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Received April 14, 1952. P.S.E.B.M., 1952, v80.

Modification of Gomori Method for Alkaline and Acid Phosphatase Avoiding Artefact Staining of Nucleus.* (19527)

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(Introduced by Harry S. N. Greene.)
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The histochemical phosphatase methods originally outlined by Gomori(1,2) have been critically studied in this laboratory. Consistently reproducible results with Gomori's acid phosphatase method have been obtained during the past two years by adhering to certain details ascertained to be necessary in the procedure(3). By incubating the sections in the paraffin ribbons directly in substrate, the known harmful effect of exposure of the contained enzyme to various concentrations of alcohol incident to deparaffinization and rehydration was eliminated. Not only do the resultant preparations show greater and more constant preservation of phosphatase activity but also its localization is much sharper and appears in finer detail. In addition, the apparent phosphatase activity of the nucleus as seen in conventional preparations is largely if not entirely absent, as Ruyter and Neumann(4) have shown with the alkaline phosphatase technic. The discrepancy between the results obtained from cellular fragmentation experiments and from histochemical determinations is therefore resolved. As noted by several investigators (5,6), nuclei isolated by cellular fragmentation technics show chemically only a small fraction of the total phosphatase activity demonstrable. Histochemically this is confirmed by the Menten(7) or other similar methods which use the alcoholic end of the phosphate ester to produce a color reaction by formation of a diazonium salt. This is clearly seen in illustrations in the articles by Danielli (8) and Manheimer and Seligman(9) although no statement to this effect appears in their respective papers. Novikoff(10) however, has recently emphasized this point. The results obtained with the conventional Gomori method for alkaline or acid phosphatase(1,2) using deparaffinized tissue sections are in direct contrast to these data in that all nuclei in tissues showing phosphatase activity have also shown marked "phosphatase activity". This discrepancy is eliminated by the use of non-deparaffinized tissue sections in which it is found that the nuclei show very little activity. Since all 3 methods using entirely different approaches now agree it would seem that this localization is a true finding.

The validity of this unconventional method depends upon the assumption that the substrate reaches every part of the cell in which phosphatase activity may exist. This has been put to test by observing the penetration, or loss of substances of different molecular size into or from tissue sections within paraffin ribbons.

1) *Penetration of water.* As noted by Harrison and Bunting(11), ferrocyanide in paraffin sections of kidney is immediately removed by contact of the ribbon with water. Similarly, glycogen leaches out of paraffin sections mounted on a glass slide and immersed in distilled water for 2 hours, parallel-

* Supported by a grant from the National Cancer Institute, N. I. H., U.S.P.H.S.

† This work was begun while a fellow of the Jane Coffin Childs Fund.

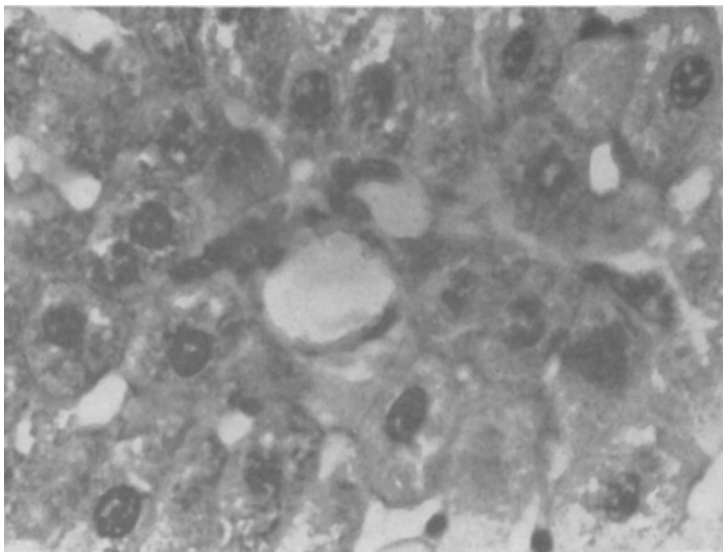

FIG. 1. Rat liver. Artefactual staining of nucleus and cytoplasm. Tissue boiled, embedded, sectioned. Sections soaked in Na_2HPO_4, then treated as routine alkaline phosphatase preparation. × 800.

ing the leaching seen in deparaffinized sections.

2) *Penetration of cobalt and or lead ions.* Paraffin sections of newborn mouse bone were mounted on glass slides and immersed directly in solutions of $CO(NO_3)_2$ at pH 9.4 and $Pb(NO_3)_2$ at pH 4.3 thus duplicating certain steps in the routine phosphatase technics. When subsequently treated with $(NH_4)_2S$ the sections showed precipitation of the metallic ions in the bone, the ions presumably having been bound to inorganic phosphate and carbonate there.

3) *Penetration of molecules* of acid and basic dyes. Excellent staining by acid and basic dyes of nuclear, cytoplasmic and noncellular structures can regularly be accomplished in non-deparaffinized sections by prolonging the exposure up to 18 hours. For the demonstration of this the following dyes and concentrations have been used: alcoholic eosin, 3%; aqueous eosin, 3%; aniline blue, 2%; Van Gieson (picric acid, acid fuchsin); light green, ¼%; paracarmine, 1-2%; basic fuchsin, ½%; toluidine blue, 0.05%; methylene blue, 2%; methyl green, 0.5%; Hematoxylin (Bullard's). It might be mentioned parenthetically that in these test preparations cytological distortion was appreciably less than in conventional deparaffinized sections. McFarland(12) advocated staining the paraffin ribbon as a short-cut in the preparation of histological material for student classes. Cowdry(13) described Mayer's stain for amyloid in which freshly cut paraffin sections are stained by immersion in 0.5% aqueous methyl or crystal violet for 5-10 minutes without removal of paraffin.

4) *Penetration of a section* under these conditions by a protein can be demonstrated by the rapid digestion by amylase of glycogen contained in liver cells in a non-deparaffinized section. Selective adsorption of the phosphatase enzymes by the nuclei has not been demonstrated by investigators. The cause of the apparent phosphatase activity of the nucleus in the sections prepared by the conventional methods is generally ascribed to the nuclear adsorption of calcium or lead phosphate resulting from phosphatase activity in the vicinity(10,14). A similar artefactual staining of the nucleus and to a lesser extent, of the cytoplasmic granules can be produced in the following manner: A mounted section in which the enzymes have been inactivated by boiling is soaked in a solution of Na_2HPO_4, then immersed half way in the alkaline phosphatase substrate buffer (without glycero-

phosphate). It is then treated successively in $CaCl_2$ in $CO(NO_3)_2$, and finally in $(NH_4)_2S$ as in the routine technic for alkaline phosphatase. In the region of the meniscus

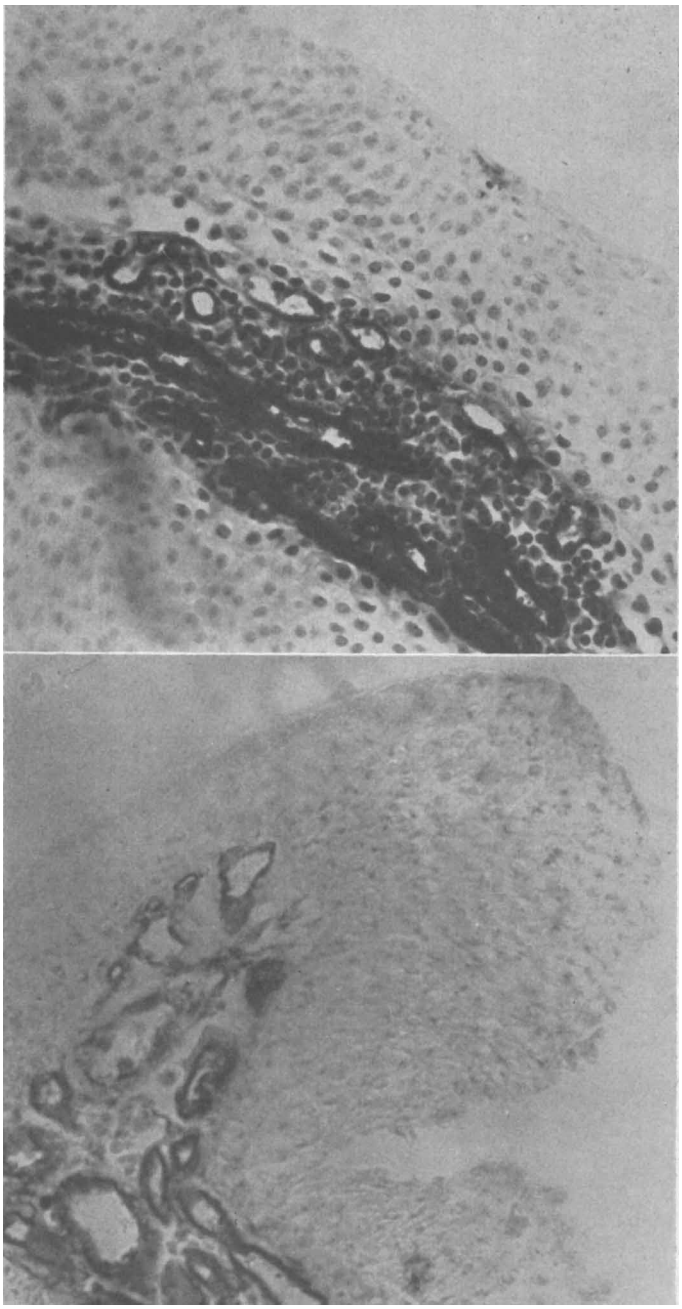

FIG. 2 (top). Human bladder papilloma fixed in 80% alcohol. Alkaline phosphatase preparation deparaffinized before incubation in substrate. Note gradient in nuclear staining from vicinity of capillaries toward periphery. Incubation time 6 hr. × 300.

FIG. 3 (bottom). Adjacent serial section to Fig. 2, incubated in substrate before removal of paraffin. Note lack of nuclear staining even adjacent to capillaries. Incubation time 6 hr. × 300.

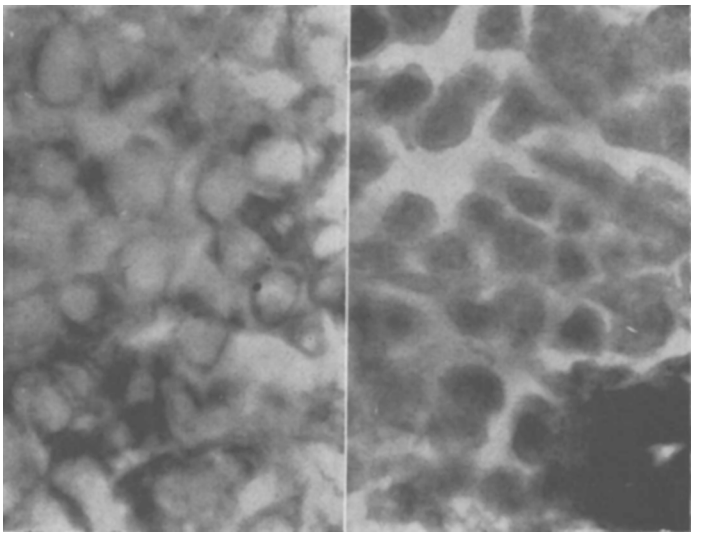

FIG. 4 (left). Rat spleen fixed in 80% alcohol. Germinal center, alkaline phosphatase preparation, incubated in substrate before removal of paraffin. Note lack of nuclear stain, presence of cytoplasmic activity. Incubation time 6 hr. × 1260.

FIG. 5 (right). Adjacent serial section to Fig. 4. Incubated in same substrate after removal of paraffin. Note the nuclear precipitate commonly interpreted as indicating nuclear phosphatase activity. Arteriole present in lower right. Incubation time 6 hr. × 1260.

which had been formed by the calcium-containing substrate buffer on the phosphate-soaked section the nuclei show a dense staining in a manner exactly similar to that seen in conventional alkaline phosphatase preparations (Fig. 1). The results are the same in this test whether the sections are or are not deparaffinized.

That much of the nuclear staining with the conventional Gomori technic is artefact is readily seen by comparing adjacent serial sections incubated in substrate, alternate sections having been deparaffinized. In the non-deparaffinized slides there is little if any diffusion within the section from points of high phosphatase activity such as capillary endothelium. In deparaffinized slides one can see a very definite gradient of staining density of nuclei from the points of high activity outwards. This is especially well demonstrated in tissues whose capillaries are widely separated such as in a papilloma of the urinary bladder (Fig. 2, 3). Martin and Jacoby(14) have demonstrated similar gradients in the nuclei of tissue having low phosphatase activity on which a tissue section having points of high activity had been superimposed. The non-deparaffinized sections may show at most a faint minutely granular precipitate associated with the chromatin network in the nucleus and the nucleolus of the fixed cell (Fig. 4, 6). This is in contrast to the dense, fairly uniform precipitate distributed throughout the entire nucleus seen routinely in the conventional Gomori technic (Fig. 5, 7). Histochemists have generally accepted this nuclear precipitate as denoting nuclear phosphatase activity. Further evidence that this is not the case is offered by careful inspection of conventional phosphatase preparations made from paraffin-embedded frozen-dried material deparaffinized before incubation. Here not all of the nuclei show the dense precipitate usually seen but many show staining only of the nuclear membrane, the nucleolus and very faint if any staining of the chromatin network.

The nuclei in frozen-dried preparations are often difficult to stain by the usual dyes just as in fresh frozen sections. This is presumably because the reactive surface groups have not been opened up and made available since there has been a minimum of denaturation of the proteins under these conditions as contrasted to the effects of the usual fixatives. The lightness of the staining of the nuclei in

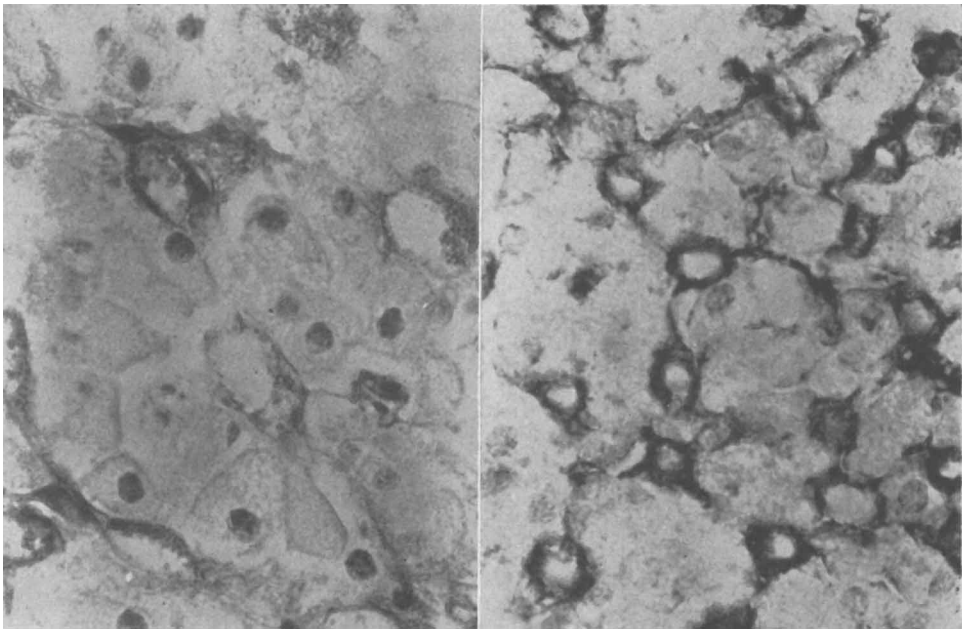

FIG. 6 (left). Rat pituitary, fixed in absolute acetone. Acid phosphatase preparation incubated after removal of paraffin. Note dense nuclear precipitate. Incubation time 6 hr. × 560.

FIG. 7 (right). Adjacent serial section to Fig. 6, incubated same length of time in same substrate before removal of paraffin. Note lack of nuclear staining and marked activity in capillaries. × 560.

phosphatase preparations in frozen-dried deparaffinized material contrasted with that after fixation with 80% alcohol would seem to be of similar origin, assuming that the staining here with Ca^{++} or $PO4^{---}$, or $Ca_3(PO4)_2$ is an acid-base phenomenon.

Summary and conclusion. 1. Staining sections before removal of the paraffin gives perfectly satisfactory, if not superior, results. Water and water-soluble material (dyes, substrate components, enzymes, etc.) readily penetrate the tissue structures exposed on the surface of the thin paraffin ribbon. The paraffin appears to act only as a filler which preserves the spatial relations of the various cytological elements during all staining and dehydration procedures. 2. Incubation of nondeparaffinized tissue sections in substrate gives a more accurate localization of phosphatase enzyme activity than the conventional method, and shows almost no activity demonstrable in the nuclei. It seems likely that the paraffin in the section prevents the lateral diffusion of the reaction products with their subsequent adsorption upon the nuclei. It is suggested this method be tried in other enzyme technics.

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Received April 17, 1952. P.S.E.B.M., 1952, v80.