THE LOCALIZATION OF ACID PHOSPHATASE IN RAT LIVER CELLS AS REVEALED BY COMBINED CYTOCHEMICAL STAINING AND ELECTRON MICROSCOPY

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ABSTRACT

Discrete localization of stain in pericanalicular granules was found in 10 μ frozen sections of formol-phosphate-sucrose–fixed liver stained by the Gomori acid phosphatase technique and examined in the light microscope. The staining patterns, before and after treatment with Triton X-100 and lecithinase, were identical with those previously reported for formol-calcium–fixed material treated in the same way, and it can be assumed that the stained granules are identical with "lysosomes." Examination in the light microscope of the staining patterns and lead penetration in fixed blocks and slices of various dimensions showed nuclear staining and other artefacts to be present, produced by the different rates of penetration of the various components of the staining medium into the tissue. A uniform pericanalicular staining pattern could be obtained, however, with slices not more than 50 μ thick, into which the staining medium could penetrate rapidly from both faces. The staining pattern produced in 50 μ slices was the same both at pH 5.0 and pH 6.2, and was not altered by subsequent embedding of the stained material in butyl methacrylate. Electron microscopy showed the fine structure of fixed 50 μ frozen slices to be well preserved, but it deteriorated badly when they were incubated in the normal Gomori medium at pH 5.0 before postfixing in osmium tetroxide. After incubation in the Gomori medium at pH 6.2, the detailed morphology was substantially maintained. In both cases lead phosphate, the reaction product, was found in the pericanalicular regions of the cell, but only in the vacuolated dense bodies and never in the microbodies. Not every vacuolated dense body contained lead, and stained and unstained bodies were sometimes seen adjacent to each other. This heterogeneous distribution of stain within a morphologically homogeneous group of particles is consistent with de Duve's suggestion (9) that there is a heterogeneous distribution of enzymes within the lysosome population. It is concluded from these investigations that the vacuolated dense bodies seen in the electron microscope are the morphological counterparts of the "lysosomes" defined biochemically by de Duve.

INTRODUCTION

It is now generally accepted that a full understanding of cell physiology and biochemistry ultimately depends upon knowledge of the functional and spatial organization which exists within and between various subcellular elements. Current interest is therefore turning towards the

study of the localization of enzymes within components of the fine structure of intact cells.

Over the past twenty years, the localization of enzymes in cells has been intensively studied with the light microscope, using a variety of specific staining methods (see 36). Related methods for use with the electron microscope, however, are still in a very early stage of development. In fact, considerable pessimism has been felt concerning the successful extension of staining methods in this direction, for doubt still remains concerning the validity of many of the methods currently used for light microscopy. Moreover, much greater precision of localization is required for the electron microscope than is adequate for light microscopy.

In developing enzyme staining techniques for electron microscopy, a compromise must be sought between the conflicting requirements of adequate preservation of enzymic activity and of the fine structure of cells. The fine structure should survive incubation in the chemically and osmotically active staining media, while the enzymically active sites must be precisely delineated, but not obscured, by the deposition of an electron opaque stain.

These problems have been discussed in some detail by Barrnett and Palade (4) and by Barrnett (3), and there would be no purpose in merely reiterating their general appraisal of them, for each specific application of staining methods to electron microscopy undoubtedly requires individual consideration. It is our purpose to consider some of the developmental aspects more fully, with particular reference to the localization of acid phosphatase in rat liver parenchymal cells. The choice of this enzyme has been determined by the following considerations:

a) It is now firmly established that in the rat hepatic cell the acid phosphatase is mainly located in a special family of granules, termed "lysosomes" by de Duve and his associates (11).

b) Lysosomes are of current interest in the study of cell physiology because of their suggested participation in normal and pathological processes of autolysis and intracellular digestion (9).

c) The Gomori acid phosphatase technique, when applied to formol-calcium–fixed liver, results in discrete staining of peribiliary granules (18, 33) which respond to experimental treatments in the same way as isolated lysosome fractions of rat liver (18).

d) The resolution of the light microscope is not sufficient to identify these stained granules with any of the liver cell structures that can be seen in electron micrographs.

e) The Gomori staining procedure is suitable for use in conjunction with electron microscopy, since the primary reaction product, lead phosphate, has excellent electron-scattering properties, first demonstrated by Sheldon, Zetterqvist, and Brandes (40) in osmium-fixed mouse intestine. Unfortunately osmium fixation does not preserve more than 1 to 2 per cent of the acid phosphatase activity of rat liver (20, 32).

f) A method of formalin fixation for electron microscopy has already been developed (20) which preserves the fine structure of the liver cell, a high proportion of its acid phosphatase activity, and 97 per cent of its phospholipids, upon which the enzymic integrity of lysosomes depends (6, 18).

Amongst the inherent disadvantages of a Gomori type staining procedure for electron microscopy is the fact that the lead-containing reaction product can obscure the characteristic morphological features of the tissue underlying the stain, as has been observed by other authors (33). Another drawback to the normal Gomori procedure might be that, even after fixation, the acidic reaction (pH 5.0) of the staining medium would initiate the activity of cathepsins present within the lysosomes, thus causing damage to their structure. This follows from the suggestion that the fragility of isolated lysosomes at pH 5.0 and below is due to damage caused by the cathepsins (6).

In addition to modifying the basic Gomori staining procedure to counter some of its disadvantages, and to fulfill certain of the requirements for electron microscopy, a study has been made to determine the size and shape of tissue samples most suitable for staining purposes. Usually, small blocks of tissue are used, both for fixation and for staining. This results in differently fixed and differently stained zones in the tissue (13). Selection of the most reliable staining pattern from several types that may occur within one block can then rarely be made on an objective basis. On the other hand, it has been reported that frozen sections of tissue, into which staining solutions can rapidly penetrate, and throughout which uniform and satisfactory kinetic conditions of staining can be maintained (22), suffer considerable damage from the freezing, thawing, and incubation processes (13, 24).

As the result of the investigations reported here, a satisfactory staining method has been developed, for use with the electron microscope, for locating sites of acid phosphatase activity in rat liver cells.

MATERIALS AND METHODS

All reagents were of the same quality as described previously (18).

Tissue Sampling and Fixation: Small blocks (1 mm^3) of rat liver were obtained by exactly the same procedure as described in the preceding paper (20). They were immediately placed in ice cold 4 per cent formaldehyde containing 7.5 per cent sucrose and buffered at pH 7.2 with 0.067 M phosphate buffer, where they were allowed to fix for 24 hours at 0–2°C. For control studies of fine structure, the blocks were postfixed for 1 hour in Caulfield's modification (8) of Palade's fixative (34), buffered at pH 7.4.

For staining for light microscopy, the small blocks of formalin-fixed tissue were used without postfixation in osmium tetroxide, but in addition, larger blocks (approximately 8 × 6 × 3 mm) were fixed in the formol-phosphate-sucrose solution in the same way as the small blocks. Other liver samples were fixed in the formol-phosphate medium for 6 and 12 hours. After fixation, the tissues were removed, blotted, and transferred to ice cold gum sucrose (21) at the rate of 1 gm tissue per 100 ml, where they remained at the same temperature, with occasional stirring, for 24 hours.

For certain ancillary experiments, pieces of rat kidney and epididymis were fixed for 24 hours and treated in the same way as described for the liver.

Preparation of Tissues for Electron Microscopy: Small blocks of post-osmicated formalin-fixed control tissue, or blocks and slices stained as described below and then postfixed for 1 hour in osmium tetroxide, were dehydrated, embedded, sectioned, and examined as described previously (20).

Preparation of Staining Solutions: These were prepared as described by Gomori (15). To vary the pH of the staining media, the reaction of the lead nitrate stock solution was appropriately adjusted before mixing with the glycerophosphate, and the final pH value of the mixture always checked with a meter (19). All staining solutions were freshly prepared, since it has been shown that stale solutions give nuclear staining artefacts (18).

Staining Procedures: To establish suitable staining conditions for electron microscopy, a series of experiments was first carried out with the light microscope. Sections, 10 μ thick, of the formol-phosphate–fixed tissues were cut on a Leitz freezing microtome, model 1310, and incubated at 37°C for 5 to 60 minutes in freshly prepared Gomori staining media buffered at various pH values between 5.0 and 6.9. The sections were washed with water, then with dilute acetic acid, and treated with ammonium sulfide solution in the standard manner (15). They were mounted directly in glycerogel, or the nuclei were counterstained in Mayer's haemalum, and the sections dehydrated, cleared, and mounted in Canada balsam. Similar experiments were made with formalin-fixed rat kidney. Sections of epididymis were stained at pH 5.0 only. The effects of sucrose (7.5 to 30 per cent) and formaldehyde (1 to 4 per cent) on the staining patterns produced in rat liver at different pH values were also examined.

For electron microscopy, all stages in the staining operations were carried out at 0–2°C, except for the incubation period at 37°C. The small formol-phosphate–fixed blocks were given three successive washes of 5 minutes in 7.5 per cent sucrose. Some were then cut into tiny pieces whilst others were sectioned at 50 μ on the freezing microtome. The intact blocks, tiny pieces, and slices were incubated at 37°C for 1 to 30 minutes in staining media buffered at pH 5.0 and 6.2, with and without addition of 7.5 per cent sucrose. Where incubation and been carried out in the absence of sucrose, the incubated preparations were washed three times for 5 minutes with 0.05 M acetate buffer at the same pH as the staining medium. When sucrose was present in the incubation medium, the same concentration was used in the buffer wash. In other experiments of this type, 1 per cent formaldehyde was added to the incubation media and 4 per cent to the subsequent buffer washes. After such treatments, the preparations were postfixed in osmium tetroxide and prepared for examination in the electron microscope.

Extent of Staining in Tissue Blocks and Slices: The larger blocks fixed in formol-phosphate were trimmed into rectangular prisms about 6 × 4 × 1 mm in size. They were then incubated in the normal Gomori medium (pH 5.0) for 5 to 60 minutes while being agitated by magnetic stirring at 120 RPM. In other experiments of this type, stationary blocks were incubated both at pH 5.0 and at pH 6.2.

After washing three times for 5 minutes in 0.05 M acetate buffer of the same pH as the incubation medium, and then with water, the blocks were treated with dilute ammonium sulfide for 10 minutes. All these operations were carried out with stirring. The blocks were washed again with distilled water, after which slices 1 mm thick were cut from each end, in a plane perpendicular to the long axis. Frozen sections 5 and 20 μ thick were then cut in the same plane from the remaining piece of tissue, and mounted in glycerogel. The depth to which staining had occurred within the blocks was determined by measurements made on the 20 μ sections, using a filar micrometer eyepiece and a low power objective at a total magnification of 180. Readings were taken at four points along each of the stained edges of three separate sections, but ignoring the corners where

two zones of staining met. The bottom face of each stationary block was unstained.

Rectangular slices, 6 × 4 mm, of thickness 25, 50, 100, and 150 μ were cut on the freezing microtome from the larger formol-phosphate–fixed blocks that had been trimmed as described above. They were incubated at 37°C with occasional agitation for 5, 10, 15, 30, and 60 mintues in Gomori media at pH 5.0 and 6.2. They were then washed and treated with ammonium sulfide in the way described above for blocks, but the solutions were not stirred because of the fragility of the slices. Individual stained slices were embedded flat in 15 per cent gelatin, after which the gelatin block was trimmed to a rectangular shape leaving about 3 mm of gelatin on all sides of each slice. The blocks were hardened in 36 per cent formaldehyde for 12 hours, and washed overnight in running tap water. After washing, they were cut in such a way that the slices within them were halved along a plane perpendicular to their long axes. Frozen sections were then cut in the same plane at 5 and 20 μ thickness from the exposed edges of the central regions of the slices. Each gelatin section, supporting a peripherally stained 20 μ tissue section, the width of which corresponded to the thickness of the original slice taken, was mounted in glycerogel, and measurements of the stained zones were made as before. The 5 μ sections of the stained blocks and slices were used for detailed examination of the staining patterns in the light microscope.

Penetration of Lead into Blocks: Formol-phosphate–fixed blocks trimmed to 6 × 4 × 1 mm were incubated without stirring, for the same sequence of times as above, in Gomori media at pH 5.0 and 6.2 from which the glycerophosphate had been omitted.

After the appropriate time intervals, they were immediately transferred to solid carbon dioxide. The frozen blocks were attached to the chuck of a cryostat microtome so that their long axes were perpendicular to the plane of sectioning. The frozen tissue was allowed to warm up to −20°C, and about half the length of the block was cut away with a razor blade. Sections were then cut at 20 μ from the exposed central regions of the blocks. The frozen sections were dropped into a 2 per cent solution of ammonium sulfide in 70 per cent alcohol at −20°C, where they were allowed to remain for 5 minutes. They were subsequently washed in 70 per cent alcohol, passed through 50 and 30 per cent alcohols to water, and mounted in glycerogel. Measurements of the stained zones were made as described above.

Control Experiments: A number of relevant control experiments were carried out at the same time as the staining experiments.

a) The effects of freezing, cutting, and thawing were assessed by comparing the fine structure of small blocks and of frozen slices.

b) The effect on tissue preservation of incubation at 37°C was assessed by incubating the slices in the staining medium from which lead and glycerophosphate were omitted.

c) The extent of non-specific lead adsorption was

Figures 1 to 3 are light micrographs of frozen sections of rat liver stained by the Gomori procedure after formol-phosphate-sucrose fixation.

FIGURE 1

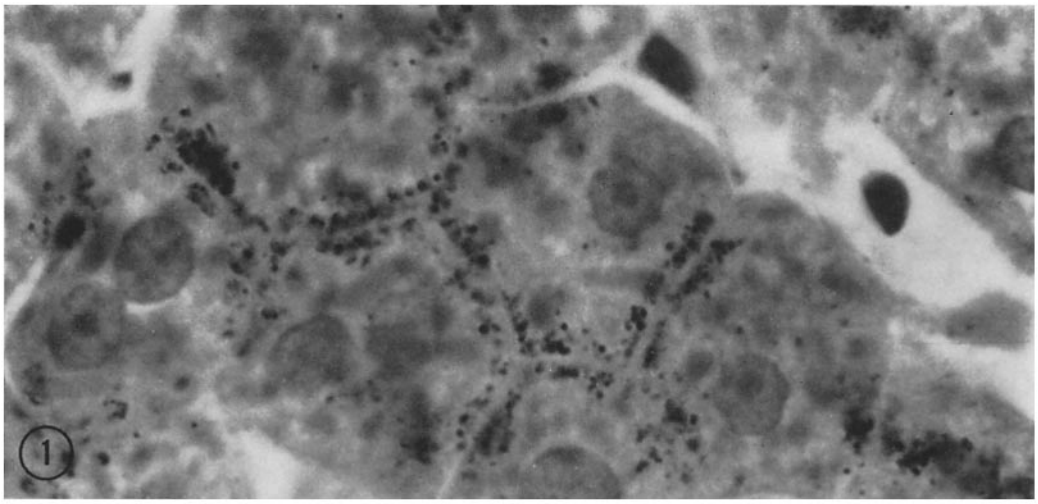
A 5 μ section of a gelatine-embedded 50 μ thick slice that had been stained for 15 minutes at pH 5.0. Stained granules, approximately 0.4 μ in diameter, are seen in the peribiliary cytoplasm of most of the cells in this field. Haemalum counterstain. × 1,500.

FIGURE 2

A 10 μ section of liver incubated for 1 hour at 37°C in a 0.1 per cent solution of Triton X-100 in 0.25 M sucrose, before staining for 15 minutes. The discrete stained granules of the type seen in Fig. 1 have disappeared and the peribiliary cytoplasm is diffusely stained. In some of the peribiliary areas there is the appearance of small unstained granules surrounded by diffusely staining material. Material which has been incubated with lecithinase, in place of Triton, has an identical appearance. × 1,500.

FIGURE 3

A 3 μ section after treatment with ammonium sulfide for 30 minutes, cut from a dehydrated, methacrylate-embedded 50 μ slice which had been incubated for 30 minutes in the Gomori medium at pH 6.2. The pericanalicular localization of stain in discrete granules has not been disturbed by the dehydration and embedding procedures. × 1,500.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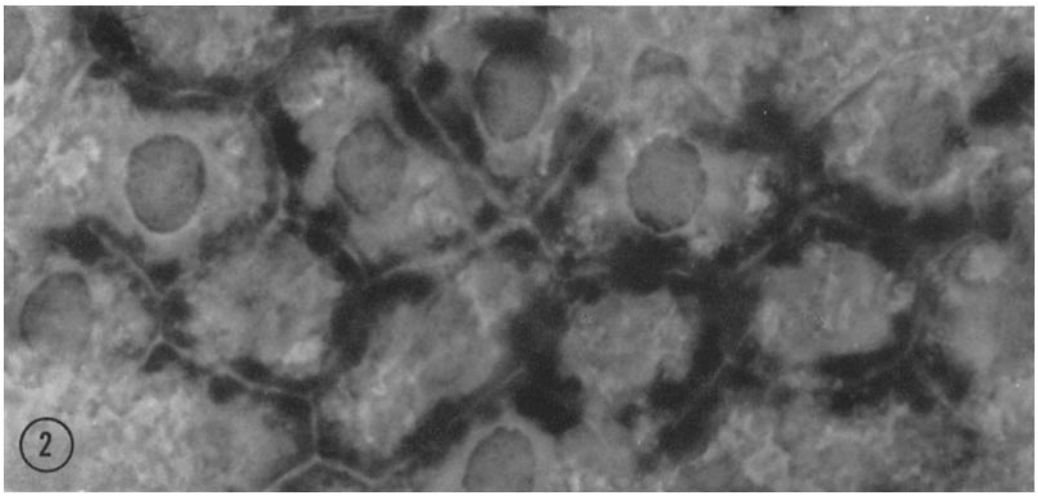
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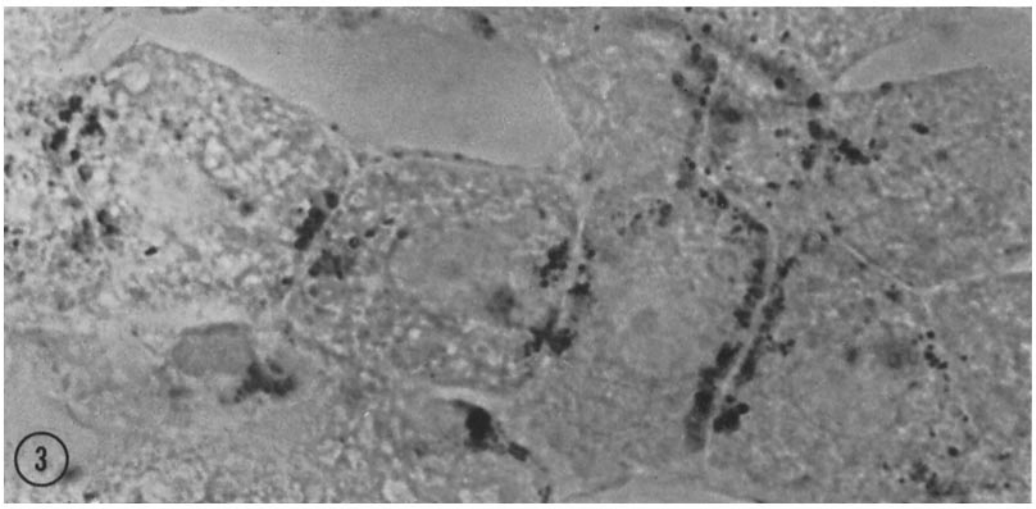
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determined in parallel with staining experiments, either by omitting glycerophosphate from the incubation medium, or by inhibiting the enzyme with 4 per cent formaldehyde (at pH 6.2 only) or with 0.01 M sodium fluoride.

d) The effects of methacrylate embedding upon the distribution of the lead salts deposited during staining were assessed by cutting 2 to 3 μ sections of embedded slices that had been incubated in the Gomori media for 15 to 30 minutes, and exposing them to the action of dilute ammonium sulfide for 30 minutes. The character of the staining pattern was compared in the light microscope with that seen in frozen sections stained in the usual way.

Ancillary Experiments: To assess the effects of lipid-disrupting procedures upon the distribution of acid phosphatase in formol-phosphate-sucrose–fixed tissue, 10 μ frozen sections were treated as follows: Some sections were incubated for 1 hour at 37°C in 0.1 per cent Triton X-100, and others in lecithinase solutions for the same time. The details of these procedures have been described previously (18). Similar experiments were done for control purposes in which the sections were treated with the Triton- or lecithinase-free media. The sections from the four experiments, and untreated sections, were separately incubated for 30 minutes at 37°C in the normal Gomori medium (15), washed, treated with dilute ammonium sulfide, and mounted in the usual way.

Optimal Staining Procedure for Electron Microscopy: The procedure finally selected as giving the most satisfactory staining results for electron microscopy is as follows:

1. Fix small blocks (1 mm^3) of liver for 24 hours at 0–2°C in 4 per cent formaldehyde containing 7.5 per cent sucrose and buffered with 0.067 M phosphate at pH 7.2.

2. Wash blocks three times for 5 minutes in 7.5 per cent sucrose at 0–2°C.

3. Cut 50 μ slices from the blocks on the freezing microtome.

4. Incubate slices for 10 to 15 minutes at 37°C in a Gomori staining medium buffered at pH 6.2 and containing 7.5 per cent sucrose and 1 per cent formaldehyde.

5. Wash slices three times at 0–2°C for 5 minutes in 7.5 per cent sucrose containing 4 per cent formaldehyde and buffered at pH 6.2 with 0.05 M acetate buffer.

6. Postfix washed slices for 1 hour at 0–2°C in Caulfield's osmium tetroxide–sucrose fixative (8) buffered at pH 7.4.

7. Dehydrate and embed in butyl methacrylate for electron microscopy.

Control slices are treated in the same way except that 4 per cent formaldehyde is used in stage 4.

RESULTS

Light Microscopy of Acid Phosphatase Staining Patterns in Formol-Phosphate-Sucrose–Fixed Tissues

Normal Staining Patterns: When 10 μ frozen sections of the fixed liver were stained for 15 minutes in the normal (pH 5.0) Gomori medium, a characteristic staining pattern of the type illustrated in Fig. 1 was obtained. The peribiliary distribution of stained granules was indistinguishable from that seen in formol-calcium–fixed liver (18). The staining pattern was substantially the same after fixation for 6, 12, and 24 hours, but with the shorter periods, the stain, though more intense, was also somewhat more diffuse.

No stain was deposited in liver sections that had been incubated in the Gomori medium from which glycerophosphate had been omitted, or to which 0.01 M sodium fluoride had been added.

After treatment with ammonium sulfide, sections cut from methacrylate-embedded liver which had previously been incubated in the Gomori medium showed that the normal pericanalicular staining pattern had not been altered by the dehydration and embedding procedure (Fig. 3).

In kidney, the staining of droplets in the cells of the proximal convoluted tubules was also the same as that previously found in formol-calcium–fixed material (18). In the epididymis there was strong staining in the Golgi region of the cells of the columnar epithelium lining the vasa efferentia and the ductus epididymis.

Effect of Treating Tissues with Triton X-100 or Lecithinase before Staining: A diffuse staining pattern was obtained with 10 μ frozen sections of the fixed liver and kidney after preliminary incubation for 1 hour at 37°C with 0.1 per cent Triton X-100 or with lecithinase solutions. In the case of the kidney, the droplets were mainly unstained, but surrounded with diffusely stained material which appeared to have been released from them. In the liver, the diffuse staining was spread throughout the cytoplasm in the immediate vicinity of the bile canaliculi (Fig. 2). When sections were incubated in control media from which Triton X-100 and lecithinase had been omitted, the subsequent staining patterns were normal. These results are identical with those previously obtained with formol-calcium–fixed tissues (18).

Effect of pH Changes on Staining Patterns: The rate

of staining in the fixed rat liver became progressively slower as the pH of the Gomori medium was raised in steps from 5 to 6.9. Longer staining times were therefore necessary at the higher pH values to obtain the same intensity of stain as that seen after 15 minutes incubation at pH 5.0, but the prolonged incubation did not alter the character of the staining pattern produced. In rat kidney, however, in which the activity of alkaline phosphatase is much greater than that of acid phosphatase (28), a change in the staining pattern could be seen as the pH of the staining solution was raised. In addition to staining of droplets in the cells of the proximal convoluted tubules, the brush border, which is the main site of alkaline phosphatase activity in the tubules (14), became stained above pH 6.5.

Effects of Adding Sucrose or Formalin to the Staining Medium: Addition of 7.5 per cent sucrose was without effect on the staining results obtained at pH 5.0 or 6.2, but with 30 per cent sucrose the intensity of stain was considerably reduced.

The tissue remained unstained at pH 6.2 after 30 minutes incubation in the presence of 4 per cent formaldehyde, while at pH 5.0, weak staining could be seen. There was still no staining reaction at pH 6.2 with 2 per cent formaldehyde even after incubating for 1 hour, but with 1 per cent, there was clear and typical staining of pericanalicular granules in the liver cells after incubation for 30 minutes.

Influence of Physical Dimensions of Tissue on Staining: Examination of frozen sections cut from liver blocks that had previously been stained at pH 5.0 and 6.2 revealed that only the peripheral zones of the blocks had stained. Within each such zone were three distinct staining patterns. The outermost layer of cells contained discretely stained peribiliary granules; farther in was a region of confused cytoplasmic and nuclear staining, and, finally, a layer of cells in which only nuclei were stained. Staining of bile capillaries was frequently encountered in the confused layer, especially in blocks that had been stained for 1 hour.

Columns 2 to 4 of Table I show the depths of staining into stationary and stirred blocks at pH 5.0, and into stationary blocks at pH 6.2, that occurred after various incubation times. It can be seen that the stained zones increase in width during the first 30 minutes, after which they remain fairly constant. There is very little difference between the widths of the stained zones in stirred and unstirred blocks. It is therefore not possible to stain beyond about 60 μ into each face of a block, irrespective of its size, and even then, heterogeneous staining patterns are produced.

Columns 5 and 6 of Table I shows corresponding results for the penetration of lead into the blocks. It would appear that lead enters the blocks by a process of passive diffusion, for, apart from a slight falling-off after 30 minutes incubation, the straight line plots of distance penetrated against the square root of the incubation time, at both pH 5.0 and pH 6.2 (Fig. 4), indicate that the penetration of lead obeys Fick's diffusion law.

When tissue slices were incubated in the staining media, the thicker slices (100 and 150 μ thick) showed a "tramline" staining effect, the centre of the slice being unstained. The distribution of stain in the two stained strips was exactly similar to that in the stained blocks of tissue. With slices 50 μ thick and less, however, the staining pattern was uniform (Fig. 1) and indistinguishable from that obtained by staining 10 μ sections.

TABLE I

*Distance Penetrated by Stain and Lead in Liver Blocks**

Incubation time (minutes)	Distance penetrated by stain (μ)			Distance penetrated by lead (μ)	
	pH 5.0 (stationary)	pH 5.0 (stirred)	pH 6.2 (stationary)	pH 5.0 (stationary)	pH 6.2 (stationary)
5	40.0 ± 2.0	49.8 ± 3.6	27.1 ± 3.1	53.3 ± 2.6	58.2 ± 2.8
10	45.0 ± 2.1	52.3 ± 2.4	35.0 ± 3.0	65.1 ± 3.9	80.5 ± 3.2
15	46.7 ± 3.2	52.4 ± 1.8	38.4 ± 2.9	77.2 ± 4.5	94.1 ± 4.1
30	47.0 ± 3.0	54.3 ± 3.4	46.5 ± 2.9	96.6 ± 4.6	131 ± 4.2
60	47.4 ± 2.9	54.7 ± 3.5	47.8 ± 3.0	120 ± 4.7	174 ± 4.6

* Results are expressed in terms of the mean of 36 measurements (stationary blocks) or of 48 measurements (stirred blocks) and the standard deviation of the mean.

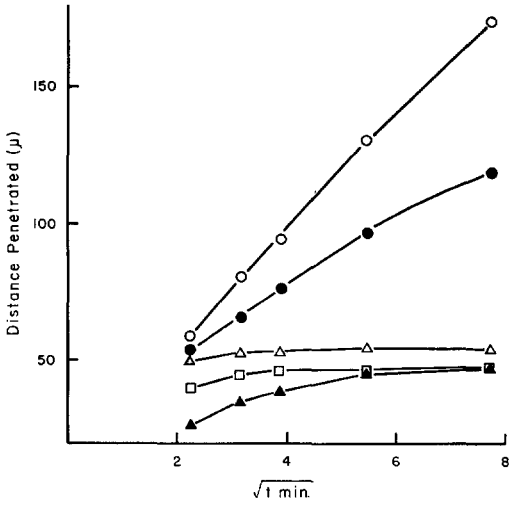


FIGURE 4

Distance (μ) penetrated at 37°C into tissue blocks by stain or by lead, plotted against the square root of the incubation time t (minutes). ○ lead, pH 6.2; ● lead, pH 5.0; △ stain, pH 5.0 (stirred); □ stain, pH 5.0; ▲ stain, pH 6.2.

Electron Microscopy

Morphology of Unstained Tissues: All the features of the liver parenchymal cell normally seen in osmium-fixed material (20, 35, 37) are well preserved by formol-phosphate-sucrose fixation. A small field showing parts of three contiguous liver cells is shown in Fig. 5. Mitochondria, rough and smooth surfaced endoplasmic reticulum, and components of the Golgi zone can be seen. In addition, a number of vacuolated dense bodies (20, 35, 37) are present, as well as two microbodies or cytosomes (37, 38). At higher magnification (Fig. 6) the microbodies show an eccentrically placed "nucleoid" with a lamellar structure (37, 38). Where the plane of sectioning does not pass through the nucleoid, they appear to have a homogeneous dense matrix, but are still easily distinguishable from other structures of similar size, such as those seen in Figs. 7 and 8. Fig. 7 shows four large vesicles or cisternae which are undoubtedly part of the Golgi complex, containing dense globular masses of material, which present an appearance similar to that previously observed in embryonic chick liver (25) and in barium hydroxide–stained rat liver (42). Two vacuolated dense bodies containing a heterogeneous matrix, and bounded by a single membrane, are shown in Fig. 8. Dense bodies containing ferritin-like grains (5, 31) were only occasionally seen.

After freezing and cutting into 50 μ slices, the fine structure of this formol-phosphate-sucrose-fixed tissue remained well preserved. Even when the slices were incubated at 37°C for 15 minutes in acetate buffer at pH 6.2, the fine structure was well maintained (Fig. 9); both the rough and smooth surfaced endoplasmic reticulum present a normal appearance, as do the other cytoplasmic structures described above.

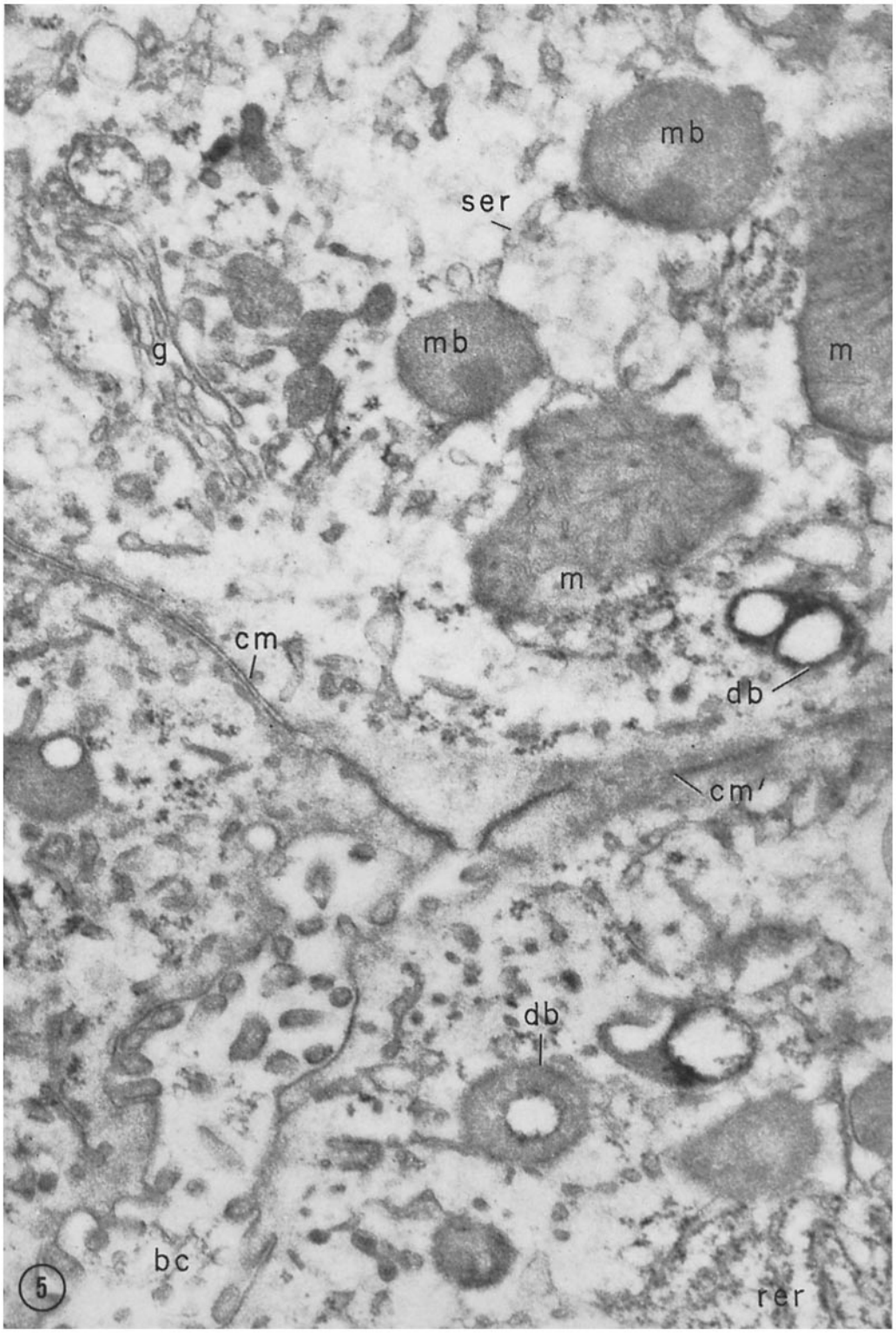
Effect of the Staining Medium upon the Fine Structure of Cells in Tissue Slices: In this section, only the effects on cellular morphology of the staining processes are considered, and not the staining patterns obtained.

After incubation of 50 μ frozen slices of the fixed liver in the normal Gomori medium (pH 5.0) for 15 minutes, extensive damage occurred to the tissue fine structure (Fig. 10). The tissue shows considerable loss of cytoplasmic continuity, but nevertheless, mitochondria, microbodies, and vacuolated dense bodies can still be identified. When the pH of the staining medium was raised to 6.2, preservation of fine structure was greatly

Figures 5 to 14 are electron micrographs of rat liver cells from tissue fixed in formol-phosphate-sucrose, and then postfixed in osmium tetroxide. Figs. 9 to 14 refer to 50 μ frozen slices of the formalin-fixed material, and in Figs. 10 to 14, staining for acid phosphatase has been interposed between the two periods of fixation.

FIGURE 5

Portions of three cells are present in the field; the opposing cell membranes are cut obliquely at *cm'*, and transversely at *cm*, and a bile canaliculus (*bc*) separates the two cells occupying the lower half of the field. A number of vacuolated dense bodies (*db*) are present, as well as two microbodies (*mb*), mitochondria (*m*), rough surfaced endoplasmic reticulum (*rer*), smooth surfaced endoplasmic reticulum (*ser*), and components of the Golgi zone (*g*). × 37,500.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improved, but when 7.5 per cent sucrose and 1 per cent formaldehyde were added to this staining medium, the results were consistently better (Fig. 11). There is no discontinuity or vacuolation of the cytoplasm, and the Golgi elements, which were not well preserved after incubation in the normal Gomori medium (Fig. 10), have much the same appearance as those seen in incubated control slices (Fig. 9).

Localization of Stain: Examination of stained blocks in the electron microscope confirmed the result previously found in the light microscope, that different staining patterns occurred from the surface of the blocks inwards. Since light microscopy had also shown that a uniform staining pattern can be obtained with 50 μ frozen slices, the use of stained blocks was discontinued.

When glycerophosphate was omitted from the staining medium, or acid phosphatase activity inhibited by inclusion of 4 per cent formaldehyde, there was no specific staining of cytoplasmic structures apart from slight staining of the ribonucleoprotein particles of the rough surfaced endoplasmic reticulum. However, all material incubated in lead-containing media showed slight residual non-specific lead deposits and weak nuclear staining. This effect was more marked at pH 6.2 than at pH 5.0 (*cf.* Fig. 14 with Fig. 10).

After brief incubation of the 50 μ slices in the normal Gomori medium at pH 5.0, lead phosphate was deposited in many, but not all, of the peribiliary dense bodies (Fig. 10). Stained microbodies were never seen. When stained for 15 minutes in the medium containing 7.5 per cent sucrose and 1 per cent formaldehyde, buffered at pH 6.2, deposits of lead phosphate were again localized only in vacuolated dense bodies near the bile canaliculi (Figs. 11 to 14.)

DISCUSSION

The acid phosphatase-containing lysosomes of rat liver have been well characterized biochemically, although the data do not unequivocally point to the existence of a single homogeneous class of particle (11). From the morphological point of view, identification of lysosomes rests upon a less firm basis, for examination of isolated lysosome fractions, in the electron microscope reveals the presence of more than one component (5, 31). Both microbodies and vacuolated dense bodies are present, and each of these morphological

FIGURE 6

A microbody surrounded by tubules of smooth surfaced endoplasmic reticulum. The single limiting membrane and eccentrically placed, lamellar nucleoid are clearly shown. × 60,000.

FIGURE 7

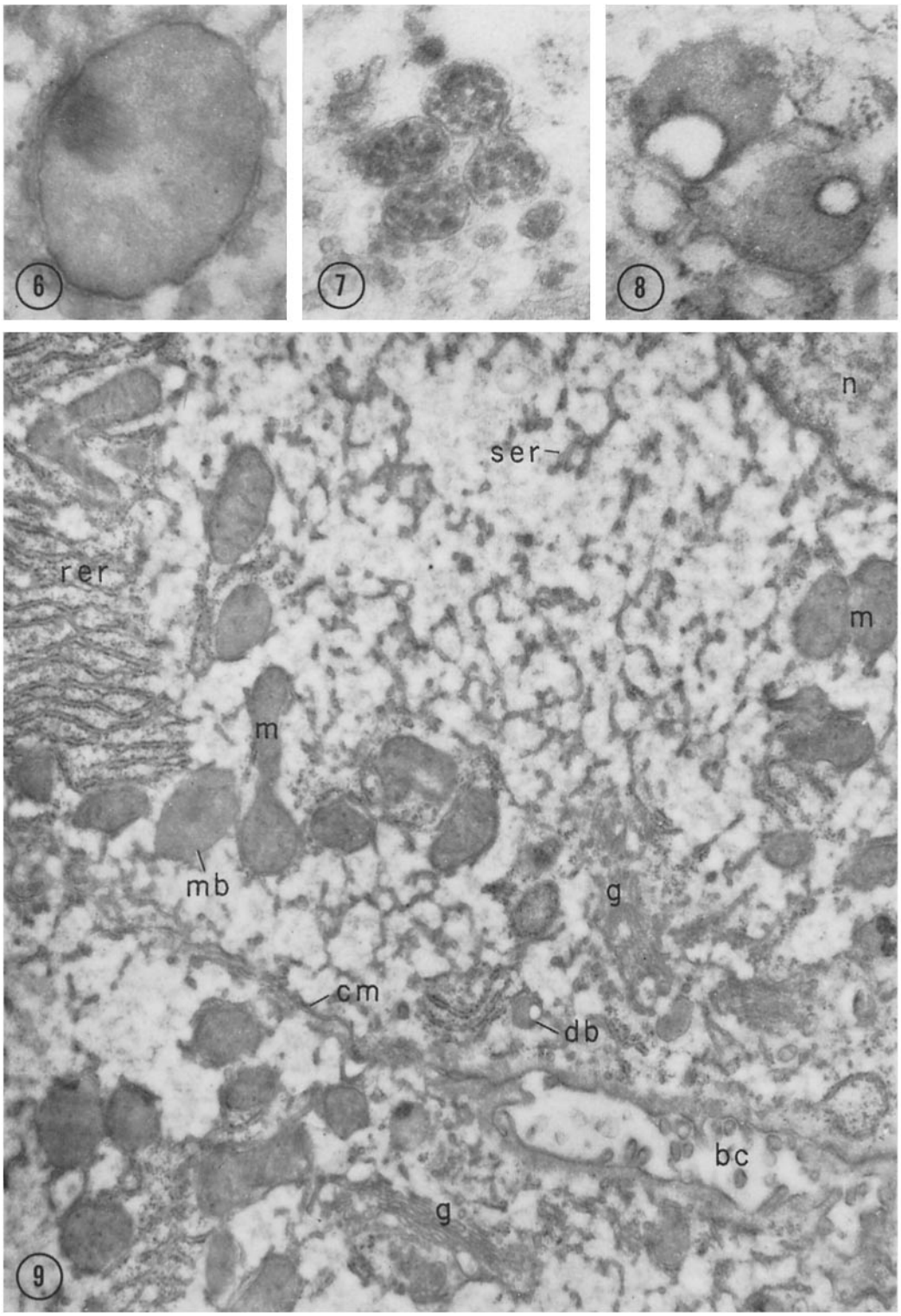
A cross-section through the Golgi region of the cell showing four large cisternae with dense non-homogeneous contents. The two cisternae at the right of the figure have a continuous limiting membrane and are connected by a narrow neck. Profiles of smooth surfaced tubules and vesicles can also be seen. × 60,000.

FIGURE 8

Two vacuolated dense bodies, containing an irregularly dense matrix bounded by a single membrane, are surrounded by elements of rough surfaced endoplasmic reticulum. × 50,000.

FIGURE 9

Part of a cell which, after treatment with formol-phosphate-sucrose fixative, was incubated for 15 minutes at 37°C in 0.05 M acetate buffer, pH 6.2, before fixation in osmium tetroxide. The nucleus (*n*) is at the top right, with the cell membrane (*cm*) and a bile canaliculus (*bc*) passing diagonally across the lower part of the field. Tubules of smooth surfaced endoplasmic reticulum (*ser*), the components of the Golgi zone (*g*), and rough surfaced endoplasmic reticulum (*rer*) are all well preserved. A microbody (*mb*), a small vacuolated dense body (*db*), and mitochondria (*m*) can also be seen. × 22,500.


6
7
8
n
ser
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9

types could correspond to the biochemical concept of the lysosome. De Duve and coworkers (5, 31) place some emphasis on the association of ferritin with some of the dense bodies of the lysosome fraction. Since ferritin-containing structures occurred with a very low frequency in the experimental animals used for the present work, it seems possible that this discrepancy reflects differences in the iron content of the diet or in the strains of animal used. Reports of the cytochemical demonstration of lysosomes in the electron microscope have not, so far, provided unequivocal identification of the structures involved (12, 30, 33), but the results were obtained by application of the Gomori staining method to formol-calcium–fixed blocks of tissue.

Although valid and precise localization of acid phosphatase activity can be obtained at the light microscope level by application of the Gomori technique to frozen sections of formol-calcium–fixed rat liver and kidney (18), it has been shown that this type of fixation gives relatively poor preservation of tissue fine structure (20). In the present series of experiments, use was made of tissue preserved in a formol-phosphate-sucrose fixative. This fixative has been shown to give preservation of tissue fine structure as good as that given by osmium tetroxide alone and to preserve acid phosphatase activity better than formol-calcium fixation (20). Furthermore, it is at least as efficient as formol-calcium (2) in preserving the lipid integrity of acid phosphatase-containing structures, as was confirmed by the change in staining patterns obtained by treating the tissue with Triton X-100 or lecithinase before staining, and the quantitative lipid analyses previously reported (20).

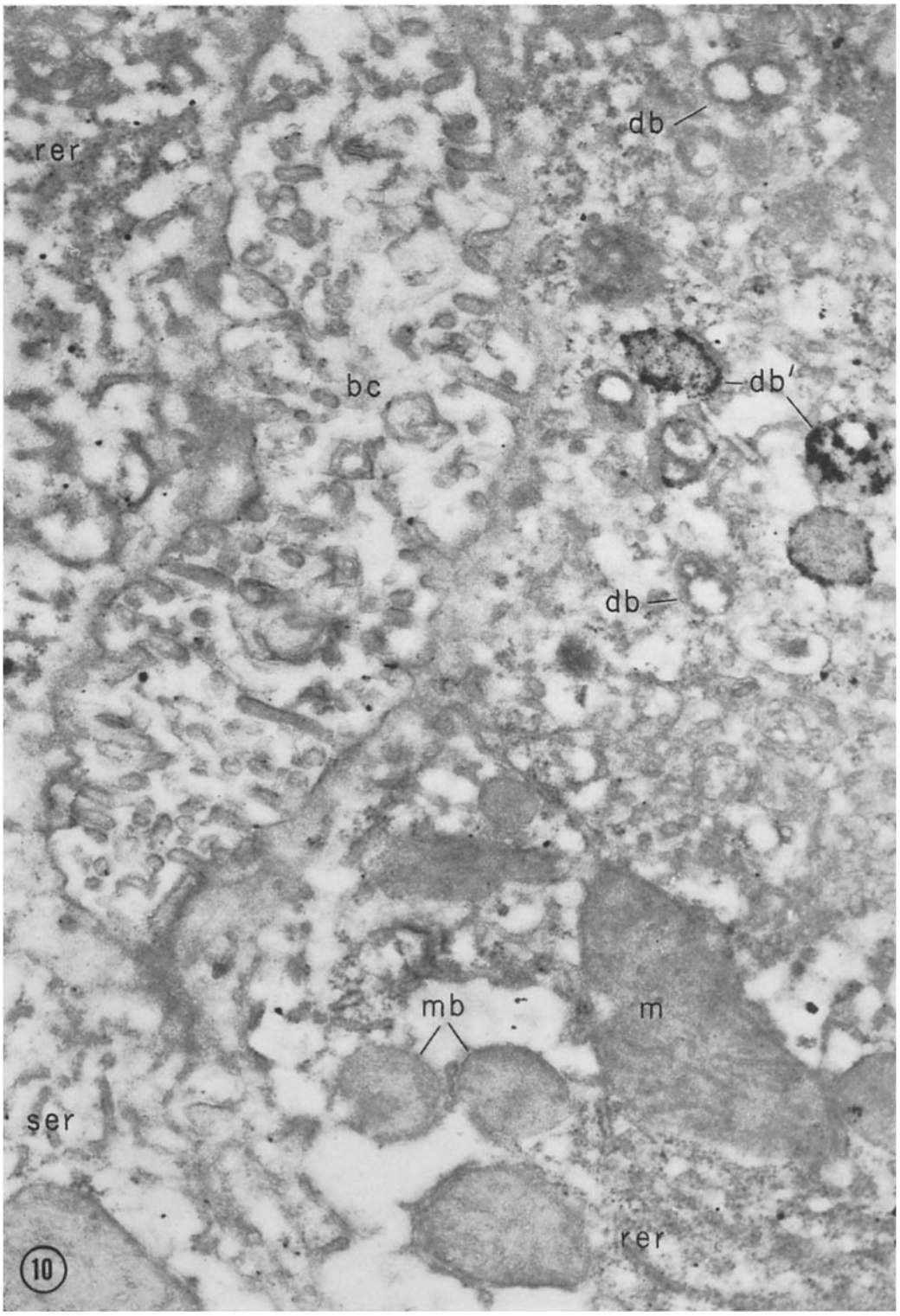
Some of the disadvantages of using blocks for staining purposes have previously been discussed (13). The present finding that discrete localization of stain is restricted to the outermost layer of cells, and that multiple artefacts are present farther in, clearly confirms that there are difficulties involved in using blocks for this type of work. No increase in the depth or uniformity of staining was obtained by stirring, although an improvement might have been expected by analogy with the increased rate of penetration of dye into textile fibres produced by stirring, which continually replaces exhausted solutions at the fibre surface with new dyestuff (41).

The unhindered passive diffusion of lead into the deeper layers of blocks showed that the solid deposits of lead phosphate offered no resistance to the entry of this component of the staining medium. Unfortunately, a technique suitable for studying the penetration of glycerophosphate could not be developed. If there is a variation in the ratio of the components of the incubation medium as they reach different levels in the block, zones of confused staining and nuclear staining would be expected, since the correct ratio is critical for valid results to be obtained with the Gomori procedure (18). That the course of staining does not follow simple diffusion laws is illustrated by the results shown in Fig. 4, although the equation expressing the relationship between the distance stained and time of staining is more complex than the one used here (22). It seems likely that the heterogeneous staining patterns and asymptotic course of staining which occur in blocks are, in fact, due to different rates of penetration of the components of the staining medium. The thickness of the tissue, and not the time of incubation, is therefore the controlling factor in the useful application of the Gomori staining procedure.

The maximum thickness of tissues throughout which a uniform and valid staining pattern would be expected can be judged to be about twice the width of the outer, discretely stained zone seen in blocks. The experiments with slices of different thicknesses confirmed this, for only slices 50 μ thick or less were uniformly stained. This result is also

FIGURE 10

Two adjoining cells separated by a bile canaliculus (*bc*) to show the effect of incubation for 15 minutes at 37°C in the normal Gomori medium at pH 5.0. The tissue is only moderately well preserved, there being considerable loss of cytoplasmic continuity; nevertheless, two microbodies (*mb*), mitochondria (*m*), and elements of rough (*rer*) and smooth surfaced (*ser*) endoplasmic reticulum can still be identified. A number of vacuolated dense bodies containing heavy deposits of lead phosphate (*db'*) are present, as well as others that have not been stained (*db*). × 37,500.


rer
db
bc
db'
db
mb
m
ser
rer
10

consistent with that of calculations which indicate that staining equilibrium would rapidly be established in slices of this thickness, for, assuming that the diffusion coefficients of the components of the Gomori medium are about 10^{-6} cm^2 sec^{-1}, and that they penetrate into both faces of the slice, the concentrations in the central plane would reach 90 per cent of those in the incubation medium in about 6 seconds (22).

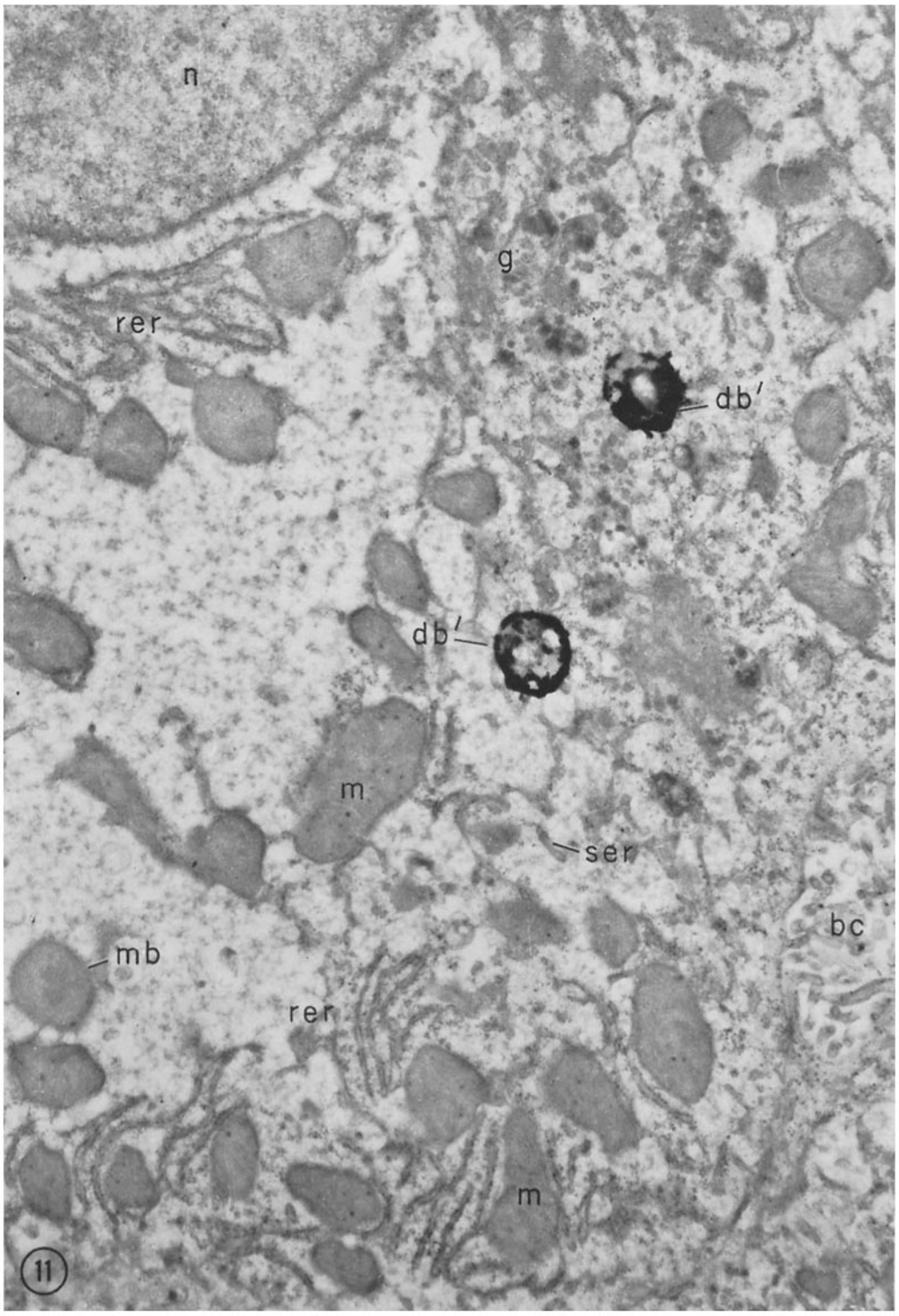
Staining of the 50 μ frozen slices under the optimal experimental conditions showed the lead phosphate product of acid phosphatase activity to be confined to a single type of cytoplasmic structure, namely, the vacuolated dense bodies. There can be no confusion between the dense masses of lead deposited in these structures as the result of enzymic activity, and the slight and irregular distribution of a granular lead deposit that was seen both in the nuclei and in the cytoplasm, together with faint staining of the ribonucleoprotein particles. This non-specific deposit was also seen in the control slices incubated in the staining medium from which glycerophosphate had been omitted, or to which 4 per cent formaldehyde had been added, and the effect was more pronounced at pH 6.2 than at pH 5.0. It is probable that this non-specific staining is related to the effects seen when tissue sections are treated with lead hydroxide (42) and is unrelated to sites of acid phosphatase activity. Non-specific lead adsorption has previously been discussed in some detail by Newman, Kabat, and Wolf (29), who found it to be greatest at pH 5.3–5.6. In the present case, the improvement in tissue preparation obtained by staining at pH 6.2 outweighed the disadvantage of an increased non-enzymic deposition of lead.

Some doubt might be felt about accepting the staining patterns produced in liver at pH values as high as 6.2, but the distribution of stain, as seen in the light microscope, appeared to be the same over the pH range 5.0–6.9. Moreover, examination in the electron microscope showed that the same cytoplasmic structures were stained at both pH 5.0 and pH 6.2, and that no other sites of activity were revealed at the lower pH level. On the other hand, rat kidney sections stained in the same way showed slight staining of the brush border of the proximal convoluted tubules at pH 6.5 and above, which suggests participation of alkaline or other phosphatases in the hydrolysis of the substrate. In view of this, routine staining for electron microscopy was not done at pH values higher than 6.2, even though, in rat liver, the ratio of alkaline to acid phosphatase activity towards glycerophosphate is about one-tenth of that in the kidney (28).

By staining at pH 6.2 it was possible to avoid, to a large extent, the damage to tissue fine structure which occurs when slices are incubated at pH 5.0. The damage found at the lower pH may be due not only to the acidity of the medium (27), but also to catheptic activity, which is negligible at the higher pH value. Further improvements were also obtained by addition of sucrose and formalin to the staining medium. Although addition of 7.5 per cent sucrose produced no detectable effect in the light microscope, examination by electron microscopy showed that damage to fine structure during incubation was reduced, and this may be the result of maintaining a constant tonicity, with respect to sucrose, throughout the fixing, washing, and staining procedures. The addition of formalin to staining media has been reported to help in maintaining tissue fine structure, (27), and the present series of experiments confirmed this observation. On the other hand, acid phosphatase activity is seriously reduced by formalin at low pH levels, but less seriously at pH 6.2 (16). The current findings indicate that, even at the higher pH, the activity of the enzyme is grossly inhibited when incubated

FIGURE 11

Part of a cell which has been incubated in the modified Gomori medium containing 7.5 per cent sucrose and 1 per cent formaldehyde, and buffered to pH 6.2. Two vacuolated dense bodies (*db'*), heavily stained with lead phosphate, lie in the cytoplasm between the nucleus (*n*) at the top left and a bile canaliculus (*bc*) at the bottom right of the field; an unstained microbody (*mb*) is also present. Despite the incubation, the tissue shows only slight deterioration. Mitochondria (*m*), rough surfaced endoplasmic reticulum (*rer*), smooth surfaced endoplasmic reticulum (*ser*), and the tubules and vesicles of the Golgi region (*g*) are all well preserved. × 22,500.


n
g
rer
db'
db'
m
ser
bc
mb
rer
m
11

in the presence of formaldehyde at concentrations of 2 per cent and above. The slight loss of activity produced by 1 per cent formaldehyde was considered to be less important than the improvement in tissue preservation obtained with this concentration.

Incubation times which give staining intensities suitable for light microscopy are not necessarily the most suitable for use with the electron microscope. For example, with the Gomori technique, sections incubated for times appropriate to light microscopy contain lead deposits which completely obscure the nature of the underlying enzyme-containing fine structure. Conversely, sections stained for shorter periods of time for electron microscopy showed only a faint, but still characteristic, staining pattern when examined in the light microscope after conversion of the lead phosphate to lead sulfide. As expected, butyl methacrylate embedding did not displace the stain from its normal location in pericanalicular granules.

The major findings which resulted from application of the staining procedure finally selected were that the vacuolated dense bodies were the only sites of lead phosphate deposition, and that on no occasion was lead found within microbodies, either at pH 6.2 or even at pH 5.0. Many sections of liver taken from different animals were surveyed before these conclusions were reached. Although the vacuolated dense bodies were the only type of structure to be stained in these experiments, the fact that lead was not deposited within all of them suggests that there is a heterogeneous distribution of acid phosphatase within this morphologically homogeneous group. It is of interest that de Duve and his coworkers (9) found, after rupture of the lipoprotein membrane of lysosomes, that release of all their hydrolases occurred in an almost perfectly parallel fashion, but that their distribution throughout the lysosome fraction, whether determined on the basis of sedimentation rates or of density, was not identical. He has interpreted this to mean that the lysosomes comprise a single population of particles within which variations in enzymic content are correlated with the size and density of the particles, but not with their sensitivity to rupturing agents. The variation in the size of the vacuolated dense bodies, and the heterogeneous deposition of lead amongst them, seen in stained sections by electron microscopy, is in accordance with this interpretation. It can be concluded that the pericanalicular vacuolated dense bodies are the morphological counterpart of the lysosomes. The microbodies appear to be a separate and distinct morphological type which may contain a different complement of enzymes.

These conclusions are in agreement with a preliminary identification of lysosomes by Novikoff, Beaufay, and de Duve (31), but are not consistent with a later view of Novikoff and Essner

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The tissue illustrated in Figs. 12, 13, and 14 was stained at pH 6.2 in the same way as that shown in Fig. 11.

FIGURE 12

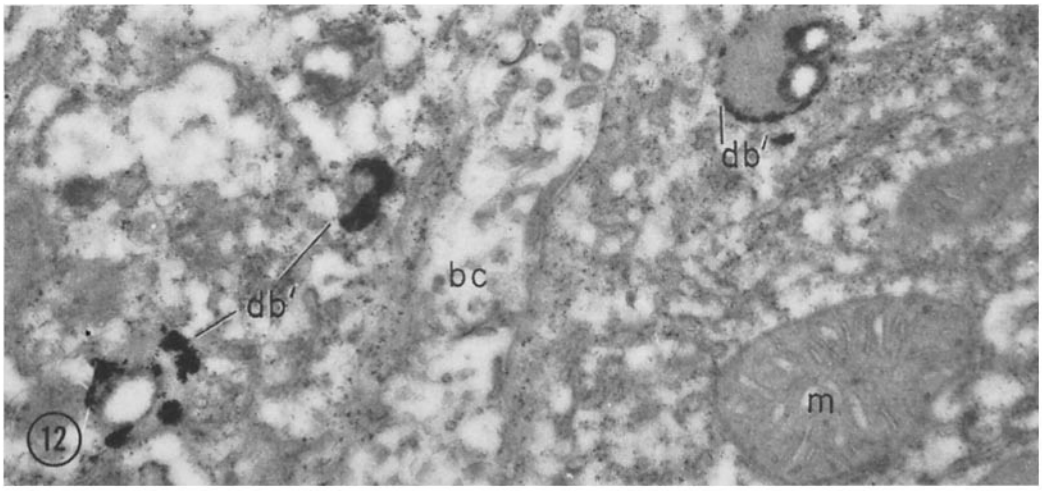
Three dense bodies (*db'*), showing deposits of the phosphatase reaction product, are present in adjacent cells separated by a bile canaliculus (*bc*). The stain is confined to the edge of the one on the right, but lies within the matrix of the larger one on the left. Only a small portion of the dense body nearest to the bile canaliculus is included in this section. A mitochondrion (*m*) is in the bottom right of the field. × 30,000.

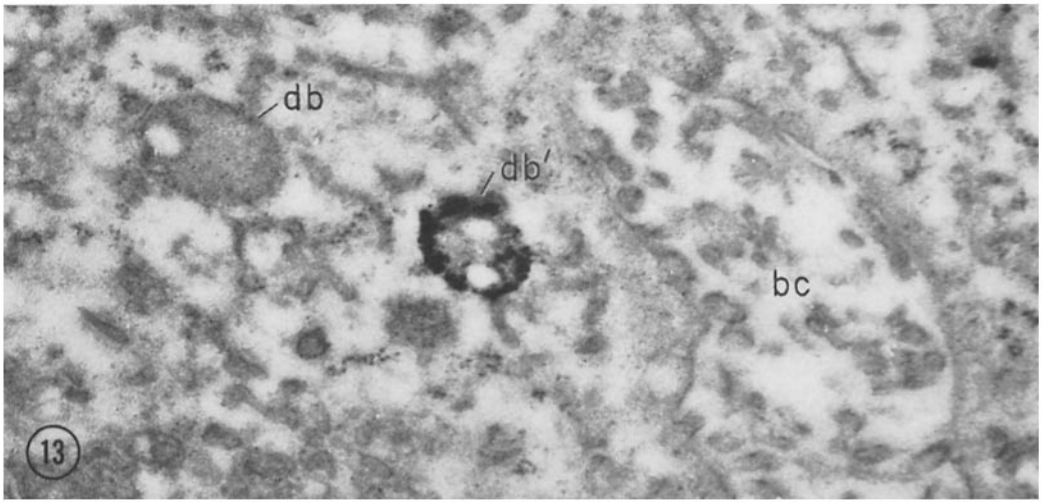
FIGURE 13

Detail of peripheral cytoplasm of two cells; to the left of the bile canaliculus (*bc*) are two vacuolated dense bodies, one of which (*db'*) contains deposits of lead phosphate, while the other (*db*) is unstained. × 30,000.

FIGURE 14

Two vacuolated dense bodies (*db'*) are shown, heavily stained with lead phosphate, adjacent to a bile canaliculus (*bc*). The lead deposit is within the matrix of these structures. × 30,000.


db'
bc
db'
m
12


db
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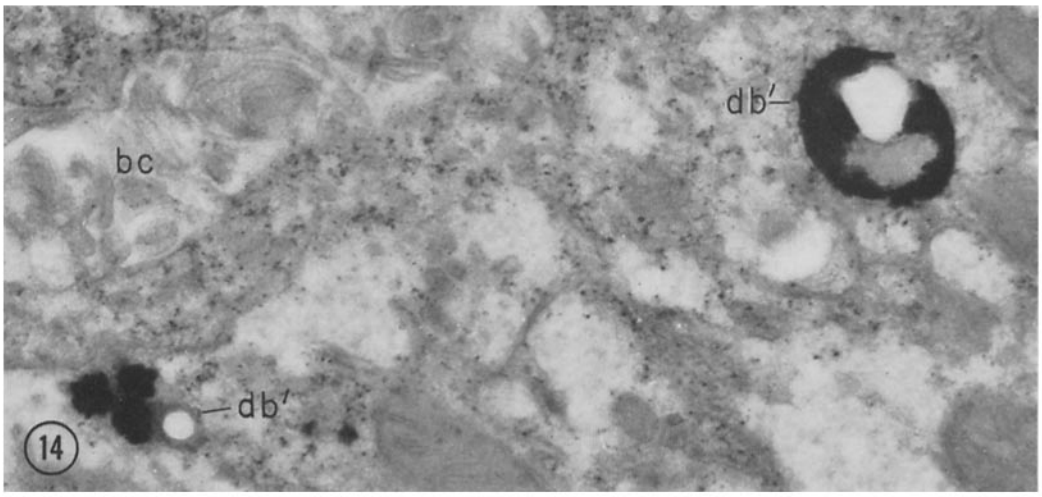
db'
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14

(33) that "most, if not all, microbodies are lysosomes." However, this refers to bilirubin-infused rat liver, and these authors also point out that size and distribution were the only basis of identification in their preparation, since the accumulated reaction product obscured the fine structure in liver stained for acid phosphatase activity. The conviction has also been expressed (30) that most bodies described by electron microscopists as microbodies (38), cytosomes (37), and large granules, are lysosomes possessing high levels of acid phosphatase activity. More consistent with the present results is the suggestion of Beaufay (5) that the microbodies may represent another newly identified group of particles containing uricase, D-aminoacid oxidase, and catalase (10).

Before the conclusions drawn from the present study can be accepted, it is necessary to return to the question of the validity of the staining method used. Inherent in all cytochemical staining techniques, where the procedure is applied to previously fixed material, is the difficulty of preserving the total enzymic activity. In the present case, 50 to 60 per cent of the total acid phosphatase activity was retained (20) and was demonstrated by incubation for short periods only, in a medium 1 pH unit removed from the optimum for the enzyme. It is obvious that, under these conditions of reduced activity, detectable amounts of reaction product may occur only at the sites which possess the highest concentrations of enzyme. It may well be asked, therefore, whether it is valid to conclude that the enzyme is present only in those structures in which lead is deposited. While there can be little doubt that the distribution of the enzyme throughout the population of vacuolated dense bodies is irregular, we would hesitate to claim that the unstained members of this group were devoid of acid phosphatase. After shorter periods of fixation, however, which are known to preserve a higher proportion of enzymic activity (39), there appeared to be no increase in the number of stained particles, but only in the rapidity of staining. It is possible, of course, that there may be differential inhibition of acid phosphatase activity during fixation, associated with different cytoplasmic sites, for more than one acid phosphatase is present in rat liver and these are known to have different susceptibilities to inhibition by formaldehyde (16). What can be said with confidence, however, is that application of the modified Gomori technique to rat liver, both at pH 5.0 and at pH 6.2, provides positive evidence for the association of acid phosphatase activity with vacuolated dense bodies, but no evidence for its association with other structures, such as the microbodies or the Golgi system. The latter might be a likely site for the enzyme, by analogy with its association with the Golgi membranes isolated from rat epididymis (26) and because some of the larger Golgi vesicles are similar in size to pericanalicular dense bodies. It is significant, however, that the Golgi zones of epithelial cells in the epididymis give a strong reaction when stained by the Gomori technique, whereas the reaction was negative in the case of the liver.

Some further comments may be made concerning the apparently irregular distribution of acid phosphatase amongst the vacuolated dense bodies. It is tempting to suggest that the stained and unstained individuals represent different stages in the development or in the functional state of lysosomes. If, as has been suggested (7, 9), one of the major roles played by lysosomes is participation in phagocytic processes, there seem to be different degrees of "readiness" in different cells for such activity. At one extreme is the example of the mature polymorphonuclear leucocyte, containing preformed granules with acid phosphatase and other hydrolase activities, which do not appear to be replenished after being discharged in response to phagocytosis of bacteria, etc. (17). Probably at the other extreme lies the ameba, for it has been suggested (23) that digestive enzymes are secreted into pinocytosis vacuoles containing protein in solution, and into food vacuoles containing ingested material. Acid phosphatase has been demonstrated in these structures by use of the Gomori staining technique (30), but possibly more significant is the observation that although very little acid phosphatase can be demonstrated by staining methods in starved *Amoeba proteus* and *Chaos chaos*, there is a very rapid appearance of the enzyme in newly formed food vacuoles after feeding (1). The relationship of the liver lysosome to these extremes is still a matter for speculation, for it is not known whether the enzymes and the morphological structure are produced simultaneously by the hepatic cell, or whether an existing morphological structure subsequently acquires, or segregates, hydrolases formed elsewhere in the cell. In view of the complexity of the system, it is therefore not possible to conclude at present

whether or not the degree of staining of the liver lysosome is related to its stage of development.

Note Added in Proof: Since submitting this paper for publication, Essner and Novikoff (*J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 773) have again reported on the results of staining formol-calcium–fixed blocks of liver by the Gomori acid phosphatase technique. They remark that an increase in the number of acid phosphatase positive bodies in stained bilirubin-infused rat liver is paralleled by an increase in the number of microbodies as seen in unstained control tissue, and they conclude that these microbodies are lysosomes. It must be noted that this conclusion is based upon a numerical correlation and not upon a definitive identification of the morphology of the stained particles. Although this correlation may be valid for bilirubin-infused animals, we have never detected stained microbodies with our normal rats.

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