STUDIES ON FORMALIN FIXATION FOR ELECTRON MICROSCOPY AND CYTOCHEMICAL STAINING PURPOSES

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ABSTRACT

A study has been made of the preservation of fine structure, phospholipids, and the activity of acid phosphatase and esterase in rat liver fixed in various solutions containing 4 per cent formaldehyde. Examination of methacrylate-embedded preparations shows that calcium-containing fixatives result in poor preservation of fine structure, whereas veronal-treated or phosphate-buffered formalin gives excellent results if the tonicity of the solutions is suitably adjusted by addition of sucrose. Formol-phosphate, to which Versene has been added, causes deterioration of cellular morphology. Phospholipids are retained almost quantitatively in tissue fixed in formol-calcium, and in phosphate-, collidine-, or triethanolamine-buffered formalin. About 50 per cent of the activity of acid phosphatase and esterase are preserved after 24 hours exposure to these fixatives at 0–2°C, and the distributions of the enzymes and of phospholipids, as judged by cytochemical staining results, are not altered by any of these formalin solutions. Consideration of the morphological and biochemical integrity of the fixed tissue suggests that 4 per cent formaldehyde, buffered at pH 7.2 with 0.067 M phosphate, and containing 7.5 per cent sucrose, is the most suitable of the fixatives for combined cytochemical staining and electron microscopical studies.

INTRODUCTION

Current interest in the combination of cytochemical staining techniques for enzyme localization with electron microscopy has underlined the need for a fixative which will preserve both the enzymic activity and the detailed fine structure of cells. Unfortunately, osmium tetroxide, which is the most widely used fixative for electron microscopy, seriously reduces the activity of many enzymes (31). It is therefore not ideally suited to enzyme cytochemistry, for while sufficient activity may remain after brief osmium fixation for some staining to occur, the results have to be interpreted with caution in view of the possibility that the enzyme may have been totally inhibited in some sites. It has recently been shown (22), however, that formol-calcium fixation gives good preservation of both the hydrolase activity and the phospholipid integrity of rat tissues and, as far as the light microscope is concerned, of cellular structures. Strong evidence has been presented (19) that valid and precise localization of acid phosphatase is obtained in frozen sections of such tissue when stained by the Gomori method (15), but little advantage would be gained in attempting to combine this type of staining method with electron microscopy if the fixative did not give adequate preservation of fine structure.

From preliminary tests with rat liver, it became clear that calcium-containing fixatives gave very

poor preservation of fine structure, even after postfixation with osmium tetroxide. On the other hand, it has been reported that buffered formalin also preserves a high level of enzymic activity (40) and gives excellent morphological preservation at the electron microscope level (42). A systematic study of various formalin fixatives was therefore undertaken to assess the effects of controlled changes in composition upon the quality of fixation, and upon the preservation of acid phosphatase activity, esterase activity, and tissue phospholipids.

MATERIALS AND METHODS

Chemical: Chemicals used in preparing the fixatives and buffers were of analytical reagent quality. The stock formalin solution (Analar grade, British Drug Houses Ltd., Poole, Dorset, England) contained approximately 36 per cent *w/v* formaldehyde and up to 10 per cent methanol as stabilizer. Use of formalin which was of a lower commercial quality, or stale, frequently gave poor fixation. Collidine was a freshly redistilled colourless fraction boiling at 170–172°C/760 mm. It was stabilized by addition of about 0.5 per cent *w/v* of chromatographic grade alumina and stored at 0–2°C. Triethanolamine hydrochloride of special buffer grade was supplied by C. F. Boehringer & Soehne G.m.b.H., Mannheim, Germany. N-Ethylmorpholine was dried over Union Carbide Molecular Sieve, type 5A, and freshly distilled before use, the fraction which boiled at 138–139°C/763 mm being collected.

Animal: Male Wistar rats of the inbred Courtauld Institute substrain were used. They weighed 160 to 180 gm and had been fed *ad lib.* on the Rowett Research Institute diet no. 86. When perfused livers were required, each animal was anaesthetized with ether and the liver exposed. The needle of a syringe containing 20 ml of ice cold 0.9 per cent saline was inserted upwards into the portal vein, the inferior vena cava was severed between the liver and the diaphragm, and the saline was steadily injected to remove blood from the organ. The liver was then excised and at once placed upon crushed ice before weighed samples were taken for enzyme and phospholipid determinations as described below.

Tissue Sampling and Fixation: Except when perfused livers were required, rats were killed by cervical dislocation. Samples of liver were immediately removed and treated as rapidly as possible by the following procedures.

Pieces about 2 × 2 × 10 mm in size were transferred to ice cold fixative and there cut into approx. 1 mm. cubes. They were then transferred to 50 to 100 ml of fresh cold fixative, which was maintained at 0–2°C for 1 or 24 hours. For light microscopy, fixed liver was treated with gum-sucrose solution (0.88 M sucrose containing 1 per cent gum acacia) (18) and used to prepare frozen sections. For electron microscopy, the formalin-fixed tissue was transferred to ice cold 1 per cent osmium tetroxide buffered at pH 7.2 and containing 4.9 per cent sucrose (8), where it was maintained at 0–2°C for 1 or 4 hours.

For the enzyme determinations, chilled perfused livers were cut into slices 2 mm thick, and not exceeding 1 cm^2 in area. Weighed amounts of the slices were transferred to ice cold fixatives (100 ml for each gram of tissue). After 24 hours at 0–4°C, the samples were removed, blotted, and treated with ice cold gum-sucrose for 24 hours to wash out the fixative under hypertonic conditions (22). Specimens were also treated with gum-sucrose for 7 days. In both cases, the enzyme determinations were made at the end of these gum-sucrose treatments. Enzyme determinations were also made upon 1 mm^3 blocks of liver that had been fixed directly in the osmium tetroxide fixative for 10 minutes and then treated with gum sucrose for 24 hours or for 7 days. Weighed, unfixed samples of the same livers were taken for control determinations of enzymic activity.

For phospholipid determinations, larger samples (2 to 3 gm) of perfused liver were fixed as for the enzyme determinations, removed, blotted, and used directly for the analyses. Weighed, unfixed samples were again used for control determinations.

During all fixation and washing operations, the contents of the vessels were gently shaken to ensure homogeneity and uniform penetration of solutions.

Fixatives: All fixatives contained 4 per cent formaldehyde, and two classes of buffers were used to adjust their pH. First, acetic, phosphoric, and diethylbarbituric acid buffers were used. The last (veronal) requires some comment, for, as reported elsewhere (20), it reacts with formaldehyde to produce a substance with no buffering capacity within the physiological range of pH values. However, since formalin "buffered" with veronal has been widely used as a fixative (7, 9, 12, 45), it was included in the present investigations. Second, buffers based upon triethanolamine, N-ethylmorpholine, and 2,4,6-collidine were examined. Being tertiary amines, they do not react with formaldehyde, in contrast to the several primary and secondary amines used to prepare certain other popular buffers. Collidine was also of interest because it is a satisfactory buffer for osmium tetroxide fixatives (6).

The tonicity of some of the fixatives was varied by addition of sucrose and, in some cases, of sodium chloride. The effects of adding Versene or ammonium reineckate were also briefly examined.

Before use, formol-calcium fixative (2) (Table I, nos. 1, 2) was shaken with approximately 1 per cent *w/v* powdered calcium carbonate, cooled in ice, and filtered, giving a solution of pH 6.5–6.8. The formol–

calcium acetate fixative (29) (Table I, no. 3) contained 2 per cent *w/v* (0.113 M) calcium acetate monohydrate, which adjusted the pH to a value of 7.2. The reineckate-containing fixative (Table I, no. 9) was made by saturating fixative no. 7 (Table I) at 0°C with ammonium reineckate.

Stock buffers (pH 7.4) used to prepare the fixatives were 0.1 M phosphate, 0.2 M collidine-HCl, 0.2 M triethanolamine-HCl, and 0.2 M N-ethylmorpholine-HCl. Guidance for preparing the last three can be found elsewhere (10). In all cases, the final pH of the buffered fixatives was adjusted to 7.2, using a sensitive pH meter.

Formalin analogues of Palade's 0.031 M veronal–acetate–buffered osmium tetroxide fixative (33) were prepared by first mixing 2.6 ml of 36 per cent formaldehyde with 5 ml each of 0.143 M sodium acetate and 0.143 M sodium veronal solutions. The alkali liberated (20) was neutralized, and the final pH adjusted to 7.2, by addition of hydrochloric acid before making up to a final volume of 23 ml. Sucrose additions were made before the final dilution.

Preparation of Specimens for Electron Microscopy: After treatment with the various formalin fixatives, the small blocks were dehydrated by successive 30 minute treatments in 30, 50, 70, and 90 per cent alcohols, followed by three similar changes of absolute alcohol. They were then embedded in butyl methacrylate. Sections cut with a glass knife on a Porter-Blum microtome (37) were mounted on carbon-coated grids (43) and examined in a Philips electron microscope, type EM 100, in the way already described (11).

Light Microscopy: After impregnation with gum sucrose, 5 μ frozen sections were cut of liver that had been treated with the various formalin mixtures, but not with osmium tetroxide. The sections were then mounted in 0.5 M saline and examined by phase contrast microscopy.

Similar sections were stained for 15 to 30 minutes in the Gomori acid phosphatase medium (15), or for 30 to 45 minutes by the indigogenic esterase method using 5-bromo-4-chloroindoxyl acetate as substrate (18, 23). Frozen sections of the osmium-fixed liver were cut 10 μ thick and stained for acid phosphatase or esterase for up to 3 hours. The Baker acid haematein method for phospholipids (2) was applied only to the formalin fixed tissues, but without previous impregnation in gum-sucrose.

Enzyme and Phospholipid Determinations: Measurements of acid phosphatase activity and phospholipid content of tissues were made as described previously (22), but only on the fixed tissues listed in Table II, and on unfixed control samples. The osmium-fixed material, however, was not analyzed for phospholipids since it is known (14) that reaction with osmium tetroxide renders them insoluble in lipid solvents of the type used in the analytical procedure (22).

The acid phosphatase activity of whole liver includes that of both parenchymal cells and blood components. Since erythrocyte acid phosphatase is known to be seriously inhibited by formalin (1), measurements of the survival of this enzyme in formalin-fixed whole liver, such as those reported earlier (22), might be misleadingly low. For this reason, enzyme determinations were made on the perfused organ, and are thus mainly relevant to parenchymal cell acid phosphatase.

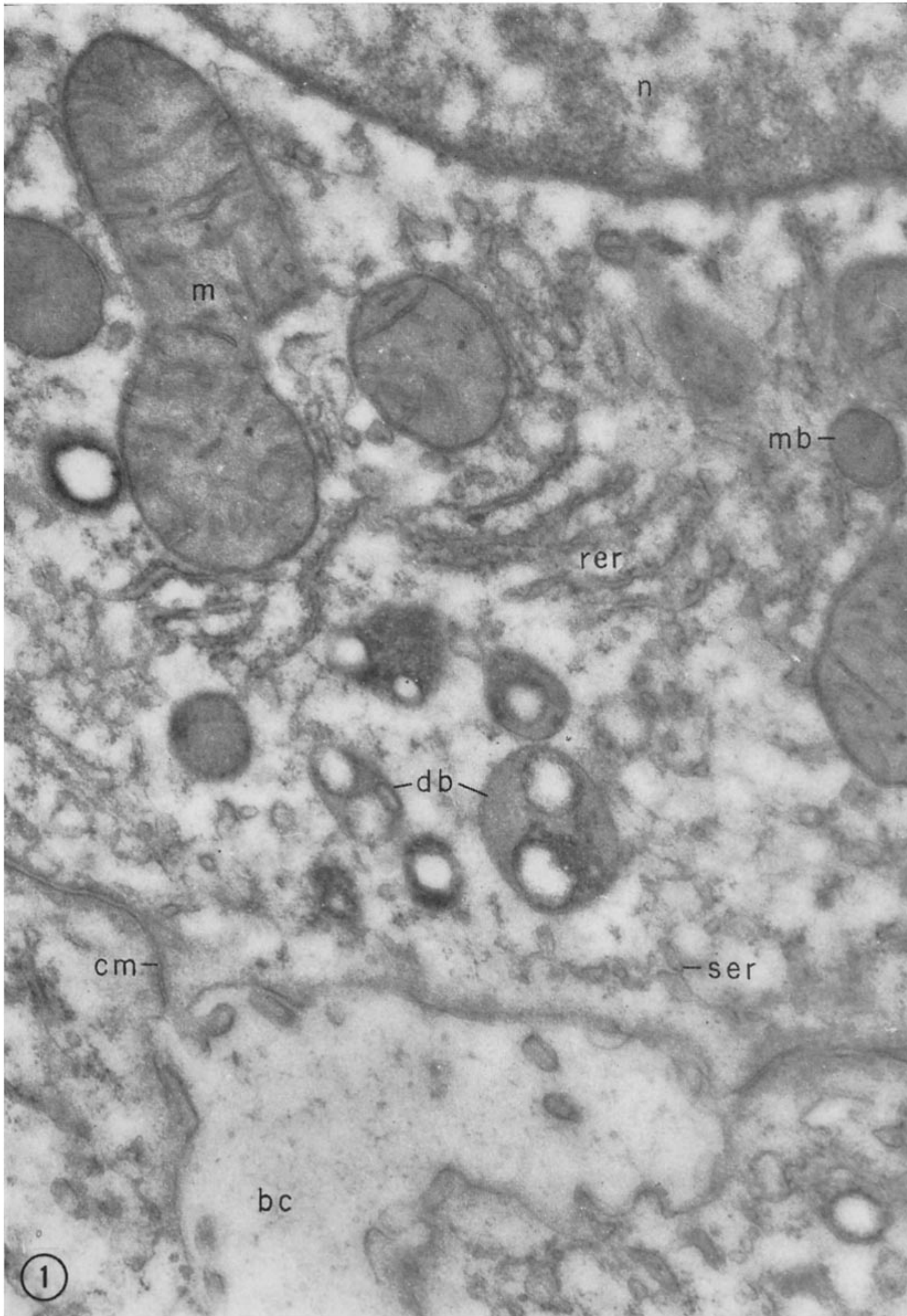
Esterase determinations were made by titration of acetic acid released from 6.2×10^{-3} M solutions of indoxyl acetate. The substrate solution also contained 0.1 per cent crystalline bovine plasma albumin as stabilizer, and tissue homogenate equivalent to 1 mg (control tissue) or 2 mg (fixed tissue) of liver, in a total volume of 3 ml. Before adding the enzyme preparation, the solution was adjusted to pH 7.4 with alkali and allowed to equilibrate at 37°C for 5 minutes. During the enzymic reaction, the medium was stirred, and the pH was maintained at 7.4 by automatic titration with 0.025 M sodium hydroxide solution, using a Radiometer (Copenhagen) Titrator type TTT1 and Titrigraph type SBR2. The volume of alkali consumed during the period 5 to 40 minutes from the time of addition of the homogenate was taken as a measure of esterase activity.

RESULTS

Morphology: Distinct differences were found in the consistency and ease of cutting of frozen sections of tissues treated with the different formalin fixatives. The majority were soft, but with calcium-containing fixatives they had a firm rubber-like consistency and cut more easily than the rest.

When the sections were examined by phase contrast microscopy, there appeared to be little difference in the quality of fixation, although pronounced cellular vacuolation could be seen in tissues subjected to the Versene-containing or N-ethylmorpholine–buffered fixatives. When tissues were examined in the electron microscope after postfixation, marked differences in the quality of fixation were immediately apparent. The preservation of fine structure was better after fixation for 24 hours in the formalin-based fixatives than after 1 hour. Conversely, better results were obtained after postfixation with osmium tetroxide for 1 hour than for 4 hours, when extraction effects in the tissue were apparent.

Part of a rat liver parenchymal cell fixed in osmium tetroxide (8) is shown for reference purposes in Fig. 1. The characteristic appearance of



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1

TABLE I

*Tissue Preservation by Various Fixatives Based upon 4 Per Cent Formaldehyde**

Fixative no.	Additions to fixatives			Comments
	Buffer	Sucrose (%)	Salts	
1‡	—	—	1% $CaCl_2$	Did not embed
2	—	—	1% $CaCl_2$	Poor fixation
3	0.113 M calcium acetate	6.1	—	Poor fixation
4	0.031 M veronal	—	—	Swollen Golgi cisternae
5	0.031 M veronal	5.0	—	Good fixation
6	0.067 M phosphate	—	—	Swollen Golgi cisternae, mitochondria, and endoplasmic reticulum
7	0.067 M phosphate	7.5	—	Excellent fixation
8	0.067 M phosphate	7.5	1% Versene	Poor fixation
9	0.067 M phosphate	7.5	Saturated reineckate	Did not embed
10	0.075 M collidine	7.5	—	Swollen Golgi cisternae
11	0.075 M collidine	7.5	0.27% NaCl	Swollen Golgi cisternae
12	0.075 M triethanolamine	7.5	—	Swollen Golgi cisternae
13	0.075 M triethanolamine	7.5	0.27% NaCl	Not examined in electron microscope

* All fixatives were buffered at pH 7.2, except nos. 1 and 2, the pH of which was 6.5–6.8. The tissues were fixed for 24 hours at 0–2°C and postfixed in osmium tetroxide for 1 hour.
‡ Not postfixed.

liver fixed in this way has frequently been described (*e.g.* 13, 36, 38). Tissue fixed in formol-calcium at pH 6.5–6.8 would not embed satisfactorily with the dehydration and embedding procedures used, unless it was postfixed in osmium tetroxide (Table I, nos. 1, 2). Even after such treatment, however, very poor preservation of fine structure is found in this material (Fig. 2). It can be seen that there is a lack of continuity in the cytoplasm and that holes are present in the nucleus and in some of the mitochondria. A microbody (39) can be seen, but other types of peribiliary dense bodies (36, 38) cannot be identified with certainty. In general, such tissue is characterized by an unusually clumped appearance, together with a loss of detail in cytoplasmic structures. For example, it is frequently difficult to identify the components of the Golgi zone and to distinguish between rough and smooth surfaced endoplasmic reticulum. Amongst the characteristic features seen in formol-calcium–fixed liver are vacuolated structures composed of irregular membranous boundaries, containing variable amounts of heterogeneous material. These structures have been observed only with calcium-containing formalin fixatives, and it is concluded that they are fixation artefacts.

Raising the pH of the formol-calcium fixative to 7.2, by using calcium acetate instead of the chloride (Table I, no. 3), did not improve tissue preservation. When liver was fixed in veronal-treated formalin (Table I, no. 4), the tissue preservation was considerably improved, although the smooth surfaced endoplasmic reticulum and

All the figures are electron micrographs of thin sections of rat liver parenchymal cells.

FIGURE 1

A small part of the periphery of a cell fixed in osmium tetroxide. A bile canaliculus (*bc*) occupies the lower part of the field, and the edge of the nucleus (*n*) can be seen at the top. Vacuolated dense bodies (*db*) adjacent to the bile canaliculus can be distinguished from a microbody (*mb*) at the upper right edge of the field. The cell membrane (*cm*), rough surfaced endoplasmic reticulum (*rer*), smooth surfaced endoplasmic reticulum (*ser*), and mitochondria (*m*) are also present. × 40,000.

the cisternae of the Golgi complex were swollen. Increasing the non-ionic tonicity of the fixative by addition of 5 per cent sucrose (Table I, no. 5), approximately that concentration used by Caulfield (8) to modify Palade's fixative (33), resulted in very good preservation of fine structure, including elements of the smooth surfaced endoplasmic reticulum and of the Golgi zone. The over-all appearance of the tissue was almost indistinguishable from that of tissue fixed in osmium tetroxide alone.

Replacement of veronal by phosphate buffer (Table I, no. 6) also gave good fixation, but in this case, addition of 5 per cent sucrose did not prevent swelling of the ends of the Golgi cisternae (Fig. 3). Tests with sucrose concentrations of 6.1, 7.5, and 8.5 per cent showed that 7.5 per cent (Table I, no. 7) consistently gave good preservation. A detail of a liver parenchymal cell fixed in this way, followed by postfixation in Caulfield's fixative (8) for 1 hour, is shown in Fig. 4. Mitochondria, rough surfaced endoplasmic reticulum, and part of the nucleus are present. Vacuolated dense bodies, and tubules and vesicles of the smooth surfaced endoplasmic reticulum around the bile canaliculus to the lower right of the field, are well preserved and have the same appearance as with osmium tetroxide fixation alone (*cf.* Fig. 1). A junction of the type previously described (34, 38) between tubules of rough and smooth surfaced endoplasmic reticulum can be seen at the top right of Fig. 4. In another example of the same material (Fig. 5), two microbodies can be seen in addition to many of the structures appearing in Fig. 4.

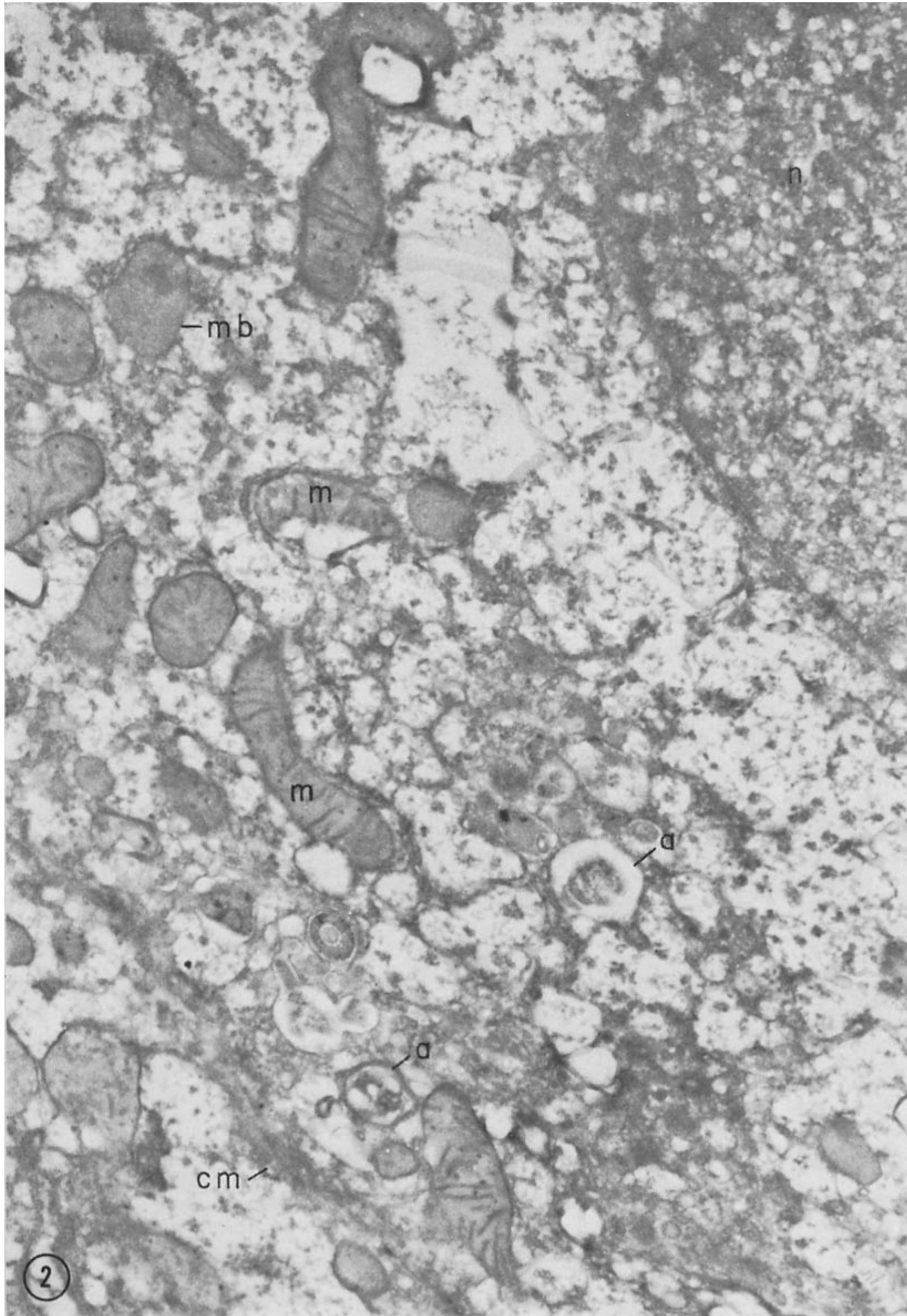
The versene-containing fixative (Table I, no. 8) gave poor tissue preservation, and vacuoles appeared throughout the tissue as in formol-calcium–fixed material. The fixative containing ammonium reineckate (Table I, no. 9) gave tissue that would not embed satisfactorily. Collidine- or triethanolamine-buffered formalin (Table I, nos. 10–12) gave substantially well preserved fine structure, but caused swelling of elements of the smooth surfaced endoplasmic reticulum and the cisternae of the Golgi zone. Liver fixed in the N-ethylmorpholine–buffered fixative (Table I, no. 13) was not examined in the electron microscope, since it was morphologically poor in the light microscope and gave weak and unsatisfactory staining results.

Enzyme and Phospholipid Staining Patterns: When frozen sections were stained to demonstrate the distribution of acid phosphatase and esterase, all but one of the formalin-fixed tissues gave the same results as those previously published (18, 19, 23), *viz.*, both enzymes were found to be associated with pericanalicular granules in the parenchymal cells of the liver. The exception was tissue treated with the N-ethylmorpholine–buffered fixative, which gave a barely perceptible non-localized reaction for acid phosphatase, and a diffuse, weak reaction for esterase. Tissue fixed for 10 minutes in osmium tetroxide did not stain for acid phosphatase, even after incubation for 3 hours. After the same incubation time, a weak staining reaction was obtained for esterase, which was more discrete and localized with these frozen sections than had previously been obtained (32) with blocks stained before sectioning. With the Baker acid haematein procedure, mitochondria were the predominant sites of staining in the formalin-fixed tissues.

Enzymatic Activity and Phospholipid Content of Tissues: The results are summarized in Table II. The highest acid phosphatase activity was found in tissue preserved by formol-phosphate-sucrose fixation, whereas the highest esterase activity was retained by the triethanolamine-buffered fixative. Collidine-buffered formalin more seriously

FIGURE 2

The field shows part of a cell from tissue first treated for 24 hours with formol-calcium and then postfixed for 1 hour in osmium tetroxide. The nucleus (*n*) lies at the top right and the cell membrane (*cm*), sectioned obliquely, at the bottom left. The tissue is poorly preserved, holes have appeared in the nucleus and between the remnants of the endoplasmic reticulum, and other fixation artefacts (*a*) can be seen. Some of the mitochondria (*m*) are damaged, and there is no clear distinction between rough and smooth surfaced elements of endoplasmic reticulum. It is not possible to identify the components of the Golgi region, but a microbody (*mb*) at the top left is well preserved. × 20,000.



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2

TABLE II

*Retention of Enzymic Activity and Phospholipid in Fixed Rat Liver**

Fixative‡	Acid phosphatase		Esterase		Phospholipid§
	24 hours gum-sucrose	7 days gum-sucrose	24 hours gum-sucrose	7 days gum-sucrose	
Buffered osmium tetroxide (10 minutes fixation only)	1.5	1.9	9	11	Not determined
Formol-collidine (11)	32	35	43	44	99
Formol-triethanolamine (12)	43	50	49	55	91
Formol-calcium (1)	46.5	50	41	43	96
Formol-phosphate (7)	50	56	46	47	97

* Mean of duplicate determinations on perfused rat livers fixed for 24 hours at 0–2°C and expressed as percentages of values for unfixed control tissues.

‡ Numbers in brackets refer to fixatives listed in Table I.

§ Determined on fixed tissues not subjected to gum-sucrose treatment.

inhibited acid phosphatase, but had little effect on esterase activity. Neither enzyme was more than slightly active following osmium tetroxide fixation.

Retention of phospholipid was remarkably good in the cases studied (Table II), and similar to that found in unperfused liver (22).

DISCUSSION

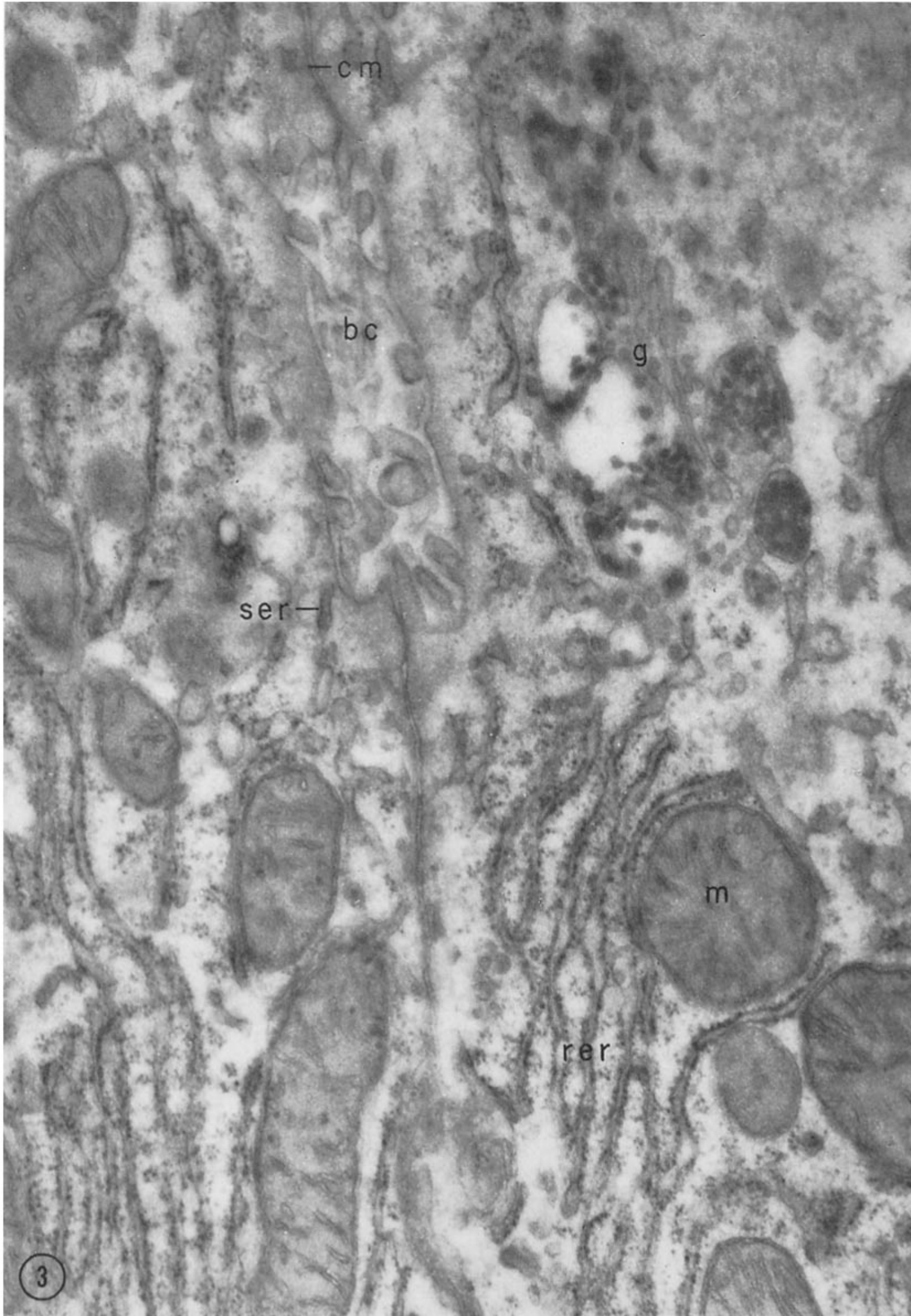
When tissues are subjected to staining without previous fixation, details of fine structure are frequently lost. It is then difficult to determine accurately with which structures the enzyme is associated. Some tissues, such as cardiac muscle, will withstand this treatment to a certain extent, but even these show damage (4). When tissues are fixed before staining, a serious reduction in enzymic activity may occur. Staining patterns must then be interpreted with caution, for, as mentioned above, there may have been selective inhibition of the enzyme in different sites. This is emphasized by the occurrence of more than one acid phosphatase in rat liver, with different susceptibilities towards inactivation by formalin (16). Further difficulties of interpretation may arise when poorly penetrating fixatives are used, for different staining patterns may be produced in differently fixed zones of the tissue. Uniform preservation of the tissue by rapidly penetrating fixatives is therefore of considerable importance.

Osmium tetroxide is a slowly penetrating fixative, and also seriously inactivates many enzymes (Table II) (12, 31). Interest has therefore recently been focused upon formalin as a suitable fixative for combined cytochemistry and electron microscopy (21, 24, 28, 42, 45). This fixative has a higher rate of penetration than most other fixatives (3) and has already been reported to preserve tissue fine structure (42) and the activity of several enzymes (22, 30, 40). Tissues fixed in formalin are therefore very suitable for cytochemical staining experiments, although for electron microscopy, a further period of treatment in osmium tetroxide results in better fixation than that given by formalin alone (44). It also appears to protect tissues from damage during embedding (42).

Fixation in formol-calcium was introduced (2)

FIGURE 3

Adjacent areas of two cells treated first for 24 hours with phosphate-buffered formalin containing 5 per cent sucrose, and then postfixed in osmium tetroxide for 1 hour. The opposing cell membranes (*cm*) pass centrally through the field from top to bottom and are separated in the upper half by a bile canaliculus (*bc*). At the top right, elements of the Golgi component (*g*) are seen, with swollen cisternae containing dense globular masses of material. Rough surfaced endoplasmic reticulum (*rer*), smooth surfaced endoplasmic reticulum (*ser*), and mitochondria (*m*) present the same appearance as in material fixed in the osmium fixative alone. × 40,000.


cm
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with the object of improving fixation of lipids, since calcium salts prevent formation of myelin figures when lecithin smears are exposed to aqueous fluids (27). Tissue fixed in formol-calcium has high enzymic activity together with well preserved phospholipids, and it also appears to be well preserved when examined by phase contrast microscopy (22). In the electron microscope, however, it can be seen that the fine structure is poorly preserved in comparison with that of tissue fixed in osmium tetroxide alone. The appearance of the tissue was characteristic of the fixative and was not due to overexposure of the sectioned material in the electron beam.

Reaction of tissues with unbuffered formaldehyde causes them to become more acidic (46), and it is known that such conditions will damage the tissue (28) and disrupt phospholipids (35). However, the damage seen after formol-calcium fixation could not be entirely due to acidity, for use of formol-calcium acetate at pH 7.2 did not alter the appearance of the tissue. It must therefore be assumed that the damage observed was caused by the direct effect of calcium ions on the fine structure of the cells. It was therefore encouraging to find that formol-veronal adjusted to pH 7.2 and containing 5 per cent sucrose preserved fine structure nearly as well as did osmium tetroxide alone. Direct replacement of veronal by phosphate, to ensure that an effective buffer was present (20), did not produce such good results until a further 1.1 per cent of sucrose was added. It was calculated that this amount was osmotically equivalent to the non-ionic reaction product of formaldehyde and veronal in the other fixative (20). Since the tissue was obviously sensitive to small changes in the tonicity of the fixative, the effects of other sucrose concentrations were investigated, as indicated above, and the optimum amount (7.5 per cent) was used for subsequent work.

It has been reported that Versene has a disruptive effect upon the fine structure of plant cells (25), and the rat liver used for the present work responds in a similar way to this metal-chelating agent. Ammonium reineckate, on the other hand, improves the preservation of plant cells by reducing the extraction of phospholipid by formalin (26), probably through the formation of insoluble complexes with phospholipids (41). Unfortunately, it cannot yet be said whether reineckate has a beneficial effect on fixation of animal tissue, as the embedding procedure used here failed with this material.

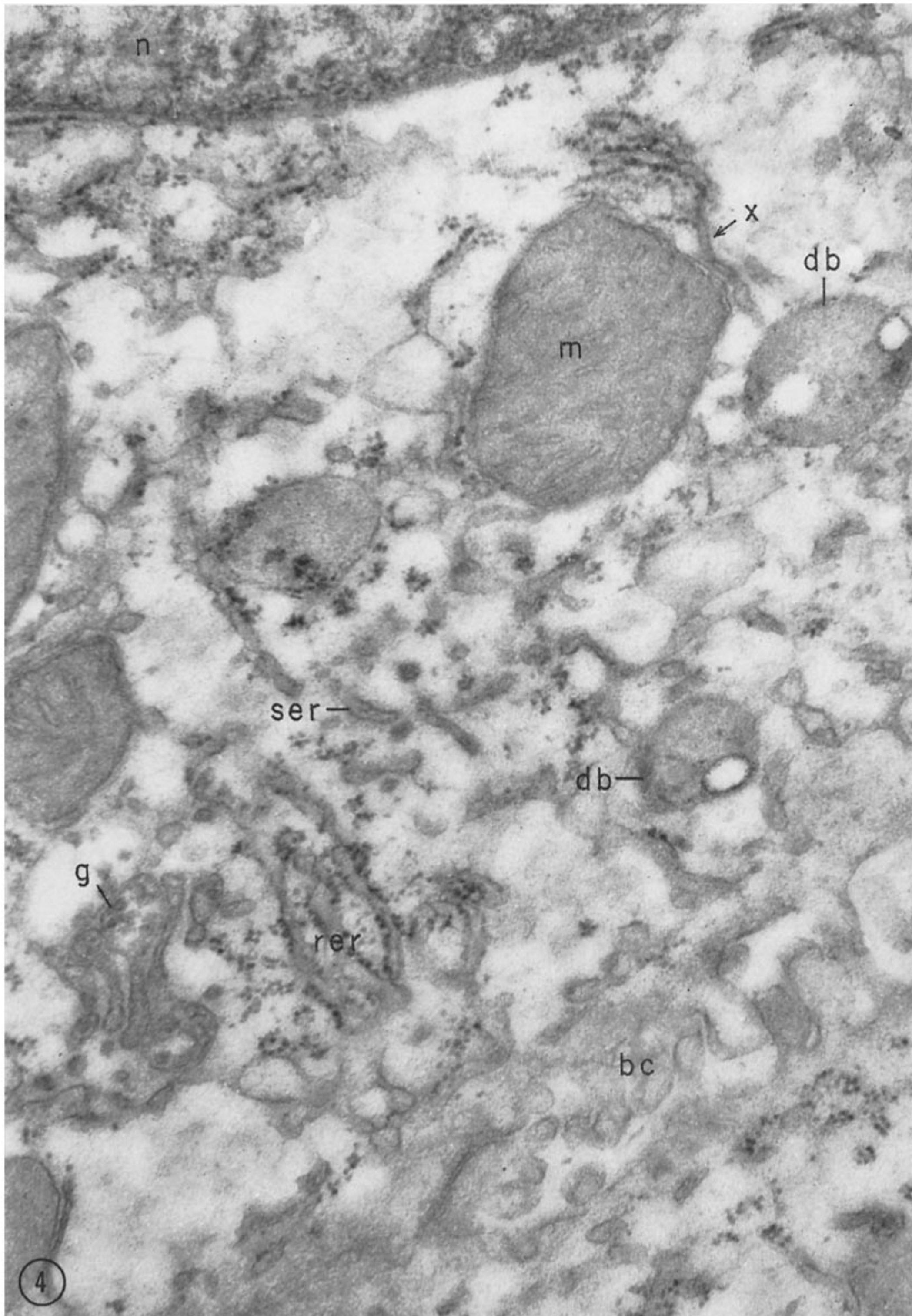
The results given by collidine-, triethanolamine-, and N-ethylmorpholine–buffered formalin were disappointing, even after modifications to the tonicity of the fixatives. The adverse effect of N-ethylmorpholine was noticeable even at the light microscope level, and it is clear that substitution of one buffer by another in a fixative cannot be relied upon to produce the same quality of fixation.

The quantitative enzyme determinations confirm previous reports (31) that osmium tetroxide preserves very little acid phosphatase or esterase activity in rat liver. The activities of both enzymes in formol-calcium–fixed perfused liver are 5 to 10 per cent lower than those found for unperfused liver (22). In the case of acid phosphatase, this was unexpected in view of the known inhibition of the erythrocyte enzyme (1) referred to above. The lower esterase activity may be due to the different assay method used in the present experiments. The previously published results (22) were obtained with a manometric technique using bicarbonate-buffered substrate solutions (17).

The enzymic integrity of the acid phosphatase–

FIGURE 4

The field shows a narrow band of cytoplasm from a cell fixed with phosphate-buffered formalin as before but with the sucrose content increased to 7.5 per cent, and then postfixed for 1 hour in osmium tetroxide. The detailed preservation of tissue fine structure obtained by this treatment is as good as that seen in material directly fixed in osmium tetroxide. The nucleus (*n*) occupies the top left edge, and a bile canaliculus (*bc*) the bottom right of the field. Two vacuolated dense bodies (*db*), components of the Golgi complex (*g*), rough surfaced endoplasmic reticulum (*rer*), smooth surfaced endoplasmic reticulum (*ser*), and mitochondria (*m*) are all present in this field. A junction (*x*) between rough and smooth surfaced endoplasmic reticulum can be seen at the top right. × 50,000.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containing granules (lysosomes) of rat liver depends upon the integrity of their lipoprotein-limiting membrane (5, 19). It is usually accepted that preservation of such membranes requires the presence of calcium ions in formalin fixatives (2). However, comparison of the properties of tissues fixed in phosphate-buffered formalin and in formol-calcium shows that the lipid integrity of the enzyme-containing structures is the same in both cases. Thus, the phospholipid analyses, and the enzyme and phospholipid-staining patterns, are essentially the same for both, and other investigations (19, 21) have shown that treatment of these tissues with Triton X-100 or with lecithinase produces the same dispersion of the enzyme. The activity of acid phosphatase is actually higher in formol-phosphate-sucrose-fixed liver than in formol-calcium-fixed tissue.

Within the limits of the present investigation, it is concluded that the formol-phosphate-sucrose fixative gives the best compromise between preservation of the morphological and biochemical integrities of rat liver. Tissue fixed in this way has accordingly been used as a basis for developing a cytochemical staining procedure for acid phosphatase for use with the electron microscope. This is described, and the validity of the method is discussed, in the following paper (21).

These studies were supported by grants from the British Empire Cancer Campaign and from the Central Research Fund of the University of London.

We gratefully acknowledge the guidance of Dr. M. A. Epstein throughout the course of the electron microscopical aspects of the work. We are also indebted to Mrs. Patience C. Barrow and Mr. H. G. Stevens for the enzyme determinations, and to Miss B. M. Blake and Mr. G. Ball for their invaluable assistance.

Received for publication, February 7, 1961.

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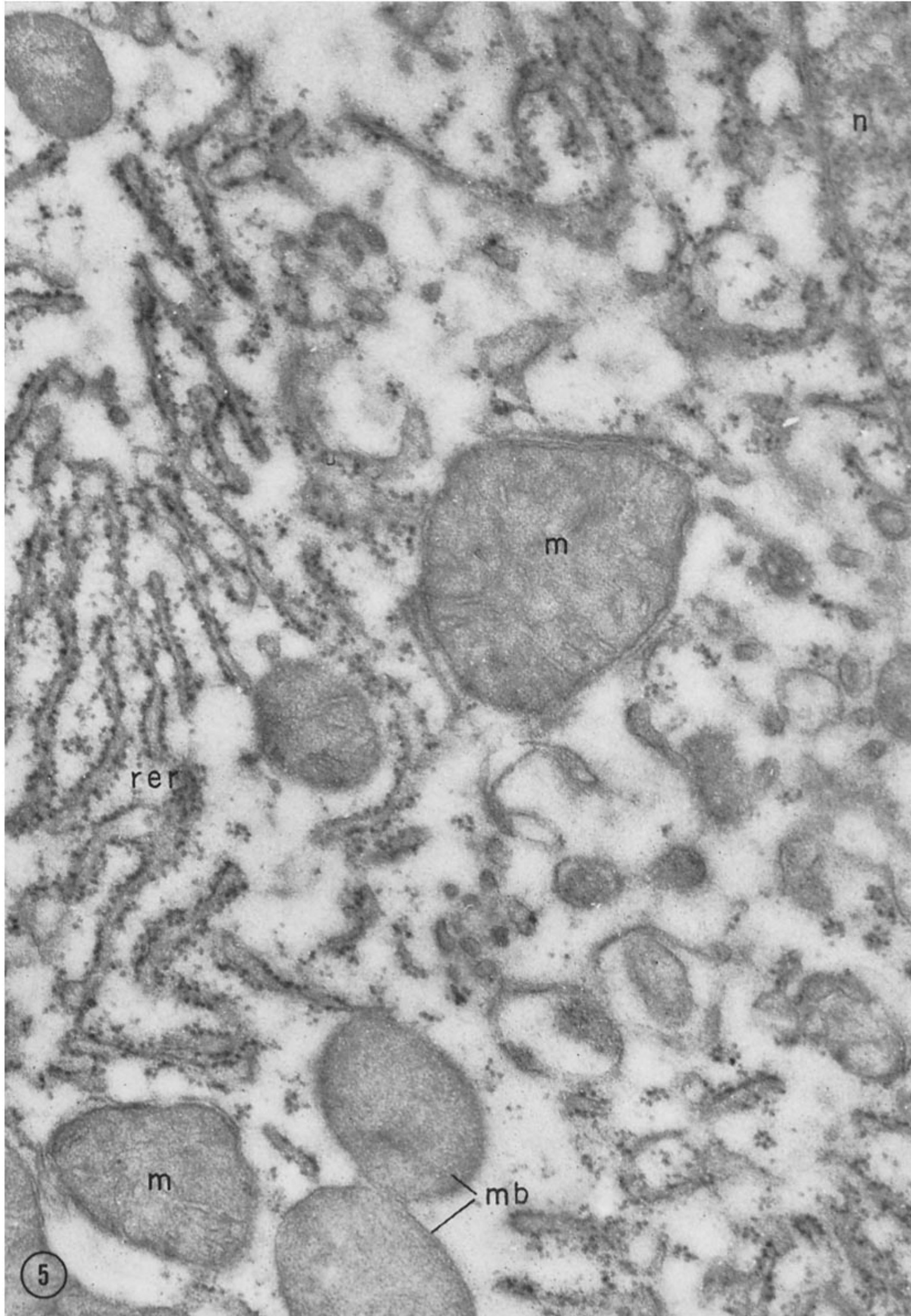
Figure 5

Detail of cytoplasm from a cell fixed in the same way as that shown in Fig. 4, to emphasize the excellent preservation of tissue fine structure obtained with the formol–phosphate–7.5 per cent sucrose fixative. The ribonucleoprotein particles of the rough surfaced endoplasmic reticulum (*rer*) are crisply defined. Microbodies (*mb*) and mitochondria (*m*) with cristae are also well preserved. × 50,000.


n
m
rer
m
mb
5

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