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Microtechnical Demonstration of Phosphatase in Tissue Sections.*†

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In preliminary experiments the phosphatase activity of aqueous extracts of dog kidney cortex made (a) of fresh ground tissue, (b) of ground tissue dehydrated with several changes of 95% and absolute alcohol, (c) of dehydrated ground tissue exposed to 56°C for one hour was determined by the Berenblum-Chain[1] modification of Kay's[2] method. These experiments have shown that phosphatase is not destroyed either by alcohol or by exposure to 56°C in completely dehydrated state, the maximum decrease in activity observed having amounted to less than 20%. Moreover, purification of phosphatase by alcohol precipitation has been successfully used by Martland and Robison.[3] It appeared possible to demonstrate phosphatase in celloidin or paraffin sections on the basis of the following principle: If tissue sections containing active phosphatase are incubated with a solution of sodium glycerophosphate or of some other suitable ester-phosphate, such as hexose-phosphate or nucleinate, at a suitable pH, at the sites where phosphatase is present, PO_4 ions will be split off. These ions may be trapped at the spot by the salts of metals whose phosphates are insoluble. A precipitate of insoluble phosphate forms which, if visible, or made visible, indicates the presence of phosphatase. A useable technic has been worked out on the basis of this principle.

Fixation. Phosphatase is dissolved or destroyed by most of the routine fixatives. In alcohol, however, it is preserved for many weeks at least. Thin slices of fresh tissue should be fixed in 95% alcohol for about one day.

Unfortunately, decalcification is impossible, because all acids destroy phosphatase completely.

Embedding. Both celloidin and paraffin embedding is suitable. If the tissue is well dehydrated, exposure to paraffin at 56 to 60°C for 2 hours will do no harm. Celloidin sections are incubated

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1 Berenblum, I., and Chain, E., *Biochem. J.*, 1938, **32**, 295.

2 Kay, H. D., *Biochem. J.*, 1928, **22**, 855.

3 Martland, M., and Robison, R., *Biochem. J.*, 1929, **23**, 237.

with the substrate solution without any previous preparation, but paraffin sections must be protected with a thin film of collodion against loss of the enzyme by dissolution. The sections are dipped in a dilute solution (about 0.5 to 1%) of collodion in alcohol-ether. The membrane is hardened subsequently by dipping the section in 90% alcohol.

Incubation. The composition of the substrate fluid can be varied considerably according to the need. As a routine, 2 stock solutions, a 2% solution of sodium glycerophosphate, and a 2% solution of calcium nitrate, both in distilled water, can be used. One part of each of the solutions is added to about 6 to 8 parts of distilled water. In general, no buffering is necessary unless one wants to study the effect of a certain pH on the process. Needless to say, buffers forming insoluble calcium salts cannot be used, such as phosphate or borate buffers. Sodium barbital, acetate, or citrate buffers may be used. Acid media should be avoided because the solubility of calcium phosphate rapidly increases with the decrease of the pH. Any substance whose action on the process is to be tested can be added to the solution except substances which form precipitates with calcium (*e. g.,* fluoride). The time of exposure should be at least 2 hours at 37°C. Some increase in the intensity of the reaction has been observed for as long as 5 hours.

The salts of some other metals besides calcium have been tried to trap the phosphate ions but all of them proved to be much inferior to calcium. The heavy metals, as a rule, greatly inhibit phosphatase activity; others, while apparently indifferent to the enzyme (cobalt, nickel), on long exposure form compounds with the tissue proteins, thus making the selective demonstration of the phosphate precipitate difficult.

After incubation the sections are washed in a dilute solution of calcium nitrate and stored in it until the next step. Distilled water should not be used for this purpose because it may dissolve some of the precipitate.

Visualization of the precipitate. Since calcium phosphate is hardly visible, it is transformed into a colored compound. One of the following methods may be used:

1. Kóssa's method of exposure to a dilute solution of silver nitrate (about 0.5%) in direct sunlight. Calcium phosphate is transformed to silver phosphate. The latter, being photosensitive, will darken in sunlight. The whole reaction takes about 5 to 30 minutes, depending on the intensity of the sunlight.- Instead of sunlight, an ultraviolet light source may be used. After the desired color is obtained, the section is rinsed in distilled water and fixed in a dilute hypo solution

(1 to 2%). The sites of phosphatase are shown in a rich golden-brown color.

2. Transfer the section to a dilute solution of cobaltous nitrate (1 to 2%) for about 5 minutes. Rinse thoroughly in several changes of distilled water. Transform cobalt phosphate into black cobalt sulphide by immersing the section in a very dilute solution of yellow ammonium sulphide (a few drops to a Coplin jar) for a few minutes. Wash.

The sections can be counterstained with any stain desired. Besides silver and cobalt the salts of several other metals have been tried but proved inferior to the methods mentioned.

Results. Lack of space precludes description at length of pictures obtained. The main points of interest are: In normal animals belonging to the same species, individual variations of pattern are very slight; there may be more or less marked differences in the phosphatase picture of the same organ in different animal species, though in most cases the type of pattern is closely similar; there is some difference between pictures obtained with different ester-phosphate substrates. The tissues and cells richest in phosphatase are, respectively: The surface epithelium of the small intestine; the transitional epithelium of the urinary bladder; both epithelium and connective tissue of the mammary gland; the convoluted and straight

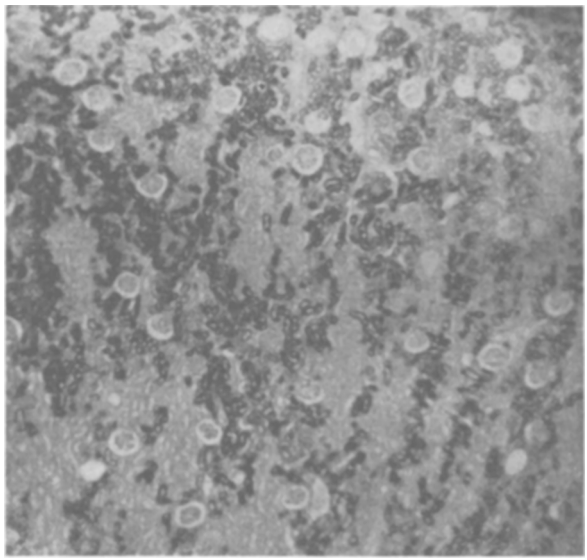

FIG. 1.
Renal cortex of the dog. Proximal convoluted tubules stained black. Magnification: 28 diameters.

FIG. 2.

Adrenal of the rabbit. Positive reaction (dark) in the cortex, especially in its deeper layers, and in islets of cortex tissue within the medulla. Medulla itself negative. Magnification: 100 diameters.

segments of the proximal convoluted tubules in the kidney; the deep layers of the adrenal cortex; the lining of the seminiferous tubules; ossifying cartilage and embryonic perichondrium; the adventitia of medium-sized arteries, and the endothelium of capillaries in certain organs. No phosphatase at all, or a minimal trace of it, is found in muscle and nervous tissue, stomach mucosa, skin, adrenal medulla, and the glomeruli and all parts of the kidney tubules distal to the transition of the proximal convoluted tubules into Henle's loops. Phosphatase pictures of liver, pancreas, and some other organs show marked differences in various animal species. The number of pathological specimens examined is too small yet to permit comprehensive statements, but the constant absence of phosphatase from young granulation tissue and from malignant epithelial tumor cells deserves mention.

The results of microtechnical demonstration and of chemical determination of phosphatase are in very good agreement.