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Histochemical Demonstration of Sites of Phosphamidase Activity.*

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In the course of experiments on the histochemical specificity of phosphatases the use of 18 out of 19 different substrates resulted in the production of identical patterns of enzyme distribution in all tissues and in all ranges of pH.[1] The nineteenth substrate, p-chloranilidophosphonic acid, included because of the existence of a specific "phosphamidase",[2-4] yielded a pattern identical with the others at pH 9 and 7, but produced strikingly different pictures at pH 5. Since this finding is a strong evidence in favor of the existence of a specific enzyme, a technic was developed for its histochemical demonstration.

Experimental. p-Chloranilidophosphonic acid (p-$ClC_6H_4NHPO(OH)_2$ was synthesized by the method of Otto.[5] The crude product was dissolved in an excess of NH_4OH, filtered, and neutralized, under ice cooling, with acetic acid. A slight excess of acetic acid was added (to about pH 4), and the solution was placed in the refrigerator. In a few hours the free phosphonic acid precipitated almost quantitatively. It was filtered under suction, washed with ice cold water, and dried *in vacuo*. The dry powder was extracted with cold absolute alcohol to remove p-chloraniline and dried. A 0.1 M stock solution was prepared by dissolving a calculated amount of the powder in an excess of 10% NH_4OH, adjusting the solution with dilute acetic acid to about pH 8 and filling it up to volume with distilled water. Such stock solutions were found to remain stable at refrigerator temperature for many weeks.

Thin slices of fresh tissue were fixed in a number of different fixatives, chilled acetone, 95% and absolute alcohol being found most satisfactory. Paraffin sections were incubated at 37°C from 2 to 48 hr in mixtures containing various amounts of substrate, Pb $(NO_3)_2$, and buffer in the range from pH 4.5 to 7.5. The best and most constant results were obtained at pH 5.4 to 5.8, the concentration of the substrate being 0.005 M, that of Pb $(NO_3)_2$, 0.0025 M and that of the buffer, 0.05 M. The presence of $MnCl_2$ in a concentration of 0.002 to 0.005 M greatly intensified the picture; Mg and Ca salts were without marked effect. Above pH 6.2 the reaction became distinctly weaker, disappearing altogether at pH 6.7. Above this pH level pictures corresponding to the distribution of nonspecific alkaline phosphatase were obtained. Below pH 5.3 confusing artifacts consisting in the Pb impregnation of various structures such as connective tissue and muscle fibers, nerves, mucin, etc. were often observed. These artifacts were present even in slides inactivated by dipping them for 5 minutes in Lugol's iodine solution prior to incubation, whereas true enzymatic reaction was completely abolished by this treatment. Since the substrate is not entirely stable around pH 5.6, the slides must be placed in the solution at an angle, the section facing downward, to avoid, as far as possible, the indiscriminate precipitation of Pb phosphate on the tissue. This maneuver will cause the precipitate to accumulate on the back surface of the slides from which it can be wiped off. Collodion coating of the slides interferes with

* This work has been done under grants from the Douglas Smith Foundation for Medical Research of The University of Chicago, and from the Pathology Study Section of the U. S. Public Health Service.

1 Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1949, to be published.

2 Walschmidt-Leitz, E., and Koehler, E., *Biochem. Z.*, 1933, **258**, 360.

3 Ichihara, M., *J. Biochem.* (Japan), 1933, **18**, 87.

4 Bredereck, H., and Geyer, E., *Z. physiol. Chem.*, 1938, **254**, 223.

5 Otto, P., *Ber. deutsch. chem. Ges.*, 1895, **28**, 617.

the reaction; in fact, if the tissues happened to be embedded through collodion the latter must be removed before incubation by passing the slides through an alcohol-ether mixture or acetone.

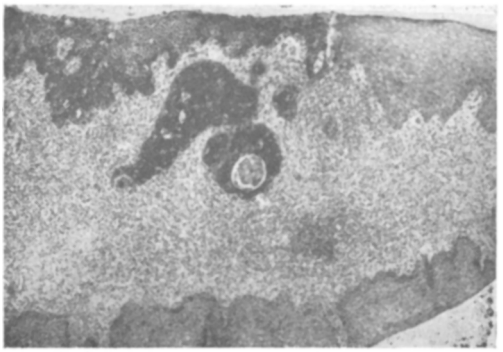

FIG. 1.
Squamous carcinoma of the cervic uteri. Note abrupt start of reaction at the edge of the malignant change.

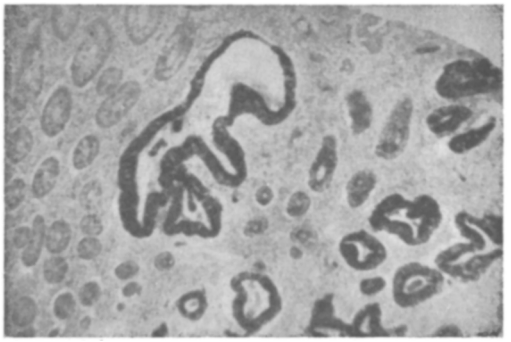

FIG. 2.
Adenocarcinoma of colon. Intense staining of carcinomatous glands; normal mucosa unstained.

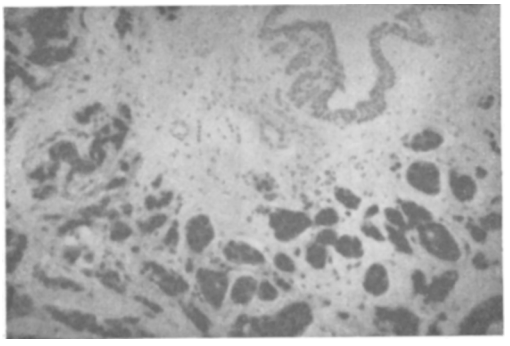

FIG. 3.
Scirrhous carcinoma of breast. Note unstained ducts.

The procedure to be described is recommended.

1. Incubate slides at 37°C for 10 to 24 hr in the following mixture:

To 50 ml of 0.05 M maleate buffer pH ± 5.6 (5.8 g of maleic acid + 62 ml of N NaOH in 1000 ml) add 1 to 1.5 ml of 0.1 M Pb $(No_3)_2$ solution and a few drops of a 10% solution of $MnCl_2$; shake until the initial precipitate dissolves. Add 2 ml of 0.1 M phosphonate stock solution. Place mixture in a 45 to 60°C oven for about 30 minutes until the turbidity consisting of $Pb_3(PO_4)_2$ settles. Filter mixture into a Coplin jar. Support latter in an inclined position (at an angle of about 30°); place slides in it with the section facing downward.

2. Rinse slides in distilled water. Wipe precipitate from backs of the slides and around the tissue.

3. Remove superficial precipitate by placing slides in a 0.1 M citrate buffer of pH 4.5 to 5. As soon as the slide appears to be completely clear around the tissue, rinse it thoroughly under the tap. This differentiation is the most critical step in the procedure since insufficient treatment of the section may leave a precipitate, appearing black in the finished section, scattered all over the slide while overtreatment may remove part or all of the enzymatic reaction product.

4. Treat slides with ammonium sulfide, etc., as in the method for lipase.[6]

Results. The dependability of the method in its present form, as far as uniformity of results is concerned, is not entirely satisfactory. Usually, long series of sections from various tissues will stain quite uniformly. Occasionally, however, partial or total failures are observed. Some sections may fail to stain altogether; others will show a patchy distribution of the reaction; still others may show a reaction at an abnormal site such as the nuclei instead of the cytoplasm. It is a curious fact that the reaction will remain selective for the same cell of group of cells, regardless of differences in its intracellular localization. Some but not all of these irregu-

[6] Gomori, G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 362.

larities may be caused by improper fixation since they may show up in only one or two amidst a large number of perfectly well stained consecutive serial sections. Nonspecific impregnations may also occur; they can be recognized as such by incubating inactivated slides (treated for 10 minutes with Lugol's solution or with any N mineral acid) as controls. The inactivated sections will show the same artifacts.

In successful slides, however, there is a typical localization of the reaction, varying somewhat with different species. The enzyme is present mainly in the cytoplasm (often in a granular form) but also in some of the nuclei. Moderate amounts are found in many tissues such as the liver, where its distribution often but not invariably follows that of glycogen; in the secretory portions of the renal tubules; in the adrenal cortex; in the small intestine where its localization is similar to that of alkaline phosphatase; in the epithelial lining of the bronchi; in the beta cells of the pancreatic islets of the mouse (not constantly); in the lachrymal gland of the hamster; and many others. However, a very intense reaction, much stronger than at the sites mentioned, is obtained in two tissues: the grey matter of the central nervous system (especially that of the cerebellum), and in malignant tumors. Twenty-four adenocarcinomas of the gastrointestinal tract, 4 squamous carcinomas, 2 hypernephromas and a number of other epithelial tumors such as embryonic carcinomas of the testis, cancers of the prostate, breast and bronchi, Walker rat carcinomas No. 256 and a butter yellow tumor stained very intensely and selectively. The reaction may start quite abruptly at the border of the malignant change, or the normal tissue may show a slight to moderate staining up to a distance of 1 or 2 mm from the edge of the neoplasm. In a few cases where the neoplasm showed various degrees of atypia in its different portions, the most atypical portions showed the most intense staining. So far not a single carcinoma has been found out of a total of 64 cases which did not show a sufficient reaction to set it off sharply against its environment. With sarcomas the results were much less constant. Some of them did not stain at all; others stained in an uneven, patchy way, and only a few stained as uniformly as carcinomas did. Two papillomas of the bladder, questionably malignant, stained moderately heavily. Granulomas such as sarcoidosis and human tuberculosis usually did not stain or showed a faint to moderate reaction in the giant cells and, rarely, even in the epitheloid cells. Tubercles in the guinea pig stained rather heavily.

Summary. A histochemical method for the demonstration of sites of phosphamidase activity is described. Small amounts of the enzyme are present in many normal tissues; large amounts are found in the grey matter of the central nervous system and in malignant epithelial tumors.

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Inhibition of the Shwartzman Phenomenon by Local Application of Bromobenzene and Other Solvents.*

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When an intradermal injection of toxin derived from certain Gram-negative microorganisms is followed, after a period of approximately 20 hours, by an intravenous injection of similar bacterial toxin, a gross hemorrhage involving the entire injected skin area occurs. This hemorrhagic reaction has become known as the Shwartzman phenom-

* This work was aided by a grant from the Life Insurance Medical Research Fund.