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A Tetrazolium Method for Non-Specific Alkaline Phosphatase

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Summary. A technique for the histochemical demonstration of non-specific alkaline phosphatase was developed using a medium containing indoxyl phosphate and a tetrazolium salt, Nitro B.T. The tetrazolium salt was reduced to diformazan by the hydrogen ions released by the formation of either indigo or indigo white by reaction of the enzyme on the indoxyl phosphate.

The localization in the organs investigated was similar to that obtained by the standard azo dye and lead techniques.

Introduction

An indoxyl technique for the histochemical demonstration of non-specific alkaline phosphatase based on oxidation of indoxyl substrates to indigo dyes, was introduced by Seligman, Heyman and Barnett (1954). It was based on a similar technique for the demonstration of esterases (Barrnett and Seligman, 1951; Holt, 1956).

However, the localization of the phosphatase was different from that given by the Gomori (1941) lead technique. Holt (1954), using 5-bromo indoxyl phosphate instead of indoxyl phosphate, obtained essentially the same localization as that with the Gomori method. Holt (1956) emphasised that the production of indigos varied with pH. As the pH becomes more alkaline the production of indigo decreases and the production of colourless dehydroindigos increases. McGadey (1967), based his indoxyl acetate tetrazolium method for non-specific esterases on this principle (Fig. 2). The hydrogen relased by the formation of indigo or dehydroindigo reduced the tetrazolium salt to diformazan at the enzyme site.

In this paper this principle is again employed in an indoxyl tetrazolium method for non-specific alkaline phosphatase using 5-bromo-4-chloroindoxyl phosphate as substrate (Fig. 1).

Materials and Methods

Testis, kidney, small intestine and pancreas were removed from albino rats of the CR strain and snap frozen on carbon dioxide snow. 10 μ sections were cut off each of the organs in a cryostat maintained at -25° C and attached to clean dry coverslips by momentarily thawing. They were then air dried at room temperature. Incubation Medium:-2,5 mg 5-bromo-4-chloroindoxyl phosphate dissolved in 0.5 ml dimethyl formamide. 10 ml 0.2 M veronal acetate buffer pH 9.5. 5 mg nitro B.T.

The concentration of substrate and the pH of the buffer used were obtained from pilot concentration and pH incubation which ranged from 0.1—1.0 mg/ml and pH 8.5—10.5 respectively. Sections of the above organs were incubated in the medium for 10—20 min. They

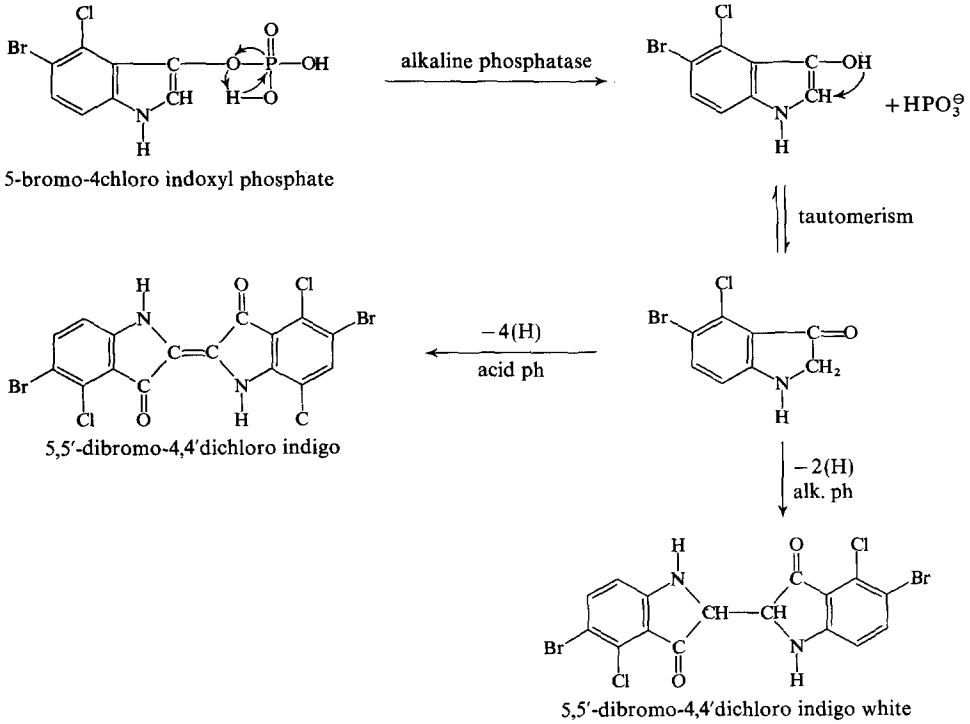


Fig. 1 shows the reaction of the indoxyl substrate in the production of hydrogens for the reduction of the tetrazolium salt

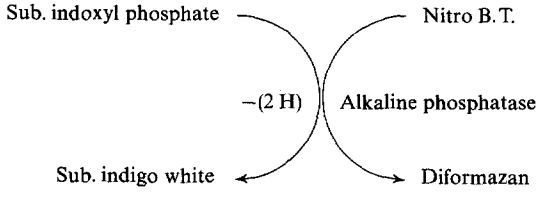
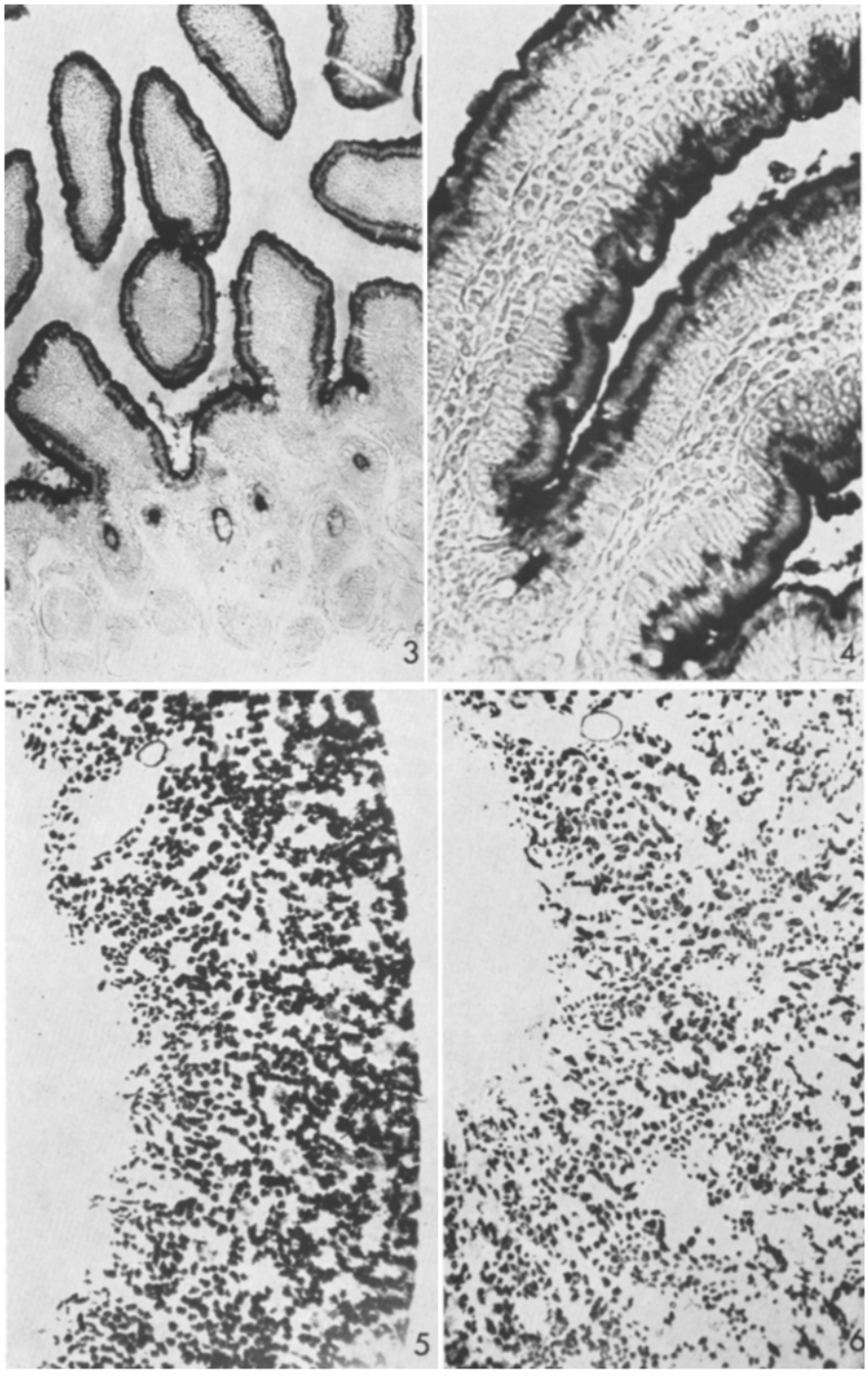


Fig. 2. General mechanism of the reaction

were then washed thoroughly in running water, fixed in 10% formalin for 30 min, dehydrated, cleared and mounted in D.P.X. Intense blue deposition of diformazan was obtained in the sites of enzyme activity.

Results

The localization found in all tissues examined was essentially the same as for the Gomori lead technique (Figs. 3 and 5) and the azo dye technique. In kidney strong deposition was observed in the proximal convoluted tubules



Figs. 3—6

(Fig. 6). The alpha cells of the pancreatic islets were positive and also the blood vessels. Small intestine deposition was localized to the brush border and the Golgi region of the surface epithelium (Fig. 4). In the testis the enzyme was localized in the basement membrane of the seminiferous tubules.

Discussion

The oxidation of indoxyl substrates by non-specific alkaline phosphatase results in the production of indigo and dehydroindigo, Holt (1956), with the release of hydrogen which reduces the tetrazolium salt to diformazan (McGadey, 1967; Fig. 2). The main source of hydrogen will probably be from the formation of the dehydroindigo as the optimum pH for the reaction is 9.5.

As seen from the results this technique gives essentially the same localization as that obtained by the Gomori and the azo-dye techniques. Drawbacks which these other methods have do not occur with this one. In the Gomori technique the deposition of non-specific lead may occur if prolonged incubation takes place and nuclear staining may also result. In the case of the azo dye techniques the final reaction products are soluble in alcohols and therefore cannot be mounted in synthetic media; also the coloured products are not stable for long periods. This method does not have these drawbacks as the diformazan is insoluble in alcohols and can be dehydrated, mounted in a synthetic medium and thus a permanent specimen obtained.

This reaction worked on formol calcium fixed, aceton dehydrated paraffin sections of rat small intestine.

This principle was also employed in an attempt to produce a similar method for acid phosphatase, but without success. This was possibly due to the fact that the acid pH does not favour the reduction of the Nitro B.T. to diformazan or because the initial reaction product indigo, is itself a coloured precipitate and inhibits the secondary reaction product being formed. The latter is perhaps the correct reason.

References

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Fig. 3. Azo-dye method for the alkaline phosphatase using α-naphthyl phosphate coupled with Fast Blue BB. in small intestine

Fig. 4. Indoxyl tetrazolium method in small intestine showing identical localization in the brush border and Golgi area as that with the azo-dye technique

Fig. 5. Gomori lead technique for alkaline phosphatase in the proximal convoluted tubules of rat kidney

Fig. 6. Indoxyl tetrazolium method in rat kidney showing the same localization as with the Gomori technique

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