INTRACELLULAR LOCALIZATION OF ACID PHOSPHATASE

A COMPARATIVE STUDY OF BIOCHEMICAL AND HISTOCHEMICAL METHODS*

BY G. E. PALADE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATES 36 TO 38

(Received for publication, August 23, 1951)

In a preceding article (1), the distribution of acid phosphatase activity was studied in a number of fractions separated from rat liver homogenates by differential centrifugation. It was found that less than 5 per cent of the enzymatic activity separates with the nuclear fraction, while over 90 per cent remains associated with the cytoplasmic components. Among the latter, the mitochondria and the microsomes showed the greatest activity. These results were found to be in sharp disagreement with the histochemical data obtained by various authors using the test proposed by Gomori (2). According to the current interpretation, this test indicates that the acid phosphatase is predominantly a nuclear enzyme (Moog (3)). For liver cells in particular, the concentration of the enzyme in the nuclei has been repeatedly stressed (4-7). In addition, its presence in some peribiliary material, supposedly connected with the Golgi apparatus (5, 8), and in the bile capillaries (5) has been described.

Because of this obvious disagreement, it was considered desirable to run in parallel biochemical determinations and histochemical tests on the same material. This was done in a series of 10 experiments on rat livers. For each liver, the activity of the respective homogenate and of the nuclear and cytoplasmic fractions isolated therefrom was tested biochemically, while the histochemical test was performed on sections. No correlation was found between the biochemical and the histochemical results. Additional experiments showed that the histochemical test is not reliable for intracellular localization studies. The precipitation pattern it gives does not reflect, as generally assumed, differences in enzyme concentration among the various cell components, but instead differences in their lead phosphate affinity. The interpretation of the results is further hampered by artifacts produced during the acetone fixation of the tissue.

* Aided in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.
Methods

Liver Fractionation.—In each experiment, a single adult albino rat of the Carworth Farm Wistar strain was used. The animal was killed by a blow on the head and allowed to bleed out. The liver was immediately removed and, after taking off a small piece for the histochemical test, the rest of the organ was passed through a tissue masher. The liver pulp thus obtained was first homogenized in a small amount of 1.46 M (50 per cent) sucrose solution and thereafter in the amount of 0.88 M (30 per cent) sucrose necessary for obtaining a final dilution of 1 gm. to 10 ml. homogenate. Except for the final dilution employed, the homogenization and the subsequent fractionation by differential centrifugation were performed as indicated in a previous article (1) to which the reader is referred for details.

From the original homogenate (LH0), the connective tissue debris and most of the unbroken and partially broken liver cells were separated by a first centrifugal run of 10 minutes at 1200 × g. The partially purified homogenate thus obtained (LH1) was used as reference material for the subsequent fractions, for reasons previously explained (1). The LH1 was centrifuged twice for 10 minutes at 1200 × g. The sediments were resuspended in 0.88 M sucrose, combined, and labeled nuclear fraction (NF). The supernatant constituted the cytoplasmic fraction (CF). All fractions were brought up to the original LH1 volume with 0.88 M sucrose in order to make their activities directly comparable per unit volume. As will be shown later, the acetone fixation required for the histochemical test destroys or damages beyond recognition all the cytoplasmic components normally resolved by the light microscope. The comparison between biochemical and histochemical results is thus necessarily limited to nuclei and cytoplasm. For this reason no subfractions were isolated from CF.

Biochemical Determination of Enzyme Activity.—In each experiment, the activity of LH1, LH2, NF, and CF was tested on 0.08 M Na2α,β-glycerophosphate buffered at pH 4.5 with acetate buffer. The assay system and the incubation conditions have already been described (1). The amount of phosphorus split by each fraction during 60 minutes at 38° C. was determined by the Berenblum-Chain method (9). In two experiments, the activity of all fractions was tested in parallel on the Na2 α + β-glycerophosphate mixture, commonly used in histochemical technique, and on pure Na2 β-glycerophosphate, and found to be the same on both substrates. The amount of inorganic P that contaminates these substrates is approximately 5 times greater in the α + β mixture. In order to avoid high blanks, pure Na2 β-glycerophosphate was used for the rest of the experiments.

Histochemical Test.—In each experiment, the liver piece reserved for the histochemical test was cut in small blocks of approximately 2 × 2 × 3 mm. The latter were fixed in acetone first at 0° C., for 24 hours, thereafter at room temperature for an equal period. The fixed liver blocks were cleared in benzene, embedded in paraffin, and cut in sections 5 to 6 μ thick.

In the first two experiments, the sections were incubated at pH 4.5 in the glycerophosphate- acetate buffer-lead nitrate mixture recommended by Gomori in his original paper (2). In the last 8 experiments, the improved Gomori formula (10), containing 0.01 M substrate, 0.05 M acetate buffer, and 0.004 M lead nitrate was used (pH 4.5 to 4.6). In all experiments, the incubation period was restricted to 5 to 6 hours at 38° C., and the lead phosphate precipitated in the sections was rendered visible by conversion to lead sulfide. In two experiments, the histochemical test was carried through in parallel with Na2 β-glycerophosphate and Na2 α + β-glycerophosphate as substrates. No difference in results was observed.

1 In the International centrifuge, type SB-1, with horizontal yoke No. 233, turning at 2700 R.P.M. at 0° C.
2 Eastman Kodak (catalog No. 3532), or Nutritional Biochemicals Company.
3 In all histochemical tests, the Eastman Kodak Na2 glycerophosphate (catalog No. 644) containing 20 to 28 per cent α isomer was used.
Inactivation.—In each experiment, a number of heat-inactivated sections were incubated together with the usual active ones. The inactivation was obtained by keeping the sections mounted on slides in a thermostat at 70° C. for at least 18 hours. Shorter treatment often resulted in incomplete inactivation. Folded or thick sections were particularly difficult to inactivate. Temperatures over 90° C. or 100° C. destroyed not only the enzymatic activity, but also the ability of the sections to fix lead phosphate. The fact that sections heated at 80° C. lose their ability of fixing lead has been already reported by Newman et al., (11).

Coating.—Some inactivated sections were coated with celloidin by dipping the respective slides 2 to 3 times in a 1.5 per cent parlodion solution. The thick film was hardened in 70 per cent ethanol. It formed a sheath around the slides for more than 3/4 of their length. During incubation, the upper limit of the film was kept above the level of the incubating mixture.

Cyto logical Techniques.—The various preparations used for biochemical determinations were systematically checked with the light or phase-contrast microscope. For this purpose, thin layers of fresh preparations mounted between slide and coverslip were used. Photomicrographs of such preparations were taken whenever possible. For studying and photographing minute particles that in fresh preparations are animated by Brownian movement, the suspensions were first fixed by mixing with an equal volume of 2 per cent OsO4 in 0.88 m sucrose (final OsO4 dilution, 1 per cent). After 4 to 24 hours' fixation, smears were prepared from this mixture and allowed to dry. They were stained with aniline-acid fuchsin according to Altmann's technique for mitochondria (12).

The effects of acetone fixation were followed on disc preparations (13). These were obtained by pressing between slide and coverslip a small amount of fresh liver pulp or a section of fresh liver cut by hand. The coverslip was secured only at the corners with vaseline drops. The preparation was immediately immersed in cold acetone which penetrated the capillary space between slide and coverslip and started to diffuse into the tissue. Such a preparation can be taken out of the fixative at any time and examined under the microscope. If the disc is thin enough, the morphological changes induced by the fixative can be observed and their development followed.

OBSERVATIONS AND RESULTS

The State of Preservation of the Material

The intracellular location of a given substance can be determined either "in situ," i.e. by a histochemical method, or in fractions isolated by differential centrifugation. The former approach implies, as preliminary steps, fixation, embedding, and sectioning of the tissue, while for the latter mechanical tearing of the cell membranes and suspension of the liberated particles in an artificial medium are necessary. It is generally assumed that the amount of damage suffered by the cell components is much greater in the latter instance, but no comparative observations bearing on this point are available. For this reason it was considered desirable to compare in this study the state of preservation of the material used for biochemical determinations with that of the material on which the histochemical tests were performed.

As already known, visible cell components, e.g., nuclei, mitochondria, and lipid inclusions are preserved in a life-like appearance in hypertonic sucrose homogenates (14, 1). This is illustrated by Fig. 1 which shows a group of liver
cells in an LH₁ preparation. The nuclei appear turgid and homogeneous. Their nucleoli are frequently visible. The very numerous mitochondria are predominantly rod-like or filamentous. The lipid inclusions appear as refringent spherical droplets, bright or dark according to their position in relation to the focal plane. All these cell components retain their characteristic morphology and their dye affinities after mechanical rupture of the cell membrane and their subsequent suspension in hypertonic sucrose. This is demonstrated by Fig. 2 which is the micrograph of an LH₁ smear fixed with OsO₄ and stained by the Altmann procedure. The nuclei, the mitochondria, and the lipid inclusions are unchanged. The material visible in the background consists of both the microsomes and the soluble components of the cytoplasm. They have been precipitated in irregular aggregates by the fixative and thus brought well above the limit of resolution of the light microscope. The same quality of preservation is indicated by Figs. 3 and 4. Fig. 3 is a phase-contrast micrograph of a fresh nuclear fraction resuspended in a small amount of 0.88 M sucrose. Most of the nuclei maintain their intracellular appearance. A few, 1 to 2 out of 10, show broken and wrinkled membranes, but their gelated content does not appear to spread in the medium. Fig. 4 shows a smear of an OsO₄-fixed, Altmann-stained cytoplasmic fraction. The background material, as in LH₂, consists of precipitated microsomes and ground substance.

A comparison of the 4 pictures of this series indicates how complete a separation one can obtain by differential centrifugation. The cytoplasmic fraction (CF) contains only cytoplasmic components; its separation appears to be always satisfactory. This is not the case with the nuclear fraction (NF) which is regularly contaminated by cell fragments, "cell membranes," and a certain amount of mitochondria. The "cell membranes" are sheets of amorphous material, 0.5 to 1.0 μ in thickness, which probably represent the membranes of two adjacent liver cells together with the lamina of interposed cement.

In summary, the preservation of the cell components in homogenates and fractions appears satisfactory in terms of structural appearance, at least down to the limit of resolution of the light microscope. By differential centrifugation of sucrose homogenates, a reasonably pure cytoplasmic fraction can be obtained, while the nuclear fraction remains visibly contaminated by cytoplasmic components.

By the same criteria, the state of preservation of the material used for the histochemical test is less satisfactory. As Figs. 5 to 12 show, the cytoplasm appears precipitated in a coarse network. In this precipitate the mitochondria cannot be recognized. The nuclear content is also coarsely precipitated, but the nucleoli are still visible. In numerous cases, unusual bodies of polymorphic appearance are found inside the cells, especially along the bile capillaries. They range in morphology from granules to highly complicated structures (Fig. 8). In favorable cases, i.e., when they are not crowded and only partially
impregnated, it is possible to identify among them typical myelin figures, similar to those described in classical cytology as the Golgi apparatus (13).

Fig. 5 shows such a tubular myelin figure slightly contorted and having a visible cavity. The polymorphic bodies appear to be lipid artifacts, i.e., myelin figures and myelin figure residues the morphological details of which are frequently obscured by crowding and by heavy impregnation with lead phosphates (Figs. 7 and 8). Over a large zone at the periphery of liver blocks fixed in acetone, an impressive diffusion effect, similar to that produced by ethanol fixation, can be noted. The precipitated cytoplasm is loosened from the cell membranes facing the periphery of the block and pushed against the membranes facing the center (Fig. 12).

Disc preparations demonstrated that the acetone fixation is responsible for cytoplasm and nuclear content precipitation, and for the destruction of mitochondria. At the beginning of the acetone fixation, intracellular and extracellular myelin figures developed at the expense of lipid inclusions, especially at the expense of peribiliary droplets. The myelin figures were identified by their morphology in the light microscope and by their birefringence in the polarizing microscope. As the acetone concentration rose in the tissue, the material of the myelin figures was reoriented in structures with hydrophobic surfaces (graded and vesicular structures, droplets showing polarization cross). From the latter, new, typical myelin figures developed during incubation in the usual glycerophosphate-acetate buffer-lead nitrate mixture.

All these observations indicate that the acetone fixation damages the cells and produces obvious artifacts. To this extent at least, the state of preservation of the material used for the histochemical test is less satisfactory than that of the material on which the biochemical determinations are performed.

**Biochemical Results**

The biochemical results obtained in a series of 10 experiments are compiled in Table I. They are in good agreement with data already published (1). There is an 8 to 9 per cent drop in activity between LH1 and LH2 indicating that the cell breakage obtained by homogenization came close to 90 per cent. If the activity of LH2 is made the basis of reference, the nuclear fraction accounts for only 4 to 5 per cent, while the cytoplasmic fraction separates with approximately 90 per cent of the original acid phosphatase activity of LH2. The table shows also that there are considerable individual variations in the enzymatic activity of the homogenates of the series, but that the same distribution pattern prevails in all of them, as the small standard deviation figures indicate.

**Histochemical Results**

The results of the histochemical tests performed on sections obtained from the same livers are shown in Table II. The grading is arbitrary and attempts to
quantitate the intensity of the brown lead sulfide color developed at the end of the test in the various cellular and extracellular structures. As is known, the color intensity is supposed to be proportional to the amount of lead phosphate precipitated in the respective structures. It appears therefore that the greatest amount of lead phosphate formed during incubation precipitates either in the nuclei or in the polymorphic bodies found along the bile capillaries and identified as myelin figures. In 5 cases, the nuclear localization was clearly predominant in liver cells as well as in stellate and endothelial cells. Fig. 6 illustrates such a case. Frequently the nucleoli appeared to be more heavily loaded with precipitate than the rest of the nucleus. In 2 cases, the precipitate was found to be present both in the nuclei and in the peribiliary myelin figures as demonstrated by Fig. 7. Finally in 3 cases, the precipitate was deposited in and around the myelin figures with very little or none of it in the nuclei (Fig. 8). In all cases, the cytoplasm appeared less stained than the nuclei and, in a number of cases, uncolored. The bile capillaries showed conspicuous impregnation in a single case; in 4 others, the impregnation was slight and fragmentary.

### TABLE I

**Acid Phosphatase Activity of Liver Homogenates and Fractions**

(Procedure for enzyme assay according to Palade (1); P determinations according to Berenblum and Chain (9)). Total phosphatase activity is expressed in µg. P split off from 0.08 M Na₂β-glycerophosphate in 60 minutes at 38°C and pH 4.5 by 100 mg. fresh liver pulp or each fraction separated from it.

<table>
<thead>
<tr>
<th>Liver fractions</th>
<th>Acid phosphatase activity</th>
<th>No. of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Per cent of LH₁ activity</td>
</tr>
<tr>
<td>LH₁</td>
<td>1333</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>(1008-1865)</td>
<td></td>
</tr>
<tr>
<td>LH₂</td>
<td>1224</td>
<td>91.9</td>
</tr>
<tr>
<td></td>
<td>(985-1746)</td>
<td></td>
</tr>
<tr>
<td>NF</td>
<td>55</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>(40-89)</td>
<td></td>
</tr>
<tr>
<td>CF</td>
<td>1165</td>
<td>95.2</td>
</tr>
<tr>
<td></td>
<td>(924-1573)</td>
<td></td>
</tr>
<tr>
<td>Recovery</td>
<td></td>
<td>99.8</td>
</tr>
</tbody>
</table>
Occasionally the lead sulfide colored also the liver reticulum, and fibers, presumably collagen, along the blood vessels.

Table II overlooks considerable variation. The results were never homogeneous throughout a given section. There was always a negative, uncolored rim, 150 to 250 \( \mu \) in width, at the periphery of the section. In cases in which the nuclei were dominantly positive, the first structures that appeared colored at the periphery of the block were the nucleoli, followed by the rest of the nucleus. Only towards the center was the cytoplasm colored. A similar situation was

**TABLE II**

*Histochemical Data*

The table shows the results obtained with Gomori’s test for acid phosphatase in the same series of rat livers that yielded the biochemical data which appear in Table I.

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Liver cells</th>
<th>Extracellular structures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclei</td>
<td>Peribiliary bodies</td>
</tr>
<tr>
<td>1</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>5</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td>9</td>
<td>+</td>
<td>++++</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>++++</td>
</tr>
</tbody>
</table>

For all experiments: fixation, acetone 48 hours; clearing, benzene; embedding, paraffin; sections 5 to 6 \( \mu \) thick; incubation, 5 to 6 hours.

Experiments 1 and 6 were incubated in the old Gomori’s mixture at pH 4.5 (2); all the others in the new one at pH 4.5-4.6 (10).

Grading: -, no color; +, light brown; ++, brown; ++++, deep brown; ++++, almost black.

found in cases with dominant positive reaction in the peribiliary myelin figures. Beside this concentric pattern, the intensity of the reaction was frequently uneven in the central zone of the sections. Moreover, in some cases, the dominant reaction shifted from nuclei to peribiliary bodies or vice versa from region to region. When the test was repeated, variations in intensity and sometimes variations in the type of reaction were found in sections coming from the same block.

From the preceding it appears that: (a) the histochemical test does not reveal a consistent pattern for the intracellular distribution of the enzyme; (b) some lipid artifacts show intense positive reaction; (c) the type and intensity of the reaction are subject to considerable variation.
Experiments with the Histochemical Test

These findings, together with the lack of agreement between the biochemical and the histochemical data, justify the hypothesis that the lead phosphate formed during incubation does not precipitate necessarily where the acid phosphatase is located. In order to test this hypothesis, an experiment of the following type was carried out in each case:—

Sections obtained from the same region of a liver block, fixed in acetone, and embedded in paraffin, were cut and mounted on slides as usual. Some of them were inactivated by heat as already described. Such heat-treated sections will be referred to as "inactivated sections" and thereby distinguished from the usual, unheated ones designated as "active sections." Slides pertaining to the same experiment were divided in 3 groups, each consisting of one or two pairs of slides. In each pair, there was a slide with active sections and another with inactivated ones. Group I was incubated in the usual glycerophosphate-acetate buffer-lead nitrate mixture. Group II was incubated in 40 ml of the same mixture to which 0.3 to 1.0 ml of the respective cytoplasmic fraction was added. The slides of group III were first coated with celloidin, as described, and incubated thereafter as group II. For all groups, the incubation lasted 5 to 6 hours at 38° C., with intermittent shaking. Slides belonging to the same pair were processed together. Group I served as a control for: (a) the type and intensity of the reaction given by the respective liver and (b) the quality of the inactivation. This procedure allowed a comparative study of the precipitation pattern obtained on the same material in a number of different situations, such as: (a) when the enzyme was present in the sections (group I—active sections); (b) when it was present only in the incubating medium (group II—inactivated sections); (c) when it was present in the same location but unable to get in direct contact with the sections because of the heavy celloidin coating (group III—inactivated sections).

Figs. 9 to 12 illustrate the results of such an experiment in a case with the nuclear type of reaction. Fig. 9 shows the precipitation pattern in an active section incubated under the usual conditions for the histochemical test (group I, active). The heat-treated section of the pair (Fig. 10) demonstrates that the inactivation was satisfactory. Fig. 11 shows an inactivated section from group II. The reaction is positive and of the same nuclear type as in the active section shown in Fig. 9. Fig. 12 demonstrates that the same pattern is obtained in an inactivated section from group III, i.e., a section coated with celloidin before incubation. The celloidin coating was thick enough to be seen and peeled off before covering the sections with a cover glass. The same precipitation pattern appeared in the active sections of groups II and III, only the reaction was more intense. In all sections of the last two groups no negative peripheral rim was observed.

Similar results were obtained in the other cases. Only the experiments in which the inactivation proved to be satisfactory were taken into consideration. In most of them, the type of reaction showed by the active section of group I was reproduced on the inactivated sections of groups II and III. In some of the cases, there was a partial and, in a few, a total shift of the reaction from the nuclear to the peribiliary type or vice versa. As in the usual histochemical test, a
certain amount of variation in the intensity of the reaction was found in each series of sections obtained from the same liver.

Although variable in type and intensity, these results seem to show that the lead phosphates impregnate preferentially the same structures both in the active sections of groups I, II, and III, and in the inactivated ones of groups II and III. In all of them the nuclei and the myelin figures gathered the largest amount of precipitate; less was found in the bile capillaries and relatively little in the cytoplasm. Only the impregnation of the reticulum and of the collagen fibers appeared to be more frequent in the inactivated sections incubated with the enzyme added to the medium.

DISCUSSION

The present experiments demonstrate that biochemical methods and histochemical tests, applied in parallel on the same material, namely rat liver, give different pictures for the intracellular distribution of acid phosphatase. According to the former, the acid phosphatase is a cytoplasmic enzyme, while according to the latter, it is mainly nuclear. In detail, the biochemical data indicate that the activity of this enzyme is almost entirely located in the cytoplasmic fraction with but little present in the nuclear fraction. The activity of the latter could be accounted for by its visible contamination with cytoplasmic components. The histochemical test shows that the enzyme is mostly concentrated in the nuclei and in some peribiliary polymorphic bodies, with much smaller amounts in the bile capillaries and in the cytoplasm. It will be remembered that: (a) the peribiliary polymorphic bodies are myelin figures developed at the expense of lipid droplets; (b) in previous experiments (1), such lipid droplets separated from liver homogenates did not show any significant activity; (c) the bile capillaries are located within the "cell membranes" which contaminate regularly the nuclear fraction, i.e., a fraction poor in enzyme according to the biochemical method. These considerations render the discrepancy between the biochemical and histochemical data even greater. Under such circumstances, it appears that at least one of the two methods is not reliable. The methods for intracellular enzyme localization could fail either because of the inadequate preservation of the cellular material or because faulty procedures for the demonstration of the enzyme are used. These two aspects will be presently discussed in relation to the methods applied in this study.

1. State of Preservation of the Material.—As indicated in the preceding observations, cell components, e.g. mitochondria, that according to biochemical determinations contain an appreciable amount of enzyme (1, 15), are destroyed during the acetone fixation. The mitochondrial enzyme may be liberated in the process and precipitated, together with the enzyme present in the microsomes and the ground substance, on the various structures that resist acetone treatment. Contrary to the general belief, according to which redistribution of
Intracellular acid phosphatase

Various substances is avoided in fixed material, it appears therefore that there are chances for secondary localization in the acetone-fixed cells.

In terms of light microscope morphology, the state of preservation of cell components seems to be far better in sucrose homogenates and fractions separated therefrom by differential centrifugation. Accordingly, and as far as adequate preservation goes, the biochemical approach appears to be preferable for the study of the intracellular distribution of acid phosphatase. For other enzymes, the issue has to be decided, as in the present case, by comparing the state of preservation of the material used in the two approaches. It has to be pointed out, however, that fixatives such as ethanol or acetone that are used in the majority of histochemical techniques damage considerably the cell structures and produce various types of artifacts.

2. Procedures for the Demonstration of the Enzyme.—In both methods, the activity of the enzyme is demonstrated by the phosphate it splits from the substrate. In the biochemical method, this is done quantitatively by direct determinations. In the histochemical test, the liberated phosphate radical is combined with lead to form insoluble lead phosphates. The latter are assumed to precipitate on the spot, thus marking the sites where the enzyme is present. The precipitate is rendered visible thereafter by conversion to lead sulfide and its amount evaluated qualitatively or semiquantitatively. It will be noted that in the biochemical approach the possibilities of redistribution by diffusion are limited to the homogenization-separation steps and that they may involve only the enzyme. In the histochemical test, these possibilities persist throughout fixation and incubation and may affect both the enzyme and the phosphates produced by its activity.

The experiments done with the histochemical test clearly indicate that diffusion processes are to be taken into account in the formation of precipitation patterns. The fact that the same pattern is obtained in active, conventionally incubated sections, and in inactivated sections incubated with the enzyme present in the medium, shows that the original localization of the enzyme does not condition the distribution of the precipitate. This could be explained by a selective adsorption on certain cell structures either of the enzyme or of the phosphates. The second alternative seems to be at work because the usual precipitation pattern is obtained on inactivated sections coated with a thick film of celloidin and incubated thereafter with the enzyme added to the medium. Under such circumstances, it is very probable that the enzyme cannot reach the section while the phosphates do. This leads to the conclusion that the lead phosphates do not precipitate on the spot, but diffuse in the medium and precipitate at locations removed from the formation sites. The precipitation appears to be of a preferential type: certain cell structures, such as nuclei, or certain artifacts, such as myelin figures, gather most of the phosphates. The end result is that enzyme sites and precipitation sites do not
necessarily correspond as it is assumed in the interpretation of the histochemical test. In the range of cellular dimensions at least, the precipitation pattern does not reflect differences in the enzyme concentration among various structures, but reflects differences in their lead phosphate affinity.

It appears therefore that in the histochemical test the preservation of the material is poor and the procedure for demonstrating enzyme sites is misleading. Differential centrifugation of tissue homogenates, followed by quantitative biochemical procedures, allows direct enzyme activity determinations on more satisfactorily preserved material. It is believed therefore that, in the present situation, the latter has to be preferred for the study of the intracellular distribution of acid phosphatase.

The validity of the histochemical test for acid phosphatase has been questioned in the past because: (a) the enzyme present in the sections was found to be unexpectedly resistant to various treatments; (b) lack of correspondence between precipitation patterns obtained in fresh and fixed material; (c) discrepancy between histochemical and biochemical findings in sectioned nerves. In some of the papers cited, diffusion processes, involving the enzyme or the products of its activity, have been invoked for the explanation of the findings. In fact, the possibility of diffusion phenomena has been envisaged by Gomori because of the measurable solubility of lead phosphate at pH 4.5; but it was supposedly prevented by modifying the ratio between the various components of the original substrate-buffer-lead nitrate mixture. The present experiments demonstrate that this modification does not actually prevent the diffusion of lead phosphates.

In the related histochemical test for alkaline phosphatase, the intervention of diffusion phenomena was discussed by Lison and clearly demonstrated by Jacoby and Martin although the latter were not able to decide whether the diffusion affects the enzyme or the phosphates. Since the preliminary results of the present work were published in abstract, Novikoff, using similar techniques, has arrived at similar conclusions in connection with the test for alkaline phosphatase. Recently Gomori recognized that in this test the positive reaction shown by the nuclei is an artifact presumably due to the affinity of desoxyribonucleic acids for phosphates. Because of the positive reaction given by myelin figures, the present work suggests that other compounds, such as phospholipids and fatty acids, may share in this affinity for phosphates. In relation to the positive reaction shown by intracellular myelin figures, one could wonder whether the impressive impregnation of bile capillaries regularly obtained in the alkaline phosphatase test is not due to ethanol-induced myelin figures that migrate through the bile capillaries—a phenomenon clearly visible in ethanol-treated homogenates.

The "phosphate affinity" of the nuclei was first found by Danielli in his studies concerning the alkaline phosphatase test but the finding was not con-
sidered disturbing for the reliability of the method. Very little can be said about the nature of this affinity. It will be remembered that various structures such as injured axons (17) and especially cell nuclei (11, 28) were found to become impregnated selectively with lead at the pH of the histochemical test or close to it, in the absence of substrate or enzyme. This “lead effect” (11) suggests that various structure-bound acids form lead salts that act as “seeds” for the precipitation of lead phosphate. In this respect, these histochemical tests are not unlike silver and other metallic impregnation methods for which such a mechanism has been proposed (29).

SUMMARY

The acid phosphatase activity of rat liver homogenates and of nuclear and cytoplasmic fractions isolated therefrom (by differential centrifugation) was determined biochemically in a series of experiments. For each liver, Gomori’s histochemical test for acid phosphatase was run in parallel.

No correlation was found between the biochemical and the histochemical results. According to the former, the enzyme appears to be almost entirely (95 per cent) located in the cytoplasm, while according to the latter, the acid phosphatase is predominantly concentrated in the nuclei and in some peribiliary polymorphic structures identified as myelin figures.

It was found that the precipitation pattern obtained in the histochemical test does not reveal, as generally assumed, differences in enzyme concentration among the various cell structures, but actually reveals differences in their lead phosphate affinity. The usefulness of the histochemical test for intracellular localization studies was found to be further limited by considerable fixation damage and formation of myelin figure artifacts.

The biochemical approach is to be preferred because of better preservation of the material and direct and more reliable methods for the demonstration of enzyme activity.

The technical assistance of Miss Sally Rutstein is gratefully acknowledged.

BIBLIOGRAPHY

EXPLANATION OF PLATES

The photomicrograph reproduced in Fig. 3 was taken with an American Optical phase-contrast microscope using a dark medium, achromatic immersion objective (N.A. = 1.25; 97 ×) and a 12.5 × ocular.

All the others were taken with a Bausch and Lomb research microscope and camera, using an apochromatic immersion objective (2 mm.; N.A. = 1.20; 90 ×), a 12.5 × compensated ocular, and a Kodak-Wratten filter No. B-58.

In the plates, all the photomicrographs are enlarged to × 1,600.

PLATE 36

FIG. 1. Fresh preparation of a crude liver homog enate (LH0). The photomicrograph shows a group of liver cells with visible cell limits. The nuclei of two cells appear clearly as large, nearly homogenous, circular spots. The cytoplasm is filled with rod-like, filamentous, or granular mitochondria. A few lipid inclusions appear as black or white circular spots (according to their position in relation to the focal plane) in the lowest cell and in the one above it and to the right.

FIG. 2. Smear of a partially purified homogenate (LH2). (Fixed with 1 per cent OsO₄ and stained with Altmann’s aniline-acid fuchsin.) Two nuclei can be recognized as large homogeneous gray spots. The large black body in the upper right corner is a red blood cell. The field is almost entirely occupied by fuchsin-stained mitochondria most of them rod-like. Some mitochondria are granular and a few appear vacuolized, showing a clearer center. The less numerous lipid inclusions are difficult to distinguish. They appear as dark, perfectly circular spots. (Some can be found along the left half of the lower margin of the picture.) In the original preparation, they were blackened by osmium oxides and accordingly easy to distinguish from the red-colored mitochondria. The background gray material, precipitated by the fixative in granular or reticular form, represents the microsomes and the ground substance of the cytoplasm.

FIG. 3. Phase-contrast photomicrograph of a fresh, unfixed preparation of a nuclear fraction (NF). The isolated nuclei retain their intracellular appearance. Their membranes are light or dark according to their position in relation to the focal plane. The nuclear content is almost homogeneous. Shadows (right upper corner) and nucleoli (center and left upper corner) are visible in some nuclei. The nucleus appearing at the 5 o’clock position is still enclosed in its cell membrane. Cytoplasmic contaminants such as mitochondria and lipid inclusions are visible in the background.

FIG. 4. Smear of a cytoplasmic fraction (CF). (Fixed with 1 per cent OsO₄ and stained with Altmann’s aniline-acid fuchsin.) The fraction is composed exclusively of cytoplasmic components. The mitochondria appear as dark, rod-like, filamentous, or granular bodies. The number of vacuolated mitochondria is slightly increased by comparison with Fig. 2. For lipid inclusions and background material see the legend for Fig. 2.
(Palade: Intracellular acid phosphatase)
PLATE 37

FIG. 5. Group of liver cells from a partially inactivated section. The coarse precipitation of the cytoplasm and of the nuclear content is clearly visible. The mitochondria have been destroyed. A comparison with Fig. 1 shows the amount of damage produced by the acetone fixation.

A few bodies colored by lead sulfide appear at the periphery of the cells (presumably along the bile capillaries). The one in the middle of the picture can be identified as a tubular, partially twisted myelin figure. Its central cavity is visible in a few places. The shorter myelin figure in the upper left-hand cell has a more easily visible cavity.

FIG. 6. Group of liver cells showing the nuclear type of reaction for acid phosphatase. The liver cell nuclei are intensely impregnated by lead sulfide; the cytoplasm is only slightly colored. The small round nuclei of two endothelial cells (second row from the bottom) and the elongated nucleus of a Kupffer cell show also a positive reaction. In all nuclei, the preferential impregnation of the nucleoli is apparent, especially in the upper cells which were closer to the periphery of the block.

FIG. 7. Group of liver cells showing a mixed type of reaction. The liver cell nuclei are colored by lead sulfide. More intensely colored material is concentrated along the bile capillaries which, in this case, appear mostly in cross-section. The lumen of the bile capillaries themselves is visible in some places and appears to be free of colored precipitate.

FIG. 8. Group of liver cells with a peribiliary type of reaction. The section is oriented along a certain number of bile capillaries. The precipitate impregnates almost exclusively the peribiliary bodies which appear granular or vacuolar in some cells (right center, 3 o'clock cell), and tubular or polymorphic in others. The lumen of the bile capillaries is visible in a number of places (in the center of the picture, along the upper margin of the transverse row of black polymorphic bodies and along the row that extends towards 11 o'clock). Note the very coarse precipitation of the cytoplasm throughout the field. A “negative” nucleus is visible in the cell immediately below the middle of the picture.
(Palade: Intracellular acid phosphatase)
Photomicrographs of sections obtained from the same region of a liver block are reproduced in Figs. 9 to 12.

Fig. 9. Active section incubated in the usual glycerophosphate-acetate buffer—lead nitrate mixture (group I—active). The liver cell nuclei show an intense positive reaction which is more pronounced for the nucleoli. The cytoplasm is lightly colored. The small ovoid nucleus of an endothelial cell appears in the upper left corner. The irregular body close to it is a red blood cell, badly deformed by acetone fixation. Like the nuclei, it shows a positive reaction.

Fig. 10. Heat inactivated section incubated in the same way (group I—inactivated). Negative histochemical reaction. Note the coarse precipitation of the nuclear content and of the cytoplasm. A comparison with Figs. 1 to 4 shows the difference in state of preservation between the material used for the biochemical determinations and that on which histochemical tests are performed.

Fig. 11. Heat inactivated section incubated with enzyme added to the medium (group II—inactivated). The precipitation pattern is similar to that shown in Fig. 9, only the general intensity of the reaction is slightly increased. The liver cell nuclei appear heavily impregnated, while the liver cell cytoplasm shows a moderate positive reaction.

Fig. 12. Heat inactivated section, coated with celloidin and incubated with the enzyme added to the medium (group III—inactivated). The same pattern of preferential precipitation in the liver cell nuclei is obtained as in Figs. 9 and 11. There is much less precipitate in the cytoplasm which shows obvious acetone diffusion effects. The latter appear as intracellular clear bands or zones of less condensed cytoplasm all oriented towards the periphery of the block (in this case towards the upper left corner). Similar, but less pronounced appearances can be seen in Figs. 9 and 11. Note that the orientation of the pictures is different.
(Palade: Intracellular acid phosphatase)