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Subcellular Location of Progesterone in the Bovine Corpus Luteum: A Biochemical, Morphological and Cytochemical Investigation

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ABSTRACT

The subcellular location of progesterone in the bovine corpus luteum has been investigated by biochemical, morphological and cytochemical techniques. Differential centrifugation of tissue homogenates showed that up to one-third of the total progesterone could be sedimented. Most of this particulate progesterone banded discretely at low density on sucrose gradients. Marker enzyme studies showed that it was not associated with either the microperoxisomes, lysosomes or mitochondria nor appreciably with the vesiculated subcellular components such as plasma membrane and microsomes.

Morphological examination of tissue sections showed that the luteinized cells of the bovine corpus luteum contain electron dense granules. Three types of granules were present. Some of these granules were identified as microperoxisomes and lysosomes by cytochemical techniques, but a third type of granules was also present.

Morphological and cytochemical studies of different regions of the sucrose density gradient confirmed the distribution of subcellular organelles as judged by marker enzyme techniques. Electron dense granules were observed in material fixed from the progesterone peak, while microperoxisomes and lysosomes were found in denser regions of the gradient and separate from the progesterone band.

These results support the hypothesis that some of the progesterone in the bovine corpus luteum is sequestered within electron dense granules.

INTRODUCTION

The secretion of steroids from steroidogenic tissue is usually assumed to occur by diffusion from the site of synthesis through the plasma membrane to the extracellular space and thence into the blood, where binding proteins transport the steroids to their site of action (Enders, 1973). In the corpus luteum, marked differences in morphology are observed between the midluteal phase of the cycle when progesterone biosynthesis is maximal and the luteolytic phase, when progesterone biosynthesis decreases. High rates of progesterone biosynthesis are associated with the presence of many electron dense granules, some of which are expelled into the extracellular space. The number of these granules being exocytosed is related to the secretory activity of the tissue and decreases as regression of the corpus luteum proceeds (Gemmell et al., 1974). When regression is induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), a marked decrease of progesterone secretion occurs and granule secretion ceases (Gemmell et al., 1976; Stacy et al., 1976). Both progesterone and granule production are decreased by colchicine administration to ewes (Gemmell and Stacy, 1977). These results suggest that the secretion of progesterone from the luteal cell might occur by exocytosis of hormone containing granules. Polypeptide hormones such as insulin, glucagon and the hormones of the anterior pituitary are secreted from the cell by this mechanism. Intracellular transport of granules to the cell periphery is mediated by microtubules; drugs, such as colchicine, which interfere with microtubular

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function also interfere with the secretion of these hormones (Lacy et al., 1968; Farquar, 1971; Leclerq-Meyer et al., 1974; Stephens and Edds, 1976).

In this study we have used subcellular fractionation, density gradient centrifugation and morphological and cytochemical studies to demonstrate that the sedimentable progesterone of the corpus luteum is associated with the presence of granules.

MATERIALS AND METHODS

Chemicals

All chemicals were Analar grade. Trizma 7.0, p-nitrophenol, p-nitrophenol phosphate, p-nitrophenol-β-D-glucuronide, HEPES (N-2-hydroxyethylpiperazine-N′-2-ethane-sulphonic acid), diaminobenzidine (3, 3′, 4, 4′-tetraaminobiphenyl tetrahydrochloride) and DCPIP (2,6-dichlorophenol-indophenol) were obtained from Sigma. Ficoll was purchased from Pharmacia, sucrose from Hopkins and Williams and 1, 2, 6, 7-[^{3}H]-progesterone (80–110 Ci/mmol) from the Radiochemical Centre, Amersham. Fluorescamine (4-phenylspiro [furan-2 (3), 1′-phthalan]-3, 3′-dione) was obtained from Roche and Folin-Ciocalteau phenol reagent from Fison Scientific.

Tissue Manipulations

Midluteal phase bovine corpora lutea were obtained from 2 local abattoirs. The material was placed on ice within 10 min of death of the cows. All subsequent manipulations were performed at 0–4°C. In the laboratory, some 60–90 min later, the corpora lutea were decapsulated, minced using a Climpex tissue mincer (fitted with a stainless steel grid perforated by 1.7 mm diameter holes) and weighed.

All sucrose solutions were buffered with either 0.01 M Trizma 7.0 (pH 7.5; 5°C) or 0.01 M HEPES, pH 7.4. A 10% w/v homogenate in buffered 0.25 M sucrose was obtained (H_1) with 3–5 strokes of a Potter-Elvehjem homogenizer (Thomas, Philadelphia, PA; No. C44491, 0.177 mm clearance) driven by an MSE homogenizer at quarter speed. The homogenate (H_1) was centrifuged at 600 × g_{max} for 5 min (MSE, Mistral 6L) to remove nuclei, large pieces of cell debris and incompletely homogenized and unbroken cells. After centrifugation, the supernatant (S_1) was decanted and recentrifuged at 10,000 × g_{av} for 30 min (Beckman Spinco L2-65B). Floating lipid was removed with a clean dry glass rod. The supernatant (S_2) was decanted and the pellet resuspended in buffered 0.25 M sucrose (H_2, 10,000 × g pellet) by 3 handstrokes of a Potter-Elvehjem homogenizer (No. A8427, 0.127 mm clearance) to a total volume in ml equivalent to the number of g of original minced corpus luteum tissue (after allowing for sampling). Between 1.0 and 1.5 ml of this 10,000 × g_{av} pellet (H_2) was loaded on to sucrose density gradients as described below.

A microsomal fraction (H_3, 100,000 × g pellet) was obtained by centrifuging supernatant S_2 at 96,300 × g_{av} for 30 min and resuspending the pellet in buffered sucrose. The supernatant or cytosol fraction, S_3, was retained. In some experiments aliquots of the cytosol were loaded on to sucrose density gradients as described below.

Sucrose Density Gradient Centrifugation

After evaluation of several different density gradients, linear 20–40% w/w buffered sucrose gradients were prepared using an ISCO Model 570 gradient former. The gradients were of 36 ml capacity with a 1.5 ml underlay of 50% w/w sucrose. After loading, the gradients were centrifuged in a Beckman SW-27 rotor for 2.25 h at 25,000 rpm (82,500 × g_{av}), and eluted in 2 ml fractions by upward displacement with 60% w/w sucrose using an ISCO Model 184 tube-holding and piercing mechanism coupled to a nonperistaltic pump. The sucrose concentration or density (20°/4°) of individual fractions was determined by refractometry (Bellingham and Stanley refractometer, Tunbridge Wells, U.K.). Fractions were stored at -20°C until further analysis was carried out. In some experiments, fractions of identical density from more than one gradient were pooled.

Some pooled gradient fractions were fixed directly by glutaraldehyde (see below) for morphological and cytochemical examination. The fixed material was collected by centrifugation and treated as described below for tissue sections.

Assays

Progesterone was measured by radioimmunoassay (Challis et al., 1973). Pregnenolone, 17α-hydroxyprogesterone, 20α-hydroxy-pregn-4-en-3-one, cortisol and estradiol-17β showed less than 1.5% cross reaction with the progesterone antiserum. The solvent blanks were routinely equivalent to <5 pg ml^{-1}. Recovery of progesterone after extraction was 83.6 ± 7.5%, (mean ± SD; n = 20). The progesterone level of a stock of corpus luteum S_1 supernatant pool was routinely determined as an assay control. Its value was 21.1 ± 3.3 ng/ml (mean ± SD; n = 51). The interassay coefficient of variation was 15.6%.

Protein concentration was determined in sucrose solutions buffered by 0.01 M Trizma 7.0 according to Lowry et al. (1951). Before assay, the protein was precipitated with 5% w/v trichloroacetic acid and then dissolved in 0.5 M NaOH. When the sucrose solutions were buffered by 0.01 M HEPES, the fluorimetric method of Böhlen et al. (1973) was used for protein determination. In both assays, bovine serum albumin was used as the protein standard.

Enzyme activities of sucrose gradient fractions were used as markers for subcellular constituents (de Duve, 1970; Steck, 1972; Sottacasa et al., 1974; Beaufay et al., 1974). Succinate dehydrogenase (mitochondria) was determined according to Pennington (1961), catalase (microperoxisomes) according to Aebi (1974), inosine diphosphatase (microsomes) according to Beaufay et al. (1974) and rotenone insensitive NADH-cytochrome c reductase (microsomes/outer mitochondrial membrane) according to Fleischer and Fleischer (1967) with 0.0002 M KCN rather than 0.001 M KCN. The β-glucuronidase assay (lysosomes) was modified from Fishman (1974) and Bergmeyer et al. (1974) so that the assay mixture contained 0.004 M p-nitrophenol-β-D-glucuronide as substrate

and 0.1% v/v Triton X-100 in 0.095 M acetate buffer, pH 4.5. After incubation for 4 h at 37°C, the reaction was stopped by the addition of 0.1 M glycine-NaOH, pH 10.7 to a final concentration of 0.075 M and the absorbance at 405 nm was determined. The acid phosphatase assay was modified from Bergmeyer et al. (1974) and Hübscher and West (1965). The reaction mixture contained 0.1 M sodium acetate buffer, pH 5.6, 0.012 M EDTA (disodium salt), 0.1% v/v Triton X-100, 0.008 M p-nitrophenol phosphate (disodium salt). The reaction was stopped after 30 min at 37°C by the addition of NaOH to a final concentration of 0.33 M and the absorbance at 405 nm determined.

Determination of alkaline phosphatase activity (plasma membrane) was based on the method of Hübscher and West (1965), but p-nitrophenol phosphate was used as substrate. The reaction mixture contained 0.04 M ethanolamine buffer pH 10.5, plus 0.005 M $MgCl_2$, 0.001 M NaF, 0.1% v/v Triton X-100 and 0.005 M p-nitrophenol phosphate. After incubation for 30 min at 37°C, the reaction was terminated by the addition of NaOH to a final concentration of 0.07 M and the absorbance at 405 nm was determined.

Separate standard curves using p-nitrophenol were constructed for β-glucuronidase and acid or alkaline phosphatase assays. All enzyme assays were found to be linear with respect to enzyme concentration and time and with the exception of catalase, displayed zero order kinetics; the catalase reaction was 1st order. Appropriate substrate and sample blanks were included in all assays. Whenever possible, assays were performed in duplicate.

Enzyme activities were calculated as Enzyme Units (EU) (μmoles product/min) except for succinate dehydrogenase and catalase activities which were Δ $Absorbance_{490nm}$/min and $k.sec^{-1}$ (the 1st order rate constant), respectively.

Electron Microscopical Methods

Morphology. Bovine corpora lutea were obtained from animals on known days of the estrous cycle from the A.R.C. Laboratories at Compton, Berkshire. Tissue pieces of maximum dimension 0.5 mm were fixed within 5 min of death in 3% v/v glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 and left on ice for 2 h. After overnight washing in buffer, the tissue was postfixed for 30 min in 1% w/v osmium tetroxide, dehydrated through an alcohol series and embedded in araldite. Sections were poststained with methanolic uranyl acetate and Reynold's lead citrate (Reynold, 1963) and viewed in a Philips 301 electron microscope.

Cytochemistry. Fresh bovine corpora lutea from the same source were sliced with a razor blade into approximately 20 μm sections and fixed in a) 3% v/v glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 containing 7% w/v sucrose for 3 h or b) 1% v/v glutaraldehyde in the same buffer for 30 min. Both sets of tissue were washed overnight in 0.1 M cacodylate buffer, pH 7.4 containing 7% w/v sucrose.

Tissue (a) was incubated in alkaline diaminobenzidine for 2 h at 37°C to demonstrate the presence of microperoxisomes (Novikoff and Goldfischer, 1969; Novikoff et al., 1973). Sections were also incubated with medium lacking hydrogen peroxide and complete medium containing the specific inhibitor DCPIP as a control. Tissue (b) was incubated according to Barka and Anderson as in Essner (1973) for 1 h at 37°C to demonstrate acid phosphatase activity. Control reactions (medium lacking β-glycerophosphate substrate and complete medium containing NaF as specific inhibitor) were also performed.

After incubation both tissues were postfixed in 1% w/v osmium tetroxide for 30 min, dehydrated and embedded in araldite.

Micrographs of noncounterstained sections were photographed at random and used to obtain a quantitative estimate of the numbers of total granules present which were either diaminobenzidine or acid phosphatase positive.

RESULTS

The Distribution of Progesterone and Marker Enzymes by Differential Centrifugation

The distribution of progesterone and of marker enzymes for cell organelles was determined after differential centrifugation of the homogenate H_1 (Table 1). The 10,000 × g pellet contained about a fifth of the total progesterone, confirming the earlier findings of Gemmell et al. (1974) and Kramers et al. (1975). Twenty to 30% of the total activities of acid phosphatase and β-glucuronidase (lysosomes), catalase (microperoxisomes), NADH-cytochrome c reductase and inosine diphosphatase (microsomes) and approximately 50% of the succinate dehydrogenase activity (mitochondria) were recovered in the 10,000 × g pellet.

The 100,000 × g pellet, prepared from the 10,000 × g supernatant, contained a further 13% of the total progesterone, indicating that at least 30% of the progesterone was particulate. A further 20% of the marker enzyme activities for microsomes and plasma membranes were also recovered and lesser amounts of the other marker enzymes.

About a third of the total tissue progesterone was not sedimented at 100,000 × g, but remained in the cytosol (Table 1). The presence of lysosomal and microperoxisomal marker enzymes in the cytosol fraction indicated that some organelle damage had occurred during homogenization. Thus some of the progesterone found in the supernatant may well have been released from its particulate form by our procedures. In contrast, the marker enzymes for microsomes, plasma membranes and mitochondria, which are firmly membrane bound, were almost completely sedimented.

At intermediate forces of centrifugation (between 10,000 × g and 100,000 × g) progressively greater marker enzyme activities were

TABLE 1. The distribution of progesterone, protein and enzyme activities obtained by differential centrifugation of bovine corpora lutea homogenate.[a]

Mass or activity	Percent of homogenate mass or activity		
	10,000 X g Pellet	100,000 X g Pellet	100,000 X g Supernatant
Progesterone	19.1 ± 3.6 (12)	13.0 ± 5.7 (5)	34.0 ± 7.0 (5)
Protein	17.0 ± 5.6 (5)	10.4 ± 0.9 (3)	41.6 ± 5.8 (3)
Succinate dehydrogenase	46.7 ± 7.6 (6)	3.8 ± 0.7 (3)	0 (1)
Acid phosphatase	25.2 ± 9.7 (6)	13.8 ± 7.0 (3)	27.0 ± 3.2 (3)
β-glucuronidase	30.7 ± 6.1 (6)	13.4 ± 4.7 (3)	10.1 ± 1.8 (3)
Catalase	21.8 ± 5.1 (3)	16.3 (1)	20.3 (2)
Alkaline phosphatase	21.5 ± 7.9 (4)	17.2 (2)	6.7 (2)
NADH-cytochrome c reductase (rotenone insensitive)	33.3 ± 5.0 (4)	20.4 (2)	1.7 (2)
Inosine diphosphatase	24.9 ± 7.1 (3)	19.5 (1)	8.4 (1)

[a]The 10,000 X g pellet, 100,000 X g pellet and supernatant (cytosol) were prepared and assayed. The percent of progesterone, protein or enzyme activity in each fraction is expressed relative to the homogenate, set at 100%. Values are given as the mean ± SD; the number of experiments is indicated in parentheses.

pelleted without a significant gain in progesterone enrichment (not shown). Therefore, the 10,000 × g pellet containing particulate progesterone was chosen as the starting material for the density gradient.

Distribution of Progesterone on Sucrose Density Gradients

The distribution of marker enzymes, progesterone and protein on a 20–40% w/w sucrose gradient in a representative experiment is shown in Figs. 1–3. Similar results were obtained in 7 other experiments for progesterone and in at least 3 other experiments for each of the marker enzymes. Tubes 1–8 of the eluted gradient contained about 80% of the progesterone recovered from the gradient. Some 20% of the progesterone was found in tubes 1 and 2 and thus had remained in the sample zone and had not entered the gradient. The progesterone of a cytosol aliquot loaded on to an analogous gradient was recovered in tubes 1 and 2 (not shown). These tubes also contained about 5% of the acid phosphatase, β-glucuronidase and catalase activity, presumably as a result of some enzyme leaching from the organelles. Tubes 3–7 (22.5–27.3% w/w sucrose, density = 1.094–1.113), comprised about 50% of the recovered progesterone, negligible catalase and succinate dehydrogenase activity and less than 5% of β-glucuronidase, acid phosphatase, alkaline phosphatase, NADH-cytochrome c reductase and inosine diphosphatase activities. The latter 3 enzyme activities were spread through the gradient from tube 8 onwards (sucrose density >1.116).

Progesterone levels were low from tube 8 onwards, whereas acid phosphatase, β-glucuronidase, catalase and succinate dehydrogenase activities were highest from tube 14 onwards (sucrose density >1.146). Thus the lysosomes, mitochondria and microperoxisomes were completely resolved from the particulate progesterone peak, while a small proportion of the microsomes and plasma membrane overlapped in this region.

Compared with the homogenate, the enrichment (relative specific activity) of progesterone was greatest in tubes 3–8. Maximum enrichment of 7–8-fold occurred in tubes 4–6 in the experiment shown in Figs. 4–6. In 4 other experiments the enrichment in this region ranged from 7–17-fold. The only other significant enrichments in this region were obtained for NADH-cytochrome c reductase and inosine diphosphatase, the microsomal marker enzymes. This enrichment was, however, less than 2-fold. The former enzyme is also located on the outer membrane of mitochondria. Tubes 1 and 2, which contained progesterone that had not entered the gradient, showed a much lower enrichment owing to the presence of soluble protein which did not enter the gradient.

All marker enzymes showed higher enrichments at densities greater than that of the progesterone peak, and the position of maximum enrichment usually corresponded to the position of maximal enzyme activity. The endoplasmic reticulum and plasma membrane

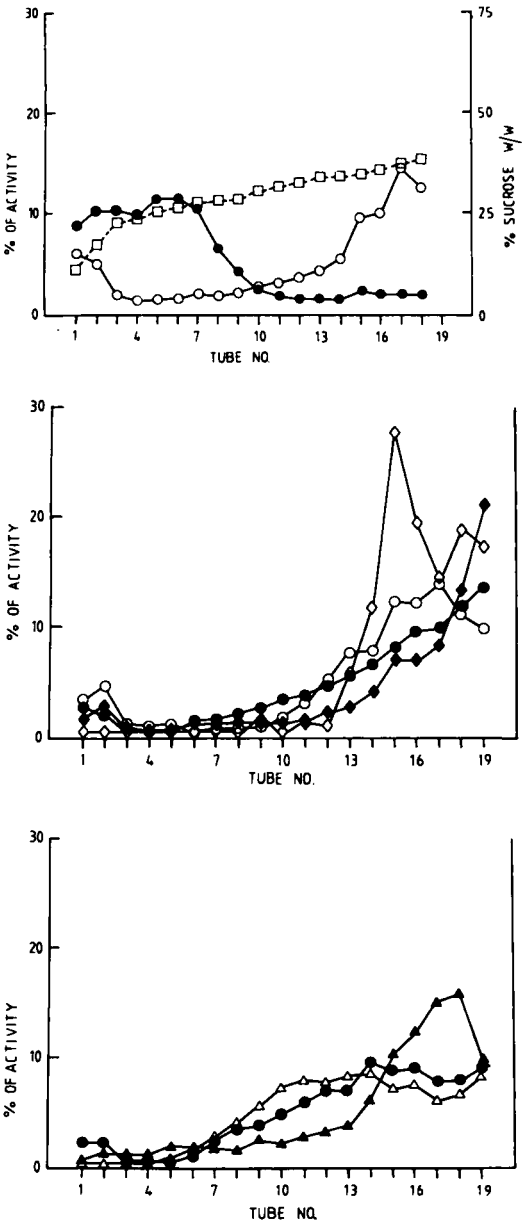
markers were spread throughout the gradient and thus not heavily enriched in any fraction. This distribution occurs because of the variation in vesicle size and density which arises during homogenization of these membranous elements of the cell. No attempt was made to separate lysosomes, mitochondria and microperoxisomes, so that the enrichment obtained in the denser regions of the gradient (2–4-fold) was less than if the gradient had been extended to effect a separation.

Prolonged centrifugation (17.5 h) of 20–40% w/w sucrose gradients gave maximal enrichments of progesterone and marker enzymes at the same densities as found for the shorter centrifugation time. Thus, by 2.25 h in a 20–40% w/w sucrose gradient, equilibrium had been attained.

Linear Ficoll gradients were also evaluated over the range 0–20% w/w and 5–25% w/w in buffered 0.25 M sucrose. They were centrifuged as above for 2.25, 18 or 20.25 h, and the resulting progesterone profiles were very similar to those obtained with sucrose gradients. However, considerable alkaline and acid phosphatase activities remained with the progesterone peak at the top of the gradient. Thus, neither prolonged centrifugation of sucrose gradients nor the use of Ficoll as density medium, improved the separation of progesterone from marker enzymes.

Morphology and Cytochemistry of the Corpus Luteum

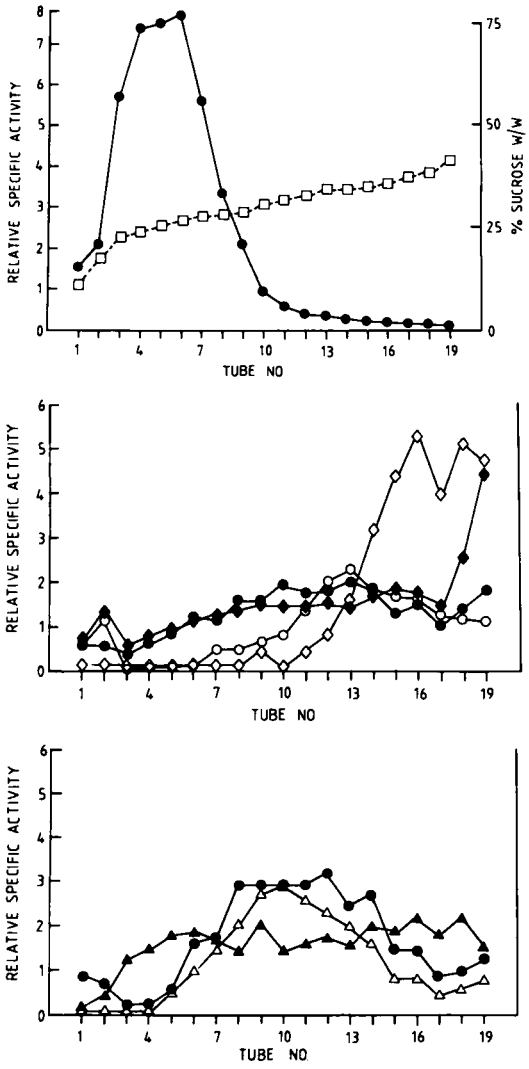
The luteal cells of the midcycle corpus luteum contained an extensive network of agranular endoplasmic reticulum characteristic of steroid secretory tissues and an abundance of electron dense granules ranging in size from



FIGS. 1–3. The distribution of progesterone, enzyme activities and protein on 20–40% w/w sucrose density gradients. Aliquots of the 10,000 × g_{av} pellet, prepared from an homogenate of bovine corpora lutea, were loaded on to 20–40% w/w sucrose gradients. Centrifugation was carried out for 2.25 h at 82,500 × g_{av}.

The abscissa denotes the tube number as eluted from the gradient. On the ordinate, the mass or activity in each tube is plotted as a percentage of the total mass or activity recovered from the gradient.

The percentage of the original homogenate mass or activity in the 10,000 × g pellet and the mass or enzyme activities in each aliquot applied to the gradient were, respectively: progesterone, 26.3%, 6.06 μg; protein, 21.4%, 20.05 mg; succinate dehydrogenase, 58.8%, 25.0; acid phosphatase, 29.8%, 0.819; β-glucuronidase, 39.3%, 0.206; catalase, 19.2%, 0.767; alkaline phosphatase, 23.3%, 0.318; NADH-cytochrome c reductase, 40.8%, 7.64; inosine diphosphatase, 28.5%, 0.534. The percentage recovery of the applied mass or activities from the gradient ranged from 80–120%. 1) ●, progesterone; ○, protein; □, % sucrose, w/w. 2) ●, acid phosphatase; ○, catalase; ◆, β-glucuronidase; ◇, succinate dehydrogenase. 3) ●, inosine diphosphatase; ▲, NADH-cytochrome c reductase; △, alkaline phosphatase.



FIGS. 4–6. The relative specific activity of progesterone and marker enzymes on 20–40% w/w sucrose density gradients. Details of the experimental procedures are given in the legend to Figs. 1–3. The results shown are from the same representative experiment.

The abscissa denotes the tube number as eluted from the gradient; the ordinate value is the relative specific activity or enrichment. The relative specific activity is the percentage of mass or activity recovered in each tube divided by the percentage of protein covered in that tube (H_1 = 100%). 4) ●, progesterone; □, % sucrose, w/w. 5) ●, acid phosphatase; ○, catalase; ◇, succinate dehydrogenase; ◆, β-glucuronidase. 6) ●, inosine diphosphatase; ▲, NADH-cytochrome c reductase; △, alkaline phosphatase.

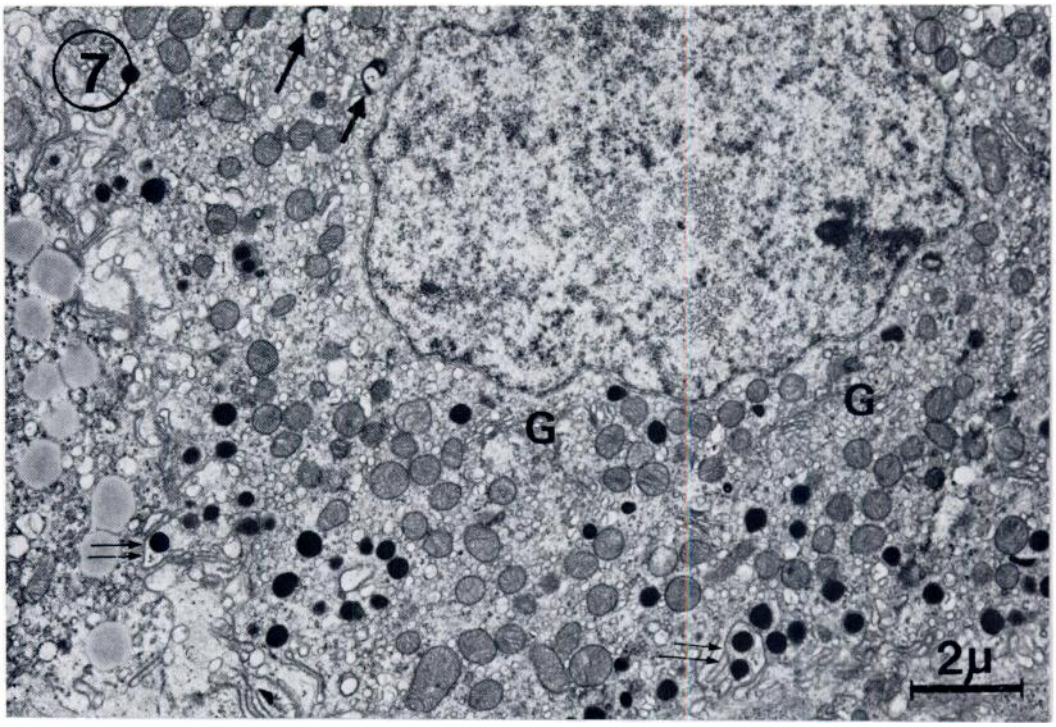
0.1–0.4 μm, some of which were irregular in shape. Many of the granules were seen at the cell periphery and appeared to be in the process of being expelled from the cell by exocytosis (Fig. 7).

Microperoxisomes and lysosomes have been identified in steroidogenic tissues (Reddy, 1973; Novikoff and Novikoff, 1973; Gulyas and Yuan, 1975; Gemmell et al., 1976) and a cytochemical study was carried out to identify these and to distinguish them from any other granules in the bovine corpus luteum. In 24 fields containing 400 granules (size 0.1–0.2 μm) a fifth of these were stained with alkaline diaminobenzidine and thus were identified as microperoxisomes. None of the positive staining granules were observed undergoing exocytosis. The nonstaining granules were generally larger (size 0.2–0.4 μm) and exocytosis of these was observed (Fig. 8). Few acid phosphatase positive granules (lysosomes) were observed in the midluteal bovine corpus luteum. Some staining was observed in the Golgi cisterni (Fig. 9).

Examination of fixed fractions from sucrose density gradients showed that electron dense granules were present in tubes 5–6 where progesterone concentration and enrichment were maximal (Fig. 10). These fractions also contained many membrane vesicles of various sizes. In contrast to tissue sections, the shape of granules from the gradient was usually irregular. The size range was consistent with that found in tissue sections. The increase in pleomorphism may have been caused by the experimental manipulations. Electron dense granules were observed in material from tubes 11–12 and tubes 17–18 of the gradient. These granules were not associated with the presence of progesterone and were subsequently shown by cytochemical techniques to be mainly microperoxisomes or lysosomes. Tubes 11–12 also contained many membrane vesicles consistent with the presence of plasma membrane and agranular endoplasmic reticulum. Tubes 17–18 consisted mainly of mitochondria plus some lysosomes and microperoxisomes.

Cytochemical studies of the fixed fractions showed that very few of the granules in frac-

FIG. 7. A luteal cell from a cow at Day 13 of the estrous cycle. Large numbers of densly staining granules are present, some of which are irregular in shape (arrows) and many are in the process of being expelled from the cell (double arrows). The cytoplasm is packed with smooth surfaced endoplasmic reticulum and many actively secreting Golgi regions can be seen (G). X 7400.



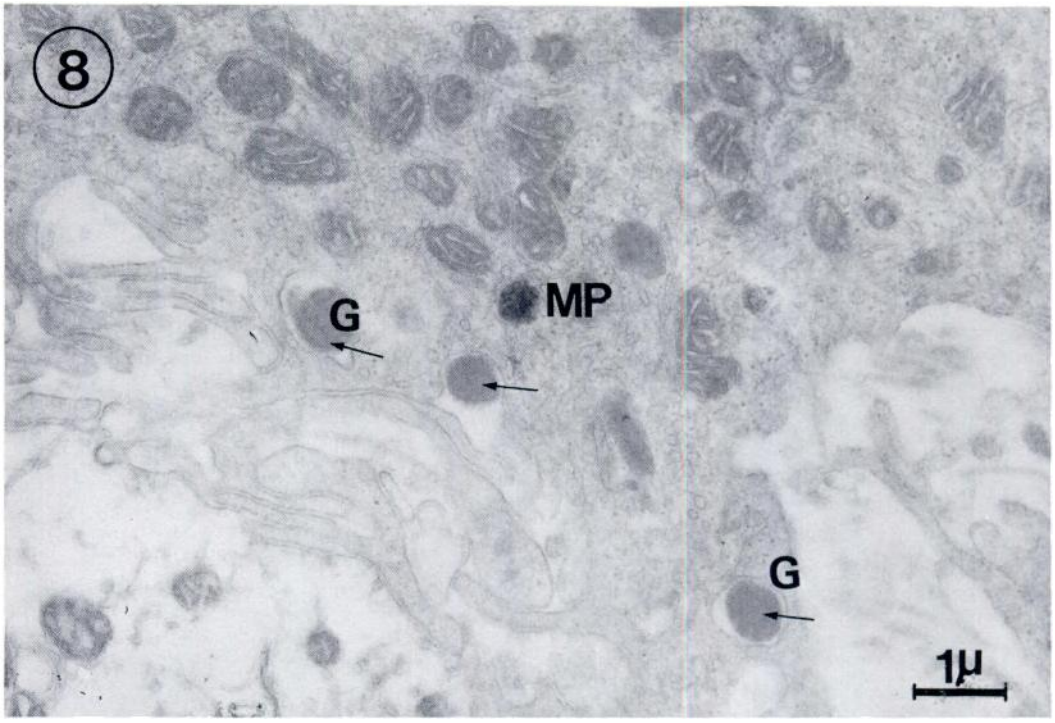


FIG. 8. Portion of a luteal cell from a cow at Day 13 of the estrous cycle incubated with alkaline diaminobenzidine to demonstrate microperoxisomes. The section is not counterstained and the dense osmiophilic reaction product can be seen over a microperoxisome (MP). Nonreacting granules can also be seen (G) some in the process of exocytosis (arrows). X 11,247.

tions 5 and 6 stained positively with alkaline DAB (not shown). In tubes 11–12 (which contained substantial catalase activity), many positively staining granules and only an occasional nonstaining granule, were observed (Fig. 11). In tubes 17–18, less microperoxisomes were observed per field, probably because of the vast number of mitochondria present in this part of the gradient. Acid phosphatase activity was observed only in material obtained from the densest region of the gradient (tubes 17–18, Fig. 12).

Thus the morphological and cytochemical findings confirmed the marker enzyme distributions. Furthermore, in those tubes where maximal progesterone levels were found, electron dense granules were also observed. The latter were neither lysosomes nor microperoxisomes.

DISCUSSION

Our results confirm and extend the findings of Gemmell et al. (1974) and Kramers et al. (1975). We have shown that at least a third of the total progesterone in the corpus luteum is particulate, since it can be sedimented by centrifugation. This figure is probably an underestimate since the 600 × g centrifugation step removed unbroken cells and large tissue fragments which would contain some progesterone.

Since both progesterone and some lysosomal and microperoxisomal marker enzyme activities were recovered in the cytosol, minor organelle damage must have occurred during our experimental procedures, most probably during homogenization. De Duve (1971) has shown that the use of a Potter-Elvehjem homogenizer can damage up to 15% of the lysosomes in a preparation of liver homogenate. It could well be that progesterone was similarly released from progesterone containing granules.

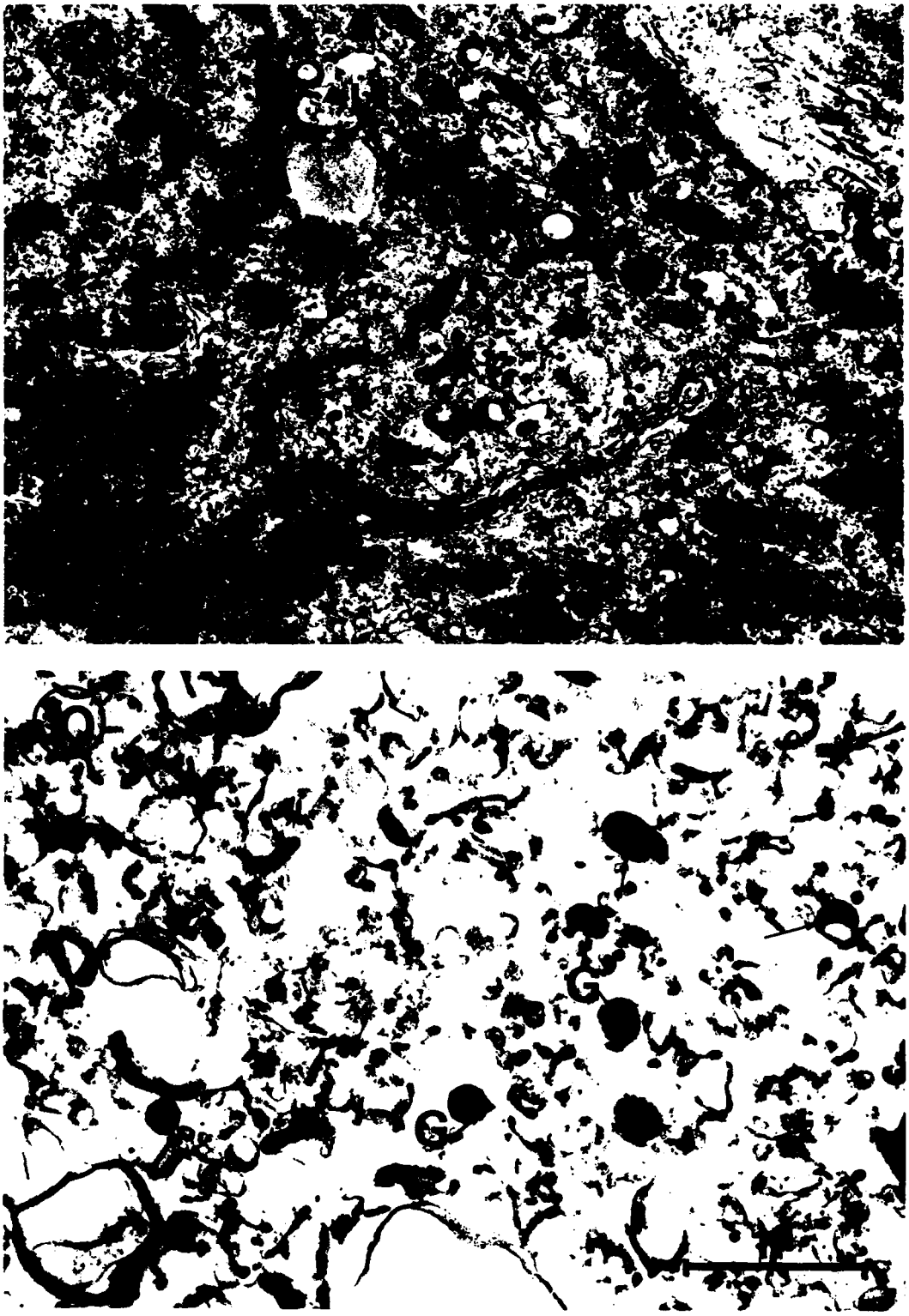
When subjected to density gradient centrifugation, most of the sedimentable progesterone entered the gradient. The portion found in the sample zone at the top of the gradient, along with some of the lysosomal and microperoxisomal marker enzymes, was presumably leached out during resuspension of the 10,000 × g pellet or during centrifugation. We established that cytosolic progesterone also remained in the sample zone at the top of the gradient. Thus any progesterone entering the sucrose density gradient must have been sequestered within an organelle or membrane.

The peak of progesterone enrichment occurred reproducibly over a narrow density range (23–28% w/w sucrose, d = 1.094–1.117) and the enrichment was considerable, up to 17-fold. Enzymatic and morphological data showed that no other cell organelles were located at this lower density, apart from some overlap with the endoplasmic reticulum. However, the microsomal marker enzymes were spread throughout the gradient, and although the total microsomal contamination was low in the progesterone-enriched fraction, the large volume of agranular endoplasmic reticulum in the corpus luteum was responsible for the considerable vesicle contamination observed in the progesterone-enriched peak. The endoplasmic reticulum marker enzymes were in fact present in greater amounts and showed higher enrichment at higher densities (31–36% w/w sucrose, d = 1.132–1.156). Thus, it is unlikely that progesterone was associated with the membrane vesicles that were visible in the electron micrographs of the progesterone-enriched peak of the gradient.

We observed electron dense granules in fractions which contained a high enrichment of progesterone; these granules were usually distorted and rather irregular in shape. The density of the progesterone-enriched granular fraction was low compared with that of other cell organelles. The low density at which progesterone banded would be compatible with the sequestration of progesterone within these granules. Granules which contain polypeptide hormones band at higher density in sucrose gradients. For example, the gastrin granules of intestinal mucosa band in sucrose at d = 1.17–1.18 (Trotman et al., 1976). The densities that we found for the other cell organelles

FIG. 9. Portion of a luteal cell from a cow at Day 15 of the estrous cycle incubated to demonstrate the presence of acid phosphatase activity. The section is not counterstained and reaction product can be seen in the Golgi cisternae and in lysosomes (L). Other granules (G) can be seen which are not positively stained. × 17,368.

FIG. 10. Fixed pellet from tubes 5–6 of a 20–40% w/w sucrose density gradient. Dense staining granules (G, arrows) are present, together with vesicles of various sizes and other membranous components. × 27,388.



of the bovine corpus luteum agree broadly with those found using discontinuous sucrose gradients (Gospodarowicz, 1973).

In our cytochemical studies, we have demonstrated that 3 types of electron dense granules are present in sections of bovine corpus luteum. Microperoxisomes and lysosomes are present. Both of these organelles have been reported previously in steroidogenic tissues (Reddy, 1973; Novikoff and Novikoff, 1973; Gulyas and Yuan, 1975; Gemmell et al., 1976). These organelles are never secreted into the pericellular space, whereas a third group of electron dense granules are secreted. We have also shown *in vitro* (Parry, Quirk and Willcox, unpulished observations) that slices of ovine corpus luteum, which are actively secreting progesterone, also secrete granules into the pericellular space. Recently, Abel et al. (1977) have reported that in similar preparations from the ewe, the number of granules exocytosed rose and fell with an increase or decrease of progesterone secretion and that concomitantly a protein was released into the incubate. In the perfused cat adrenal, ACTH stimulation caused an increase in the number of granules within the tissue along with an increased glucocorticoid production and the release of protein into the perfusate (Rubin et al., 1974; Laychock and Rubin, 1974; Gemmell et al., 1977). These authors also suggested that glucocorticoids were secreted with a protein in granule form.

In fixed fractions from sucrose density gradients, microperoxisomes and lysosomes were located in parts of the gradient other than the progesterone-enriched peak. Their location corresponded to that observed for the respective marker enzymes for these organelles. The progesterone-enriched fractions, which also contain electron dense granules, did not contain microperoxisomes or lysosomes. These cytochemical data support the contention that these electron dense granules contain progesterone. In addition, it is likely that a protein must be present within the granules to render them electron dense. A possible candidate is the secreted protein described by Abel et al. (1977). We have preliminary evidence for a progesterone-binding protein in bovine corpus luteum (unpublished data) similar to that described by Leymarie and Gueriguian (1970), but we have not yet investigated the location of this component on density gradients.

Electron dense granules in the porcine corpus luteum of pregnancy were observed by Belt et al. (1971) who suggested that relaxin was contained within these granules. However, Chamley et al. (1975) reported that high circulating progesterone and relaxin levels did not coincide during the estrous cycle of the sheep. It is unlikely therefore that relaxin is related to the concomitant granule production and progesterone secretion observed by Gemmell et al. (1974). Since an assay for bovine relaxin is not available, we cannot exclude the possibility that the electron dense granules which we observed in the progesterone-enriched regions of the gradient might also contain relaxin.

Our biochemical and cytochemical results support the concept that progesterone is localized within electron dense granules which are neither microperoxisomes nor lysosomes. Further confirmation of the hypothesis that steroid hormone secretion occurs via exocytosis of steroid containing granules must await the purification of granules in quantities sufficient to investigate their composition more fully.

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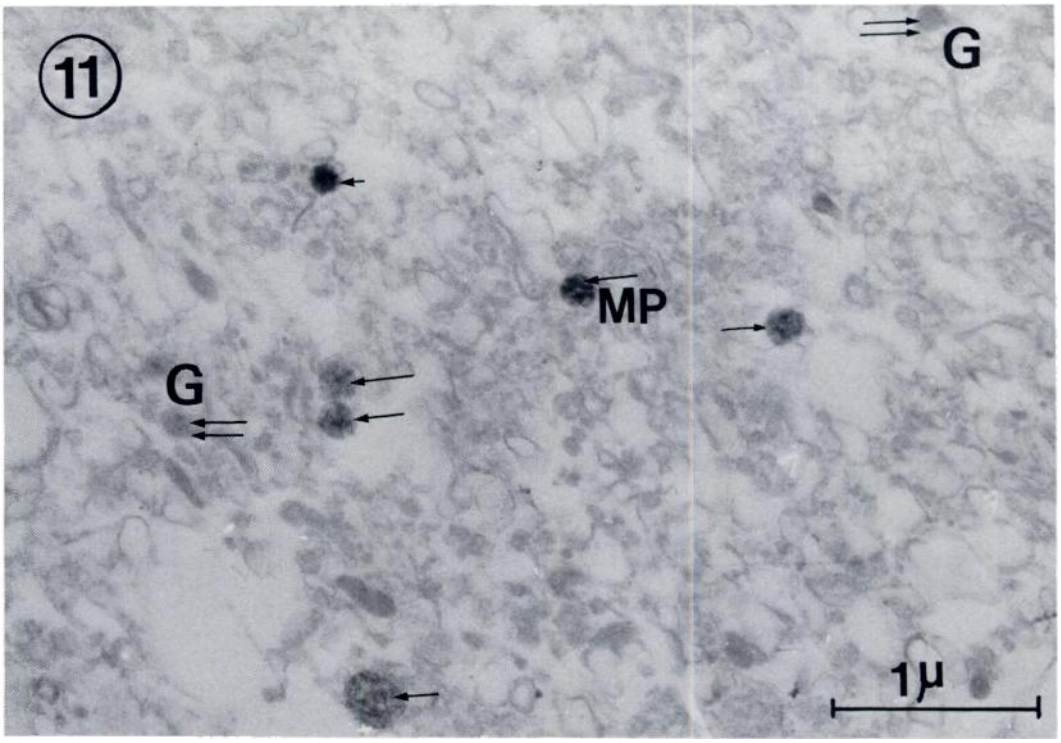
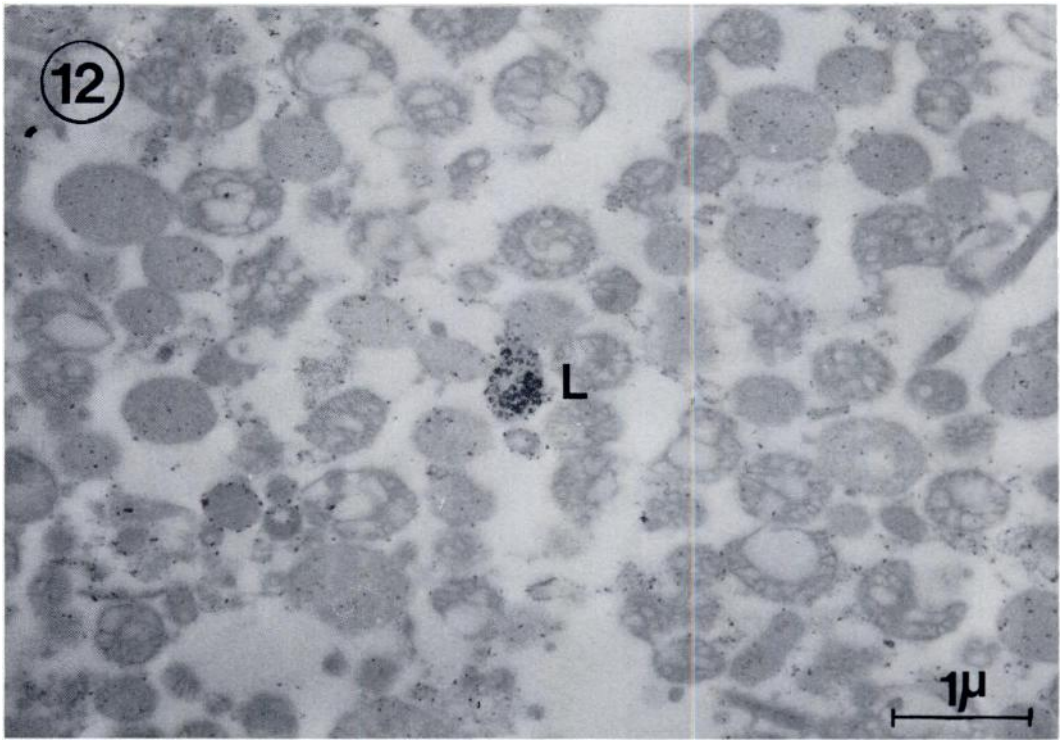
FIG. 11. Fixed pellet from tubes 11–12 of a 20–40% w/w sucrose density gradient, after incubation with alkaline diaminobenzidine to demonstrate microperoxisomes. The section is not counterstained and the dense osmiophilic reaction product can be seen over microperoxisomes (arrows). An occasional nonstaining granule can be seen (G, double arrows). Membrane vesicles constitute the remainder of the field. × 34,650.

FIG. 12. Fixed pellet from tubes 17–18 of a 20–40% w/w sucrose density gradient incubated to demonstrate the presence of acid phosphatase activity. The section is not counterstained. The dense reaction product identifies a lysosome (L); some nonbackground stain is observed. The rest of the field is comprised mainly of mitochondria. × 17,160.





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