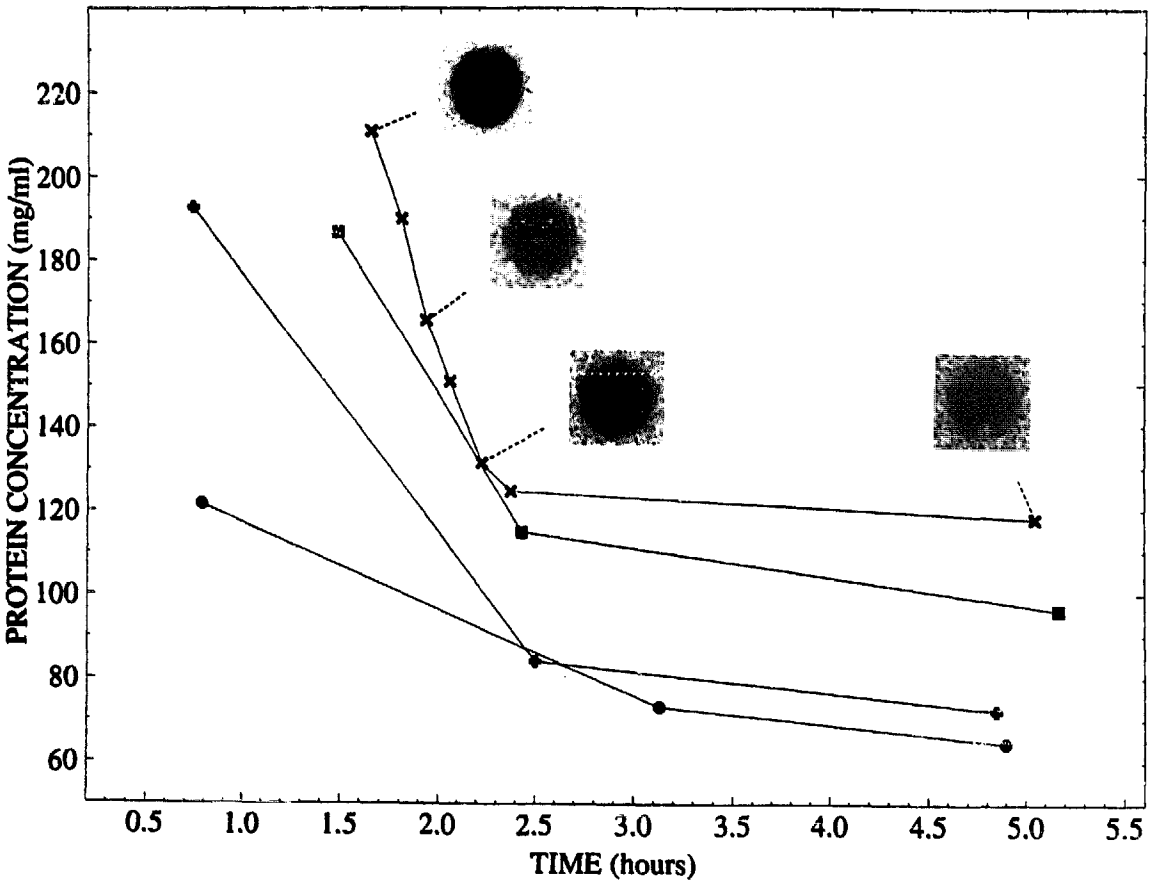

Fig. 2. The approach to a steady-state protein concentration for four granules in the same field from the same preparation. No treatment was applied to these objects other than their resuspension from the final granule sediment (see text for further description). Also shown are four images from the sequence for a particular granule (×) during the fall in protein concentration. Note that other than the interior of the granule becoming less dense *, the object is otherwise unchanged and maintains a diameter of approx. 1 μm throughout. Protein concentration declines towards a plateau with three of the four granules converging on a single steady-state value by 5 h. Image parameters: pixel size, 32 nm×32 nm; dwell time, 2 ms/pixel; $\lambda = 3.4$ nm.

* [Because we chose to display the images in this article without any image processing, it is not possible to quantitatively compare the density of the images visually. This is a consequence of the source of the X-rays. The beam is emitted from an electron storage ring. The current of this ring, and the flux of emitted X-rays, decays over time. That is, the light source is not constant, but decreases in intensity (I_0) over time. Therefore, the 'density' or darkness of images taken at different time points cannot be compared, without first correcting for the decrease in I_0. The quantitative data, corrected for I_0, is presented in the graph in this figure as protein concentration.]

creases in granule protein concentration are shown in Fig. 2 for four granules from the same sample preparation, suspended in the same medium, and viewed simultaneously over a period of several hours. Decreases in protein concentration of approximately 37–56% were observed. The final values were similar for three of the four granules in the same field and suggest attainment of a common equilibrium state. Consistent with this

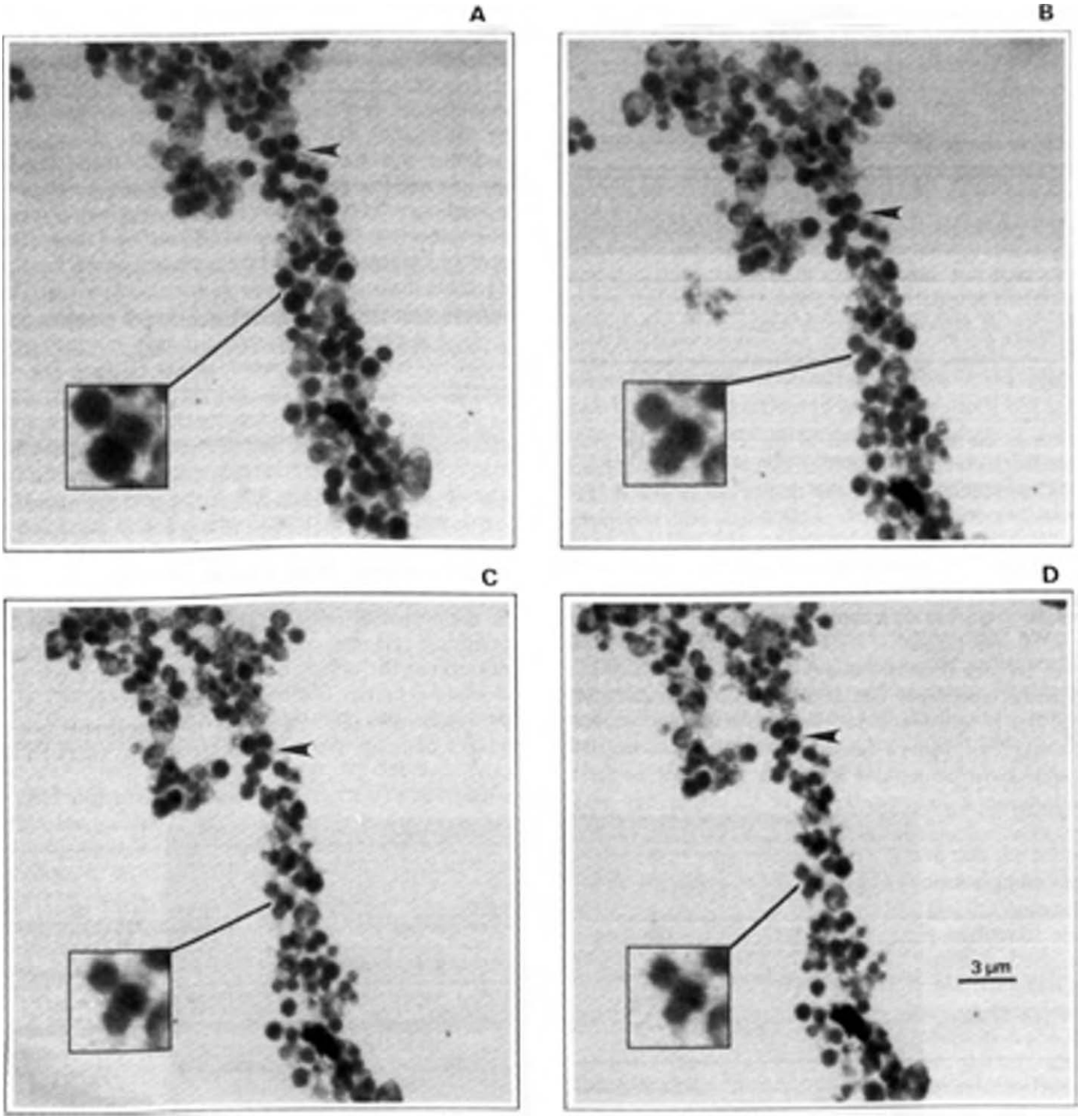

Fig. 3. The continuous release of protein from zymogen granules as a result of the perifusion of fluid. Granules decrease in size and mass over time. The initial state is shown in 3A (suspended in 0.3 M sucrose with 5 mM $NaHPO_4$ buffer (pH 6.0)), and the changes produced as a result of fluid flow are shown as they evolve at hourly intervals. Water was flowed past the objects for 20 min at the beginning of each hour. One group of three granules is shown decreasing in size in the inset. Also, the arrow denotes another group of granules shrinking. Fifty granules were analyzed. Their average volume decreased from 0.45 μm^3 (3A) to 0.38 μm^3 (3B) to 0.32 (3C) to 0.25 (3D), and protein mass decreased from 265 femtogram to 185 fg to 161 fg to 139, respectively. Protein concentration was 633 mg/ml in 3A, decreased to 516 mg/ml in 3B, but increased again in 3C to 525 and again in 3D to 591 mg/ml. Protein release from the granules was not dependent upon the initial concentration of protein within the objects; that is, release occurred from granules with a high initial concentration as well as from those with a low initial concentration. Image parameters; pixel size, 63 nm × 63 nm; dwell time 4 μs/pixel; λ = 3.5 nm.

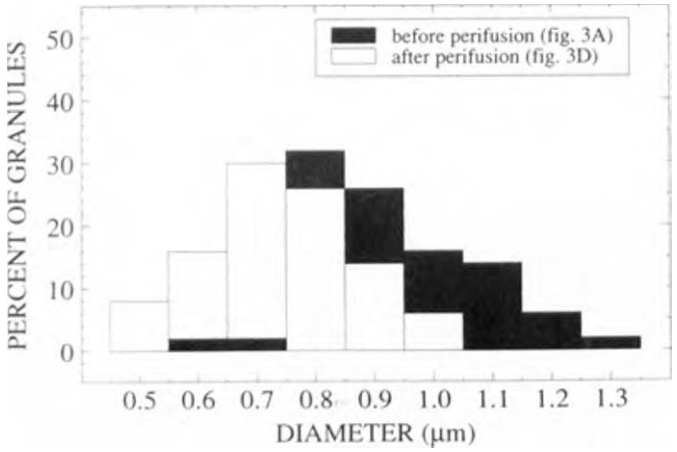

Fig. 4. Distribution of zymogen granule diameters for the 50 individual granules shown in Fig. 3; 'before perifusion' refers to Fig. 3A and 'after perifusion' refers to Fig. 3D. The column height is the 'percent granules' for each time period (black and white). They are not additive. The peak diameter shifted from 0.8 μm to 0.7 μm. Before perifusion only 4% of the granules had diameters less than 0.75 μm, whereas after, 50% of granules fell in this range.

view is the fact that one of the objects, at a lower concentration than the others initially, fell less to reach the asymptote. That is, the magnitude of the decline was proportional to the initial state and was not a uniform response.

Induced decreases in the size and protein concentration of granules: flow experiments

We also produced release of protein from granule suspensions by imposing simple equilibrium displacements by means of the perifusion of fluid. As noted above, such effects had been described in earlier, non-X-ray microscopic, work on the subject [1,23,24]. After the LBL-WC, containing granules adhered to the window of the chamber, was inserted into the microscope, an initial image of the sample was taken. Subsequently, water was flowed through the LBL-WC at a rate of approximately 1 μl/min (the granule's environment being renewed about once every 15 s). Granules remained in place throughout the experiment and were imaged at approximately hourly intervals. As the solution moved past populations of granules, changes in granule size, protein concentration and number could be observed directly.

When this was done, granules lost their protein content and became smaller. In Fig. 3, a large population of granules is shown in four separate frames taken at hourly intervals as water was flowed past them. The area of the image covered by granules can be seen to decrease over time as the granules are shrinking. The protein concentration within individual granules decreased somewhat in the middle frames, but increased again as granules continued to shrink (see Fig. 3, legend).

In this study we did not observe lysis. In a lytic process, if a particular perturbation produced the release of 50% of the protein contents of a particular granule suspension, then 50% of the granules would be lysed and their contents released and solubilized, whereas the remaining 50% that did not lyse would remain unaltered. Most, if not all, of the granules were altered by perifusion, showing a continuous reduction in their protein content. Furthermore, release from individual granules occurred relatively evenly from throughout the object, not, as we would expect if the membrane barrier had been compromised locally, at a particular locus. Some granules did disappear between images and this might reflect their lysis, although of

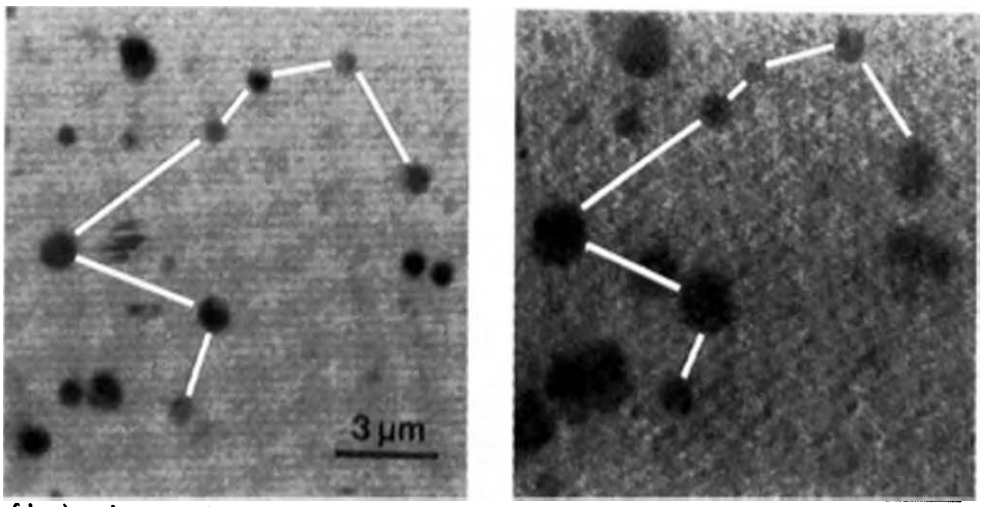

Fig. 5. The uptake of bovine chymotrypsinogen A by zymogen granules. (A) Field of zymogen granules prior to the addition of 250 mg/ml chymotrypsinogen (in 0.3 M sucrose with 5 mM $NaHPO_4$ buffer (pH 6.0)). (B) The same field 10 min after the addition of chymotrypsinogen. Granule volume, on average, increased from 0.56 μm^3 to 2.2 μm^3. The low contrast of the second image is the result of the increased density of the surrounding medium due to the addition of chymotrypsinogen. Image parameters: pixel size, 59 nm × 59 nm; dwell time, 4 ms/pixel; $\lambda = 3.6$ nm.

course it might simply mean that they had moved out of the field of view.

Increases in the size of granules after the addition of exogenous chymotrypsinogen A

The reversibility of protein release from zymogen granules has been demonstrated (in the absence of X-rays) by the uptake of previously released protein and exogenous proteins of the same type found within the granule. This has been seen both in vitro and in situ [1,24,29].

The experimental procedure used to introduce the granules into the LBL-WC was the same as in control and flow experiments. After the initial images were taken, a solution of 0.3 M sucrose (pH 6.0, 5 mM $NaHPO_4$) containing either 50 or 250 mg/ml chymotrypsinogen A (bovine, Sigma), was introduced into the LBL-WC. At the lower concentration, granules continued to decrease in size over time, as they did normally due to protein efflux (data not shown). At 250 mg/ml, however, this process was reversed, and granule protein mass and volume became larger, indicating uptake of the added enzyme (Fig. 5). The increase in granule size was quite large (on average, about 4-times), suggesting major changes in membrane organization as a result of protein uptake.

4. Discussion

The membrane permeability barrier

Thus, the measurements showing loss of protein content from isolated zymogen granules and the X-ray images taken simultaneously provide direct evidence confirming the occurrence of protein transport across zymogen granule membranes. But, before we draw the conclusion that a natural permeability exists across this membrane in vitro for these proteins, we must convince ourselves that during the isolation process the membrane bilayer has not been compromised or has even become absent, and that protein release is not simply from a suspended protein aggregate.

There are a great many reasons for thinking that the membrane barrier is present and a partial list is as follows:

(1) Zymogen granules isolated by the methods that were used here are shown to be enclosed by a bilayer membrane when examined in the electron microscope. Thus, isolation does not remove the membrane. One group [5] has suggested that the membrane is permeabilized in some fashion during isolation by this method, and that they can produce granules that lack such permeability. Studies by others have not confirmed this result [6,30]. In addition, the permeability of the granule membrane to protein has been demonstrated in situ where artifacts induced by isolation are not a question [29].

(2) Granules partially or wholly depleted of their protein contents, by techniques similar to those that we used, are still enclosed by a membrane that appears whole in thick sectioned electron microscopic stereo pairs, as well as in standard thin-sectioned transmission images [23]. Thus, emptying granules does not remove the membrane.

(3) Removal of the membrane by detergents produces the dispersion of essentially all of the protein content of the object in a matter of seconds. We performed such an experiment in the X-ray microscope by perifusing 0.05% Triton X-100 through a granule-filled chamber. This caused disappearance of the granules and their contents, leaving behind only remnants of the original objects. Thus, the contents of the granule solubilize in dilute suspending media in the absence of the granule membrane. This should not be surprising because the proteins are held in the aggregate by weak interactions (dilution completely disrupts these complexes and solubilizes the proteins).

(4) Our measurements showed a substantial restriction of protein diffusion away from the granule, even as release occurs. Permeability coefficients calculated from the flux and concentration data for individual objects provide values ranging between 10^{-9} and 10^{-10} cm/s. This is a low permeability and indicates the presence of a substantial barrier to diffusion, which, lacking other possibilities, must be the membrane.

(5) The granule edge remained sharp even as protein was released and, as noted above, the internal content remained uniform during release. It did not become diffuse and less dense towards the granule edge as we might expect if release occurred slowly from the aggregate in the absence of a membrane barrier.

Thus, there is every reason to believe that the membrane that normally encloses the zymogen granule was in place as we carried out our studies. But this does not exclude the possibility that the membrane barrier was affected in some fashion by direct or indirect effects of the X-rays, producing a membrane permeability that is not naturally present. Although far less radiation intensive than electron microscopy, the absorption of soft X-ray photons produces photoelectrons which in turn are able to ionize water and protein to produce radicals. These species, although highly reactive, will probably not travel more than 5 nm and so, the structural damage due to absorbed radiation can be expected to be relatively small for the moderate resolution demands of the current experiments (approx. 1000 photons/50 nm × 50 nm × 2 μm voxel).

However, oxidative damage spreading along a membrane could introduce an artifactual permeability, even if structural damage were otherwise minimal [31]. Al-

though such damage may well occur, the present results do not appear attributable to a radiation-induced permeability artifact in regard to either protein release, granule shrinkage (or swelling) or change in density. First, the same treatments applied here lead to the release (or uptake) of protein from the granule, with similar half-times in the complete absence of ionizing radiation [1,23,24].

Second, neither the rate of protein release nor the observed structural change (shrinkage, swelling or change in density) were correlated to imaging frequency (and hence radiation dose). For example, the fall in protein concentration over time for two objects in the same field with comparable, initial protein concentration occurred at roughly the same rate, even though one was imaged six times and the other only examined at the beginning and end of the sequence (see Fig. 2). Additionally, images of objects taken after a steady-state protein concentration is approached show only small changes in density (Fig. 2). If the membrane barrier was being compromised by imaging, then we would have expected the contents of the granules to continually diffuse out into the external solution until the object was essentially emptied, rather than maintaining a steady-state concentration.

One granule imaged 15 times at a total applied radiation dose of 64 Mrad remained intact and its mass, size and general morphological integrity maintained over time. Also, shrinkage and changes in protein concentration were observed in anaerobic environments using solutions where nitrogen was the dissolved gas and where oxidative damage should be mitigated.

Mechanism of release

These observations provide evidence for the membrane's permeability to the proteins contained within the granule, but they do not tell us how transport occurs. It has been known for a long time that at least certain water-soluble globular proteins can penetrate and cross even, low conductance, simple artificial lipid layers [32–35] and some of the proteins in the granule form specific calcium-dependent complexes with amphipathic phospholipids that greatly decrease their hydrophilicity [36]. Thus, the possibility of bilayer penetration by the protein cannot be excluded as a possible means of release. Additionally, transport may occur through large membrane pores either as a facilitated process or by classical physical diffusion. Given the relatively small fluxes, only a small number of large pores would be required, and a structure that resembles a large, about 5 nm diameter, transmembrane pore has recently been reported in zymogen granule membrane [37]. Finally, membrane porters may be involved, as well as 'helpers', such as chaperonines, unfoldases, phosphorylation, or ATP. In this light, it should be noted that the release of protein from the granule is highly temperature sensitive particularly between 20 and 25°C [38].

Physiological relevance

Release occurs from isolated granules at a rate similar to that observed for protein secretion in situ by rat acinar cells. In the experiments shown in Figs. 2 and 3, between 30 and 50% of granule protein content was released over a period of 3–5 h. In the same species in situ, feeding diminished cells of their granule volume by on average 55% in approx. 90 min [39]. If protein release is correlated in a linear fashion to the decrease in granule volume, then release would occur at about twice the rate observed for isolated granules [39]. Although it should be appreciated that release of protein from granules in vitro occurs in a very different environment from that in situ, the rough correspondence in rates suggests a potential physiological relevance for granule permeability to proteins.

In addition, there are a range of other observations in situ that are consistent with the current ones and provide a variety of evidence for a non-vesicular pathway for secretion. For example: (1) granules are observed to shrink within the cell, not disappear, when secretion is augmented with stimulant [39]; (2) labeled chymotrypsinogen added to extracellular fluid accumulates in zymogen granules within the acinar cell [29]; (3) the secretion of at least some of the 10–20 different proteins found in the zymogen granule can occur independently of each other [3,40]; and (4) secretion occurs at a highly augmented rate even when there are no zymogen granules observable within the cell [3]. We refer the reader to Ref. 3 for a discussion of these and other observations, and a summary of much of the earlier evidence on the subject.

It should be kept in mind that a physiological role for membrane protein transport in the secretion process does not mean that a vesicular secretion mechanism must be rejected. The two processes may well occur in parallel carrying out different functions. Although there has been no study of such differences, one can imagine that the vesicle or exocytosis process might be operant at the onset of digestion, rapidly providing a large bolus of enzymes for food entering the duodenum, whereas membrane transport would be the means of modulating the secretion of one or another enzyme as digestion progresses over time and in the face of more or less substrate in need of digestion. Such regulation of digestion has been demonstrated in a variety of studies [40]. Alternatively, it has also been suggested that exocytosis might serve as the mechanism of granule turnover, independent of the rate of protein secretion. That is, granules act as capacitors, discharging their contents by means of membrane protein transport, but from time to time, exocytosis occurs as the degradative limb following granule biogenesis [41].

5. Final comment

Thus, using STXM, we have observed that isolated secretion granules are not inert objects. They can change in size, as well as protein concentration; shrinking (or enlarging) in response to non osmotic disequilibrium states, as they release (or increase) their protein contents. As such, the experiments provide direct visual, as well as quantitative, evidence for the permeability of the membranes of isolated granules to their contained proteins. The ability to calculate permeability coefficients for individual granules, as well as the ability to measure mass, concentration, and flux likewise for individual zymogen granules, makes it possible to assess variations in these parameters in the population. This, when a sufficient number of granules are analyzed in this fashion, will in turn allow us to define with some quantitative precision the properties of the transport mechanism, or mechanisms contained within the membrane.

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