IMPROVED PRESERVATION OF PAROTID TISSUE FOR ELECTRON MICROSCOPY

A Method Permitting the Collection of Valid Stereological Data

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INTRODUCTION

Parotid tissue is, in many respects, the best subject for studies on the genesis of exportable proteins and on the mechanism of exocrine secretion. It has been adopted by us for combined biochemical, radioautographic, and morphometric studies on the production and fate of zymogen granules. To our knowledge, however, no detailed stereological analyses of parotid tissue have been carried out at the electron microscope level, although it seems to us that such an approach would reveal invaluable information for the elucidation of various problems relating to exocrine secretion. In particular, the fate of the zymogen granule membrane following exocytosis of the granule contents or the relocation of membrane material during the secretory cycle might be clarified.

Precise stereological analyses of parotid tissue have been hampered by the difficulty experienced in fixing the tissue. In our hands the prescribed methods for the chemical fixation of parotid tissue have produced poor results (2, 5, 9). We report here a procedure for the fixation of rabbit parotid glands under isotonic conditions which yields tissue optimally preserved for stereological analysis. Subsequently the results of our preliminary volumetric and areal analyses of acinar cells of starved rabbits are recorded.

MATERIALS AND METHODS

Parotid tissue was excised from New Zealand white rabbits (1.8–2.2 kg) anaesthetised with urethane

(1 g/kg) and was fixed in cold 1.5% w:v distilled glutaraldehyde (8) in 0.1 M Na cacodylate/HCl buffer pH 7.3 (16–20 h). Excess aldehyde was then washed out with eight changes (over 4 h) of cold 0.15 M Na cacodylate/HCl buffer and the tissue blocks were refixed in cold 1% w:v osmium tetroxide in 0.125 M Na cacodylate/HCl buffer (1.5 h). All these solutions were made to 330–350 mosmol and checked cryoscopically before use. Dehydration was carried out at room temperature in graded ethanol solutions starting with ethanol:water 3:1 v:v. Tissues were embedded in Araldite (Ciba Products Co., Summit, N. J.).

Photomicrographs of 0.5 μm sections taken using a Zeiss planapo oil immersion objective ($\times$100) were used for nuclear size determination. Electron micrographs taken in a Phillips 200 electron microscope at a nominal magnification of $\times$15,000 were used for all other stereological analyses. Photographic enlargement of micrographs was standardised with the aid of micrographs of a subdivided millimeter stage micrometer (light microscopy) and of a grating replica of 2,160 lines/mm (electron microscopy). Final print magnifications were $\times$1,750 and 30,000 respectively.

A systematic stratified random sampling technique was employed (6). The whole block faces of two tissue blocks from each animal were photographed for nuclear size determination (250–300 nuclei per animal). Mean nuclear volume was calculated from measurements of nuclear profile diameters (d). Mean corrected nuclear volume (1) was assumed to equal that of a sphere of diameter $\frac{4}{\pi} \times$ mean profile diameter where $d = 2\sqrt{ab}$ (a and b = major and minor semiaxes, respectively). 12 electron micrographs were taken at random from sections of each of nine tissue blocks per animal for electron microscope stereological analysis, (108 micrographs per animal in all). Each micrograph represented about 28.90 μm^2 of tissue section. Volume densities (Vv) were normally established by point counting, using as points the intersections of a squared lattice marked on a transparent screen which was superimposed onto each micrograph. The Vv of small, smooth membraned vesicles (ca. 30–180 nm diameter) was determined from measurement of random intercept lengths (Ll) and Vv taken to equal Ll (7). For this procedure a transparent screen marked with parallel lines was superimposed onto each micrograph and the length of line over each smooth vesicle intercepted (chord length) was measured to the nearest 0.1 mm with a subdivided eyepiece graticule. This screen was also used to obtain "line cut" data from which membrane surface densities (Sv) were calculated from the formula $Sv = 2Pl$ (10). Areas of zymogen granule profiles were measured with a planimeter (ca. 500 profiles per animal). Finally, all Vv, Sv, and Nv (numerical density) estimates were expressed on a per cell basis, using estimates of cell volume derived by combining nucleo-cytoplasmic ratios and the independently derived nuclear volume estimates.

RESULTS

Fig. 1 illustrates the ultrastructural appearance of acinar tissue prepared under isotonic conditions. The conformation of the plasmalemma can be seen to vary in different regions of the cell. The basal plasmalemma is relatively unfolded while the lateral plasmalemma is thrown into numerous complex folds. The apical plasmalemma, which is demarcated from the lateral plasmalemma by desmosomal junctions, bears a number of microvilli which project into the acinar lumen. Cell profiles often appear to have two areas of apical plasmalemma, suggesting that the duct system, in the rabbit, may branch within the acinus. Within the cells, the organelles show the markedly polarised distribution common to many exocrine secretory tissues. Towards the base of the cell a prominent nucleus is surrounded on three sides by rough endoplasmic reticulum and associated mitochondria. Often cell profiles exhibit two Golgi zones usually in a juxtanuclear position. The apex of the cell is mainly occupied by the zymogen granule population.

Several factors attest to the morphological integrity of the tissue which, in our experience, is only attained by isotonic processing. These are clearly illustrated in Fig. 2. The mature zymogen granules are discrete and homogeneous, and obvious signs of disruption are not evident. The cisternal profiles of the endoplasmic reticulum are not distended. The intercellular spaces, and in particular those between adjacent acinar cells, often become grossly enlarged in hypertonic media. Here, enlargement of these spaces has not occurred.

The results of the stereological analyses of parotid acinar cells from starved rabbits are summarized in Table I. Data from three experimental animals was processed separately, the sample sizes being sufficient to give 95% confidence limits of the means of better than $\pm$ 10%. Thereafter the results were pooled so that the values quoted here are the means of the three experiments.

Estimates of mean cell volume varied by about 38% between experiments, largely due to the variable degree of loading with zymogen granules. Granule content varied between 175 μm^3

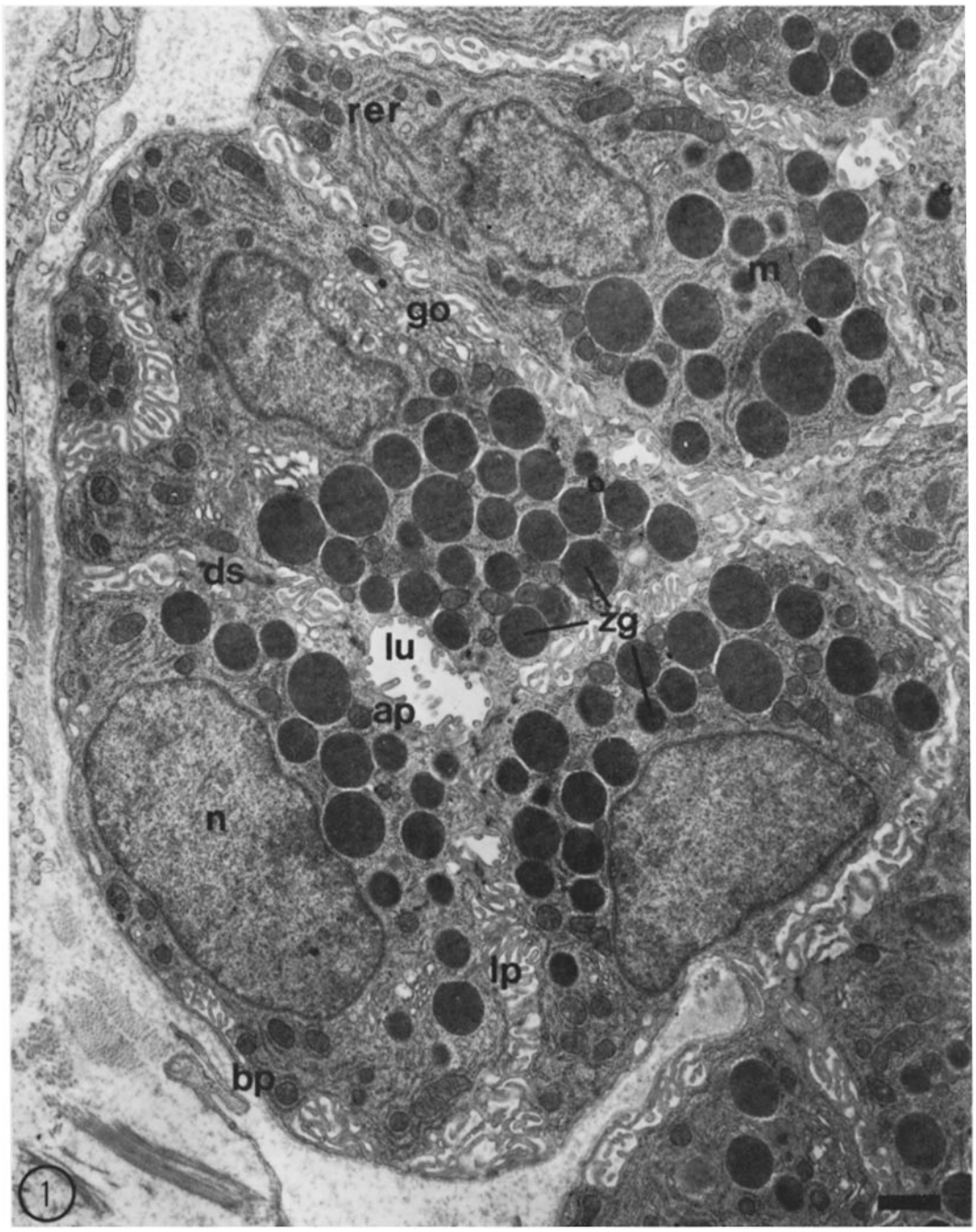

FIGURE 1 Electron micrograph of a rabbit parotid secretory acinus from a starved animal. Tissue prepared under isotonic conditions. Abbreviations: *ap*, apical plasmalemma; *bp*, basal plasmalemma; *ds*, desmosome; *go*, Golgi zone; *lp*, lateral plasmalemma; *lu*, lumen; *m*, mitochondrion; *n*, nucleus; *rer*, rough endoplasmic reticulum; *zg*, mature zymogen granules. × 9,500.

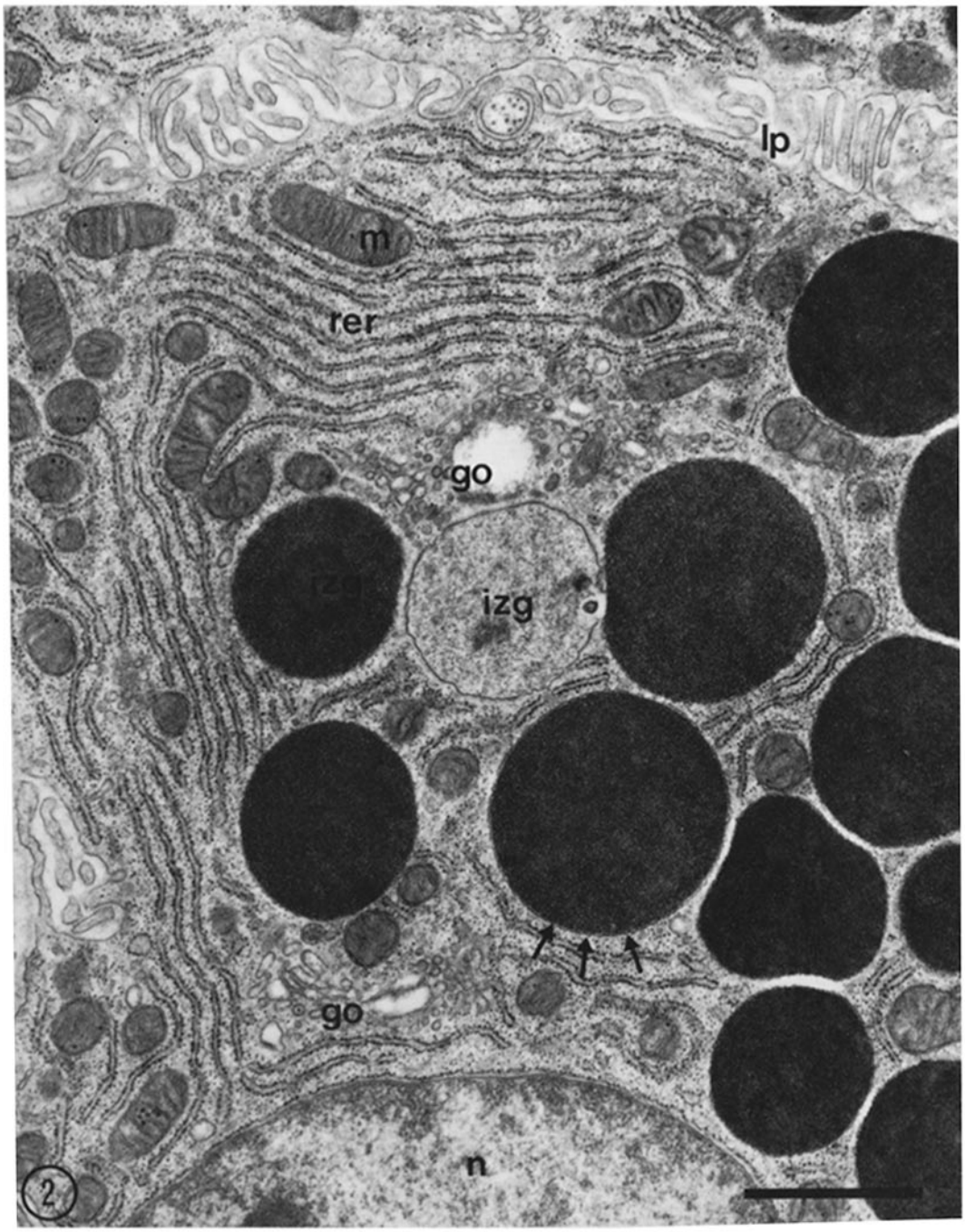

FIGURE 2 Electron micrograph of part of an acinar cell. The zymogen granules appear discrete and their limiting membrane can be resolved (arrows). The RER cisternae are not distended and the intercellular spaces are not enlarged. Abbreviations: *go*, Golgi zone; *izg*, immature zymogen granules; *lp*, lateral plasmalemma; *m*, mitochondrion; *n*, nucleus; *rer*, rough endoplasmic reticulum. × 26,000.

TABLE I

Analysis of Parotid Acinar Cells from Starved Rabbits

Item (per cell)	Value ± SE*
Cell	
Volume μm^3	1,089 ± 118
Total surface area μm^2	2,577 ± 94
Apical plasmalemma area μm^2	324 ± 24
Lateral and basal plasmalemmal area μm^2	2,252 ± 73
Nuclear	
Volume μm^3‡	116 ± 8
Surface area μm^2	165 ± 11
$\bar{D}$ μm (corrected§)‡	6.05 ± 0.02
Mean profile axial ratio‡	1.55 ± 0.01
'Roundness'‖	1.37 ± 0.01
Zymogen granule	
Volume μm^3	312 ± 94
Surface area μm^2	1,515 ± 367
$\bar{D}$ μm (corrected¶)	0.98 ± 0.04
Number/cell**	340 ± 77
Mitochondrial	
Volume μm^3	55 ± 2
Total outer limiting membrane area μm^2	672 ± 24
Mean profile area μm^2‡‡	0.15 ± 0.02
Number/cell**	399 ± 60
Rough endoplasmic reticulum	
Cisternal volume μm^3	110 ± 5
Membrane area μm^2	3,726 ± 172
Smooth endoplasmic reticulum	
Cisternal volume μm^3	13 ± 0.3
Membrane area μm^2	1,034 ± 28

* Standard error.

‡ Obtained from measurements on light micrographs.

§ $\bar{D} = \frac{4}{\pi}\bar{d}$ Abercrombie (1).

‖ "Roundness" $= \frac{\text{surface to volume ratio} - \text{actual}}{\text{surface to volume ratio} - \text{sphere of same volume}}$.

¶ Schwartz-Saltykov diameter correction procedure (see Underwood, 11).

** $Nv = \frac{k}{\beta}\frac{Na^{3/2}}{Vv^{1/2}}$ (12).

‡‡ Profile area $= \frac{Vv}{Na}$.

(187 granules) per cell and 492 μm^3 (413 granules) per cell. This variation in loading was not, however, reflected in increases in total plasmalemmal area, estimates of which remained very constant. This perhaps suggests that as the cells fill with granules some "rounding up" of the cells occurs. The values for other parameters remained consistent between experiments. Of the total plasmalemmal area, about 13% is in contact with the acinar lumen (apical) and would therefore be directly involved, together with the zymogen granule membrane, in the process of exocytosis.

Mean zymogen granule diameters were estimated from measurements of profile areas on the assumption that the profiles were circular and the granules spherical. This assumption was maintained in calculations to determine granule numbers. Thus, in the Weibel and Gomez *Nv* formula (12) the value of 1.382 was substituted for the coefficient β (value for spheres) and the coefficient of dispersion k was taken as 1.05, this being the median value of the most commonly encountered values of k. Mitochondrial numbers were estimated using the same formula on the assumption that they had a mean axial ratio of 4 (coefficient $\beta = 2.62$). Again, a value of 1.05 was substituted for the coefficient k.

DISCUSSION

It has been demonstrated that rat parotid zymogen granules remain labile after osmium tetroxide fixation and that glutaraldehyde fixation succeeds in stabilising their contents (3). Generally, hypertonic glutaraldehyde solutions have been employed for fixing parotid tissue, and Castle et al. (5) have stated that in their hands a hypertonic fixative (our estimate, greater than 1,000 mosmol) was necessary to reduce swelling, explosion, and extraction of secretory granules during processing. Such fixatives do, however, cause considerable shrinkage as shown by pyknotic nuclei and enlarged intercellular cisternae. In formulating our solutions, we took into account the observations of Bone and Denton (4) that cells are still osmotically active after glutaraldehyde fixation. These authors propose that at least 60% of the fixative's final tonicity should come from the buffer salts to avoid osmotic swelling during buffer washes. This criterion has been adhered to here. Also, to prevent undue changes in tissue volume during subsequent processing stages, dehydration was started in ethanol:water 3:1 v:v (13). In view of these precautions, the lack of obvious signs of swelling or extraction and the consistency of the estimates of the areas, volumes, and sizes of cell compo-

nents, we consider the tissue to be optimally prepared for stereological analysis.

Now that baseline stereological data is available, experiments are underway to study various aspects of the process of exocrine secretion by a combination of time sequence radioautography and stereological analysis.

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