RAPID RELEASE OF THE ZYMOGEN GRANULE PROTEIN BY OSMIUM TETROXIDE AND ITS RETENTION DURING FIXATION BY GLUTARALDEHYDE

ABRAHAM AMSTERDAM and MICHAEL SCHRAMM

From the Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

ABSTRACT

Fixation by osmium tetroxide and glutaraldehyde of zymogen granules isolated from rat parotid and pancreas was investigated. Protein determinations showed that osmium tetroxide caused rapid release of most of the soluble protein of the granule during fixation in buffered isotonic sucrose. Such granules when examined in the electron microscope after shadow casting appeared quite flat, indicating that most of the contents had indeed been removed. Numerous damaged membranes of the granules were also observed. In contrast, zymogen granules fixed by glutaraldehyde and shadow cast essentially retained the spherical shape and the protein contents. The application of the shadow-casting technique in quantitative studies on the protein content of zymogen granules is discussed.

INTRODUCTION

Numerous studies have established that the digestive enzymes secreted by exocrine glands are derived, within the cell, from the zymogen granules. There is, therefore, great interest in procedures which detect changes in the exportable protein content of the zymogen granules. One of the main procedures in recent years has been the electron microscopic study of thin sections fixed in OsO_4 (cf. 1, 2). It was shown by Palade that prolonged fixation by OsO_4 (24 to 48 hr) causes disappearance of zymogen granules and other cell structures (3). The present communication demonstrates that zymogen granules isolated from rat parotid and pancreas lose a large part of their protein content within a few minutes after addition of OsO_4. Such effects are not observed with glutaraldehyde as the primary fixative. Therefore, the preferential use of glutaraldehyde is considered for quantitative studies of the protein content of zymogen granules.

METHODS

Isolation of the Zymogen Granule Fraction

Rat parotid glands were collected at 37° in Krebs-Ringer bicarbonate medium which was continuously gassed with a mixture of 95% O_2, 5% CO_2. All subsequent steps were carried out at 0°–4°. A homogenate was prepared in 0.3 M sucrose containing 10 mM Tris buffer pH 7.6 or phosphate buffer pH 7.0, 0.5 mM EDTA and 1 μg/ml diphenyl *p*-phenylenediamine. The latter substance was added to prevent lipid peroxidation (4). The sediment obtained after centrifugation for 5 min at 250 *g* was discarded. The zymogen granule fraction was then isolated from the supernatant by centrifugation for 10 min at 1100 *g* (*see* reference 5). The sediment was washed once and

finally suspended in the above sucrose medium. Rat pancreas zymogen granules were obtained by the same procedure. The stock suspension of granules containing about 4 mg protein/ml was immediately used for fixation experiments.

Fixation Procedures

A. OSMIUM TETROXIDE: A fresh 3% solution of OsO_4 was prepared in 0.3 M sucrose containing 10 mM phosphate buffer pH 7.0, 30 mM Tris buffer pH 7.6 (to neutralize the fixative), and 0.5 mM EDTA. The final pH was 7.0. All dilutions of the fixative and of the stock suspension of zymogen granules were performed in the isotonic sucrose medium which served for isolation of the zymogen granules. Fixative was added to the zymogen granules at 4° or at 25° to give final concentrations of OsO_4 from 0.04 to 2%. The final granule concentration was about 1.5 mg protein per ml in all experiments. After 30 to 120 min, the suspension was centrifuged to remove the fixative. The supernatant was saved for protein assay and the sedimented granules were washed with 10 mM phosphate buffer pH 7.0 containing 0.5 mM EDTA. The precipitate was finally suspended in 1 mM phosphate buffer pH 7.0.

B. GLUTARALDEHYDE: The compound was obtained from Fluka A. G., Switzerland, at a concentration of 25% in water. The freshly prepared fixative solution contained 6% glutaraldehyde, 0.3 M sucrose, 10 mM phosphate buffer pH 7.0, 0.5 mM EDTA, and 3 mM NaOH (to neutralize the acid in the glutaraldehyde reagent). The final pH was 7.0. The fixative was mixed with the zymogen granule suspension to give final concentrations of 1.5 to 4%. All other procedures were as described for fixation by OsO_4.

In some experiments, the material treated with glutaraldehyde underwent postfixation with OsO_4.

Electron microscopy. Aliquots of the fixed and washed suspension were placed on collodion-coated grids and dried in a desiccator. The grids were shadow cast with platinum-palladium at a height to shadow ratio of 1:3 to 1:5 and covered with carbon by evaporation. An RCA EMU 3G electron microscope was used at 50 kv.

Protein determinations. Total soluble protein of the native zymogen granules was prepared for assay as follows. Hypotonic lysis of the granules was performed by 10- to 20-fold dilution in 10 mM phosphate buffer pH 7.0. Insoluble material, accounting for 10 to 20% of the total protein of the granules, was removed by centrifugation at 1100 *g* for 10 min. Protein released from the granules during fixation for 30 to 45 min in isotonic 1% OsO_4 was measured after removal of the granules by centrifugation.

Soluble protein was assayed turbidimetrically by addition of trichloroacetic acid (6). The validity of this procedure when performed in the presence of OsO_4 is demonstrated for serum albumin in Table I. As explained in the text, this method was not applicable to the fixation medium containing glutaraldehyde. Protein was, therefore, precipitated from the fixation medium by trichloroacetic acid and assayed colorimetrically (7).

TABLE I

Comparison of Serum Albumin Assay with and without OsO_4

Bovine serum albumin was incubated in distilled water or OsO_4 solutions for 75 min at 4° in a volume of 3 ml. Two ml of 7.5% trichloroacetic acid were then added and readings were taken immediately in the Klett colorimeter with filter 54. The composition of incubation mixtures with OsO_4 were described under Methods, with and without sucrose. The OsO_4 reagent was freshly prepared to avoid high blank readings.

	Klett readings after incubation in		
Serum albumin	H_2O	1% buffered OsO_4	1% buffered OsO_4 with sucrose
mg			
0	0	4	3
0.25	13	16	—
0.50	26	28	27
1.00	55	57	53
2.00	103	111	—

RESULTS

Observations on Osmium Tetroxide Fixation

When OsO_4 was added to rat parotid zymogen granules in isotonic sucrose at pH 7.0, either at 4° or at 25°, a dramatic clearing of the suspension occurred within the 1st min. This phenomenon persisted even when the sucrose concentration was raised to 0.8 M. Testing various concentrations of OsO_4, it was found that the clearing effect occurs throughout the range of 0.04 to 2%. Protein determinations after incubation with 1% OsO_4 showed that 80% of the soluble protein of the granule had been released into the fixation medium. Observations in the phase-contrast microscope revealed that very few zymogen granules remained in the solution. Even these were not properly fixed and appeared to have lost most of their contents, according to the electron micrographs (Fig. 1).

Since isolated zymogen granules are usually fixed as a pellet at the bottom of a centrifuge tube (cf. 8), this procedure was also tested. A pellet of

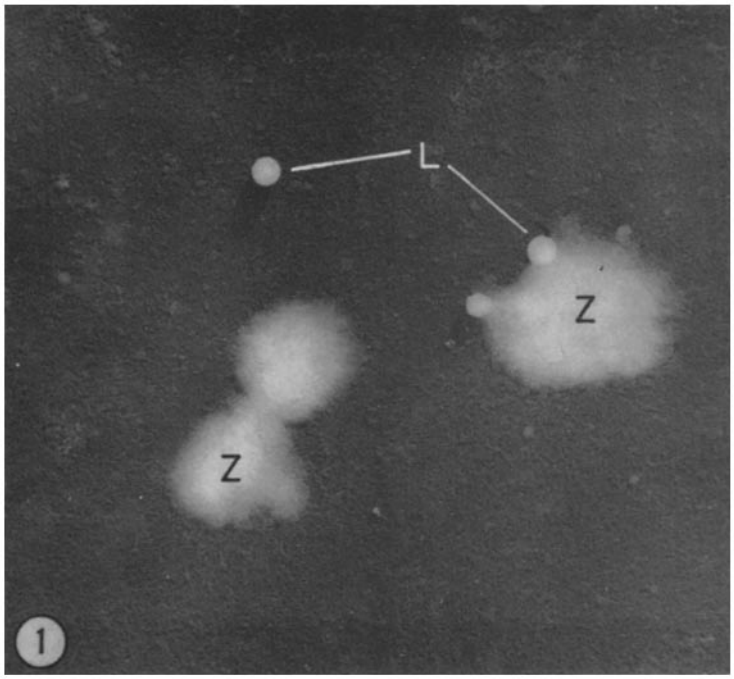

FIGURE 1 Rat parotid zymogen granules (*Z*) after treatment with 1% OsO_4 at 4° for 60 min. The periphery of the few remaining granules is poorly outlined. The material scattered in the background, on the supporting membrane, probably originated from the granules which are not properly fixed. Latex granules (*L*) are 0.264 μ in diameter. × 15,000.

rat parotid granules was layered with 1% OsO_4 at 4° until complete browning occurred (150 min.). After decantation of the fixative, the pellet was rinsed once with 10 mM Tris buffer pH 7.6 containing 0.5 mM EDTA. The pellet which was still compact was then dispersed in the above Tris buffer, recentrifuged, and finally suspended in 1 mM Tris buffer. Aliquots were examined by phase-contrast microscopy. Most of the granules appeared as quite transparent "ghosts." Electron micrographs showed flat bodies engulfed by material which had apparently leaked out (Fig. 2). It should be noted that in the original procedure designed for the preparation of thin sections (8), the pellet is not dispersed after fixation.

When rat pancreas zymogen granules were exposed in suspension to 1% OsO_4 at 4° or at 25°, there was a variable decrease in turbidity. However, quantitative assays showed that at least 75% of the soluble protein of the granules had been released into the isotonic medium during fixation. Electron micrographs demonstrated that most of the particles remained but were rather flat and partially frayed (height-to-width ratio less than 0.1). Some of the flat granules were still electron opaque (Figs. 3 and 4).

The undesirable effects of OsO_4 were not eliminated by varying the rate of fixative addition. It should be noted, however, that when OsO_4 was used in postfixation after glutaraldehyde, the granules appeared well preserved.

Observations on Fixation by Glutaraldehyde

Suspensions of zymogen granules of rat parotid and of rat pancreas showed no decrease in turbidity when fixed in 2 to 4% glutaraldehyde in the cold or at room temperature. Analysis of the fixation medium showed that no protein had been released during fixation for 60 min at room temperature. Even after washing and resuspension in the hypotonic buffer (see Methods), there was still no decrease in turbidity as compared to a control suspension in isotonic sucrose. Since native granules burst in hypotonic solution (9), the above observations demonstrate that glutaraldehyde is an effective fixative of the granules. Phase-contrast microscopy after fixation revealed a population of highly dense granules.

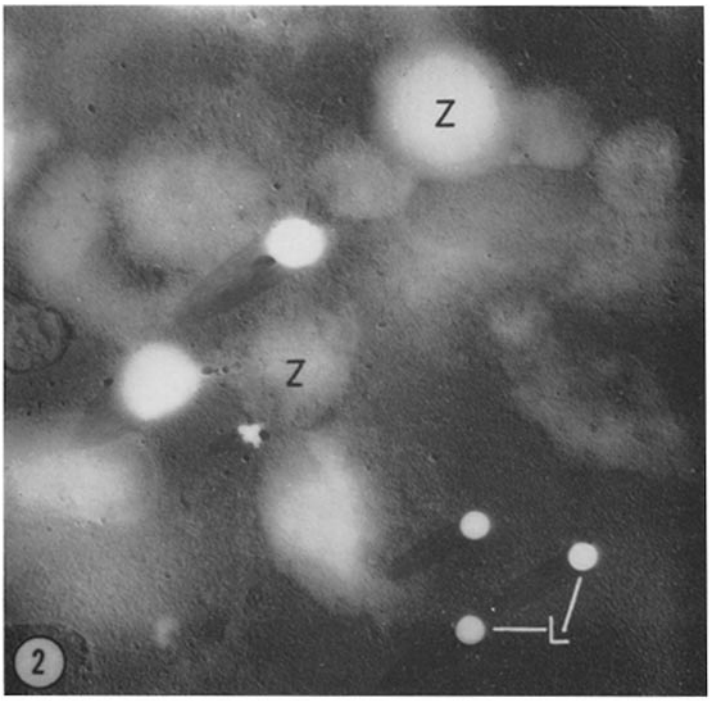

FIGURE 2 Rat parotid zymogen granules (*Z*) treated as a packed pellet with 1% OsO_4 and subsequently resuspended as described in the text. The granules have lost most of their contents which cover the background. × 14,500.

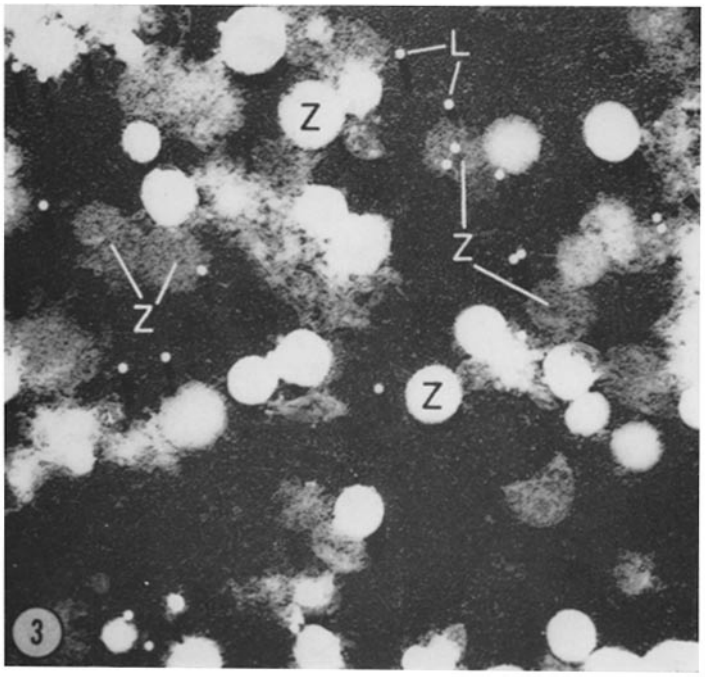

FIGURE 3 Rat pancreas zymogen granules (*Z*) after treatment with 1% OsO_4 at 25° for 75 min. All the granules are flat while some are still impenetrable to electrons. × 5,500.

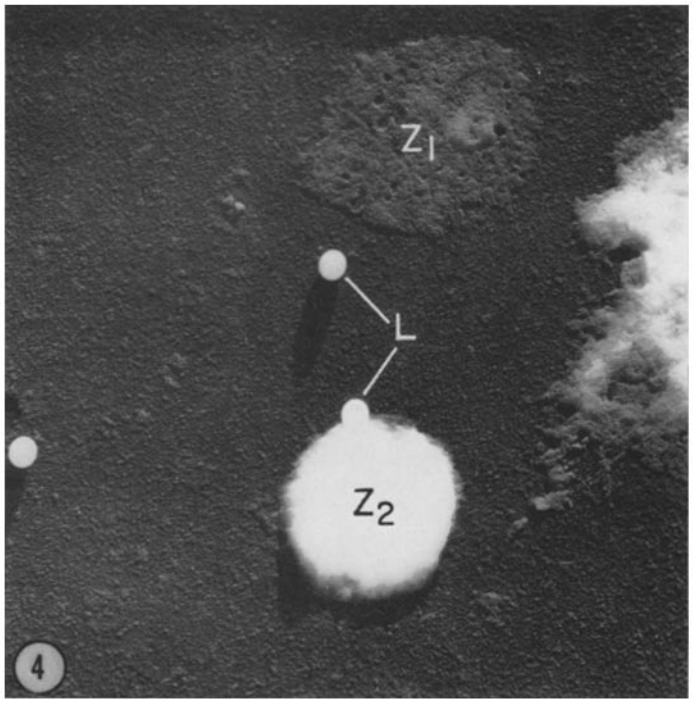

FIGURE 4 Conditions were as in Fig. 3 but for the treatment with OsO_4 which was at 4°. The thickness of the perforated granule membrane (Z_1) is about 150 A. The granule (Z_2) appears still partly electron opaque although its thickness is only about 650 A. × 14,500.

In electron micrographs, most of the granules had a height-to-width ratio approaching one. Some granules demonstrated a variable extent of flatness (height-to-width ratio) reflecting differences in the amount of material inside the granules. Figs. 5 and 6 show the parotid granules. Essentially identical electron micrographs were obtained with pancreas granules.

The shadow-casting technique proved to be a sensitive method for judging fixation efficiency. When glutaraldehyde was added dropwise to the granule suspension at 4° to a final concentration of 1.5%, it was observed that some of the contents of the granules had leaked out. The average height-to-width ratio was only about 0.5, and the circumference of the granules which was partly transparent showed protruding fibrils (Figs. 7, 8). Slow addition of glutaraldehyde at low concentration thus appears to decrease the efficiency of fixation.

Effect of the Fixatives on Serum Albumin

To determine protein released from the zymogen granules by OsO_4, a calibration curve of serum albumin was set up. It is shown in Table I that serum albumin treated with OsO_4 gave turbidimetric readings which were almost identical with those of the untreated protein. Prior to the addition of trichloroacetic acid, the solutions of serum albumin treated with OsO_4 were completely clear.

In contrast, serum albumin could not be assayed after incubation with 4% glutaraldehyde since rapid precipitation of large floccules occurred as soon as the trichloroacetic acid was added.

DISCUSSION

The fixation of the zymogen granule presents a special problem since this structure consists mainly of concentrated soluble proteins surrounded by a limiting membrane which is only 70 A thick (5). The present experiments demonstrate that incubation with OsO_4 caused rapid release of the soluble proteins from the granule. Under the same conditions but without the fixative, the granules are quite stable. It is thus obvious that OsO_4 readily reacts with the granule membrane to increase its permeability while the proteins inside the granules remain soluble and diffuse out. It is not surprising that these proteins are apparently unaffected by

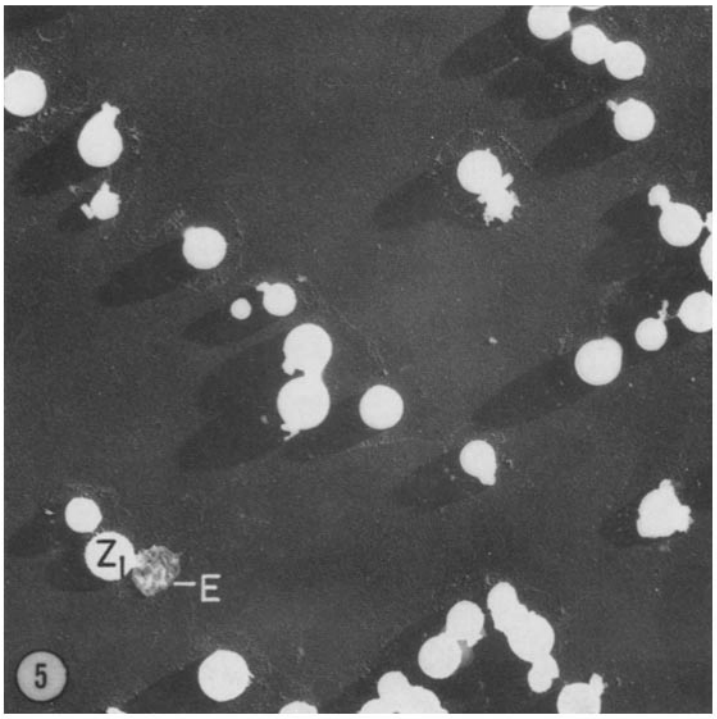

FIGURE 5 Zymogen granules from rat parotid fixed in 2% glutaraldehyde at 4° for 60 min. The average height-to-width ratio is about 0.7. A rather flat granule (Z_1) and an empty granule (E) are also shown. Protrusions and attachments on the granules as well as a few buffer crystals are observed. × 5,500.

OsO_4 since this fixative seems to be relatively inert towards many proteins (cf. reference 10). The present experiments with serum albumin also support this view.

Other cell structures containing soluble proteins may react differently towards OsO_4, depending probably on the nature of the membrane involved. For example, human erythrocytes did not release significant amounts of protein when incubated with OsO_4 (unpublished). The effect must, therefore, be evaluated for each individual system.

The action of glutaraldehyde on the zymogen granule must be based on a mechanism which is radically different from that of OsO_4 since the contents of the granule appeared efficiently fixed. It is quite likely that glutaraldehyde possessing two aldehydic groups readily converts the concentrated soluble proteins inside the granule into a cross-linked network (cf. reference 11). The remarkable stability of the glutaraldehyde-treated granule towards hypotonic media supports this assumption. In spite of the favorable properties of glutaraldehyde it is not certain yet that this fixative as used in the present work truly preserves the fine structure of the native zymogen granules.

To date, most of the electron microscope studies on zymogen granules and observations on their protein content were made on thin sections of glands fixed in OsO_4. In view of the present observation that OsO_4 causes release of protein from isolated granules, it may be suspected that this effect also occurs to an undetermined extent during fixation of whole tissue. A recent study on thin section of dog pancreas interestingly reports that the granules appeared much denser when the tissue was fixed in glutaraldehyde followed by OsO_4, as compared to fixation by the latter reagent alone (12). These observations are also in line with recent findings that in certain cell types the cell membrane becomes permeable even to ferritin (diameter of 100 A) upon fixation by OsO_4 (13, 14). Protein leakage due to fixation should, therefore, be considered when interpreting electron micrographs of zymogen granules in pellets and in intact cells. However, packing and successive barriers may re-

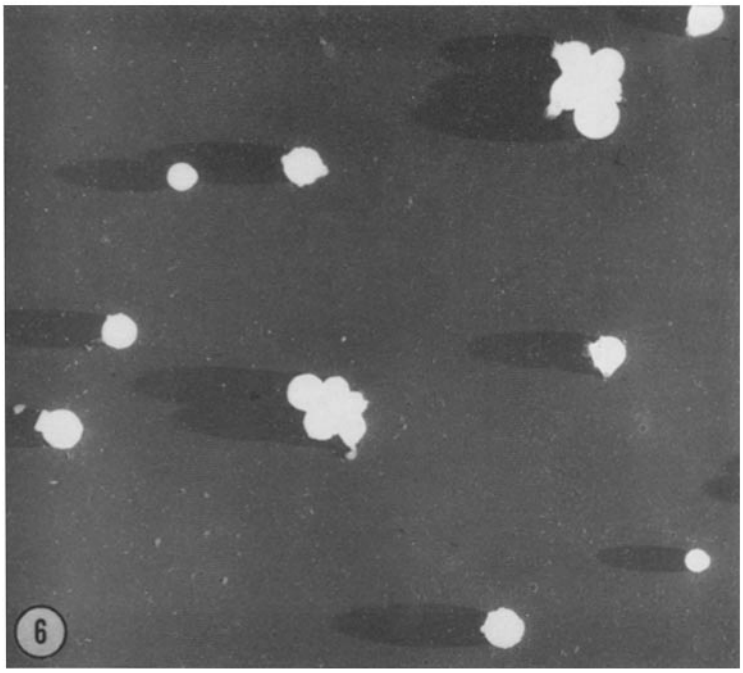

FIGURE 6 Zymogen granule preparation and conditions were as described for Fig. 5, but fixation was performed at 25°. The height-to-width ratio of the granules is about 0.9. The width of the granules is somewhat smaller than in Fig. 5, due apparently to the different fixation temperature. × 5,500.

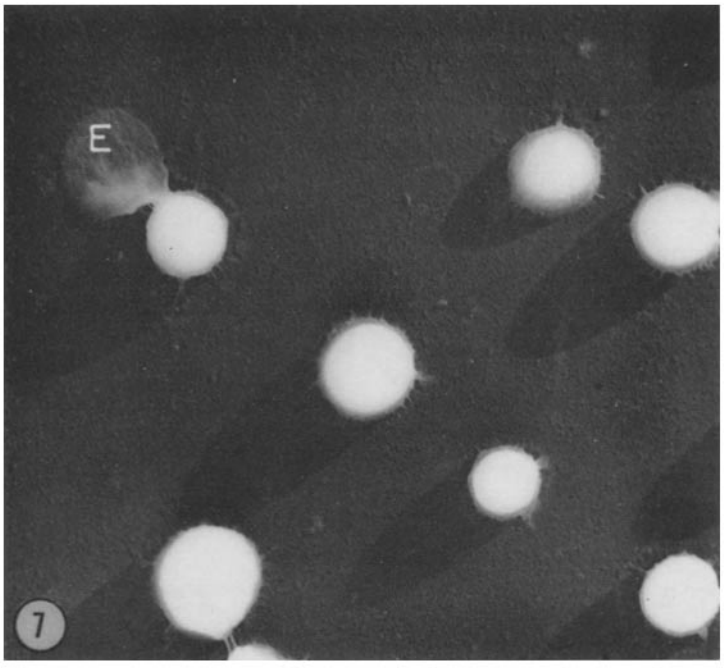

FIGURE 7 Rat parotid zymogen granules fixed by dropwise addition of glutaraldehyde to 1.5% at 4°. The circumference of the granules appears partially transparent with fibrillar protrusions. An empty granule (*E*) is also observed. × 14,500.

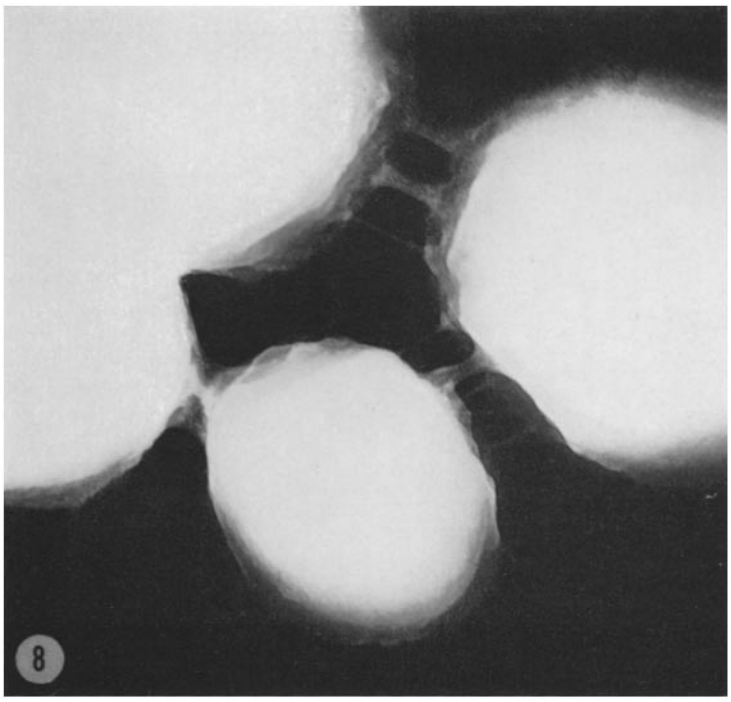

FIGURE 8 Same experiment as in Fig. 7 showing detail at higher magnification (× 56,000).

duce protein losses from the granules in these instances.

It should be borne in mind that even when fixation is satisfactory, only extreme variations in protein concentration can probably be detected by the electron opacity of stained sections, the reason being that the range in which electron opacity is proportional to the concentration of material is apparently quite limited and is usually unknown. Thus, zymogen granules which contain widely different protein concentrations may appear as of equal electron opacity in stained thin sections.

The isolated zymogen granule fraction viewed after shadow casting reveals less structural detail than thin sections of the gland. There are also possible artifacts of isolation and contaminants in such fractions. However, for the purpose of determining changes in the protein concentration of the zymogen granule, measurement of the average volume of the granule by shadow casting may serve as a fairly reliable criterion. A rough calculation shows that the shadow-cast zymogen granule fixed by glutaraldehyde is approximately spherical with an average diameter of about 1 μ. When measured by the same method, the height of an "empty" collapsed granule obtained by hypotonic lysis is about 140 A while its diameter increases roughly to 1.5 μ (*see* also reference 5). There is, therefore, a 70-fold decrease in height of the granule due to the removal of its contents. Much less drastic changes in the height and volume of the granules can readily be estimated by the shadow-casting technique. Whether such calculations apply to zymogen granules losing their contents within the cell remains to be seen. In any case, it seems worthwhile to examine the isolated zymogen granules in studies on enzyme secretion since such observations can readily be made on a large representative sample of the total population of the gland.

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