A COUPLING HISTOCHEMICAL AZO DYE TEST FOR ALKALINE PHOSPHATASE IN THE KIDNEY

By MAUD L. MENTEN, JOSEPHINE JUNGE, AND MARY H. GREEN

(From the Department of Pathology and the Renziehausen Foundation, University of Pittsburgh and Children's Hospital, Pittsburgh)

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The relatively high concentration of alkaline phosphatase in the kidney suggests for this enzyme an important physiological rôle, the elucidation of which has been attempted by both chemical and histochemical methods. The latter methods are aimed at definitely establishing the loci of the enzyme activity in the kidney tissue. The basis of the currently used technique for histochemical demonstration is the formation of silver phosphate at the site of the alkaline phosphatase in the tissues. This technique involves successively hydrolysis of the glycerophosphate substrate, precipitation of the freed phosphate *in situ* as a calcium phosphate, conversion of the calcium salt by silver nitrate into silver phosphate with its subsequent blackening by sunlight. By means of this method or its modification, Gomori (1), Takamatsu (2), Hepler *et al.* (3), and Kabat and Furth (4) have shown this enzyme in large amounts about the brush border and within the lumen of the proximal convoluted tubule. The presence in the kidney tissue of inorganic phosphates, as well as those of the nucleoprotein and the phospholipids, detracts somewhat from the specificity of this technique. However, much of the inorganic phosphate and chloride as well may be removed from tissue sections by washing in distilled water before the test is performed. The question may be raised, however, as to whether the test medium is sufficiently alkaline to render soluble any silver chloride formed.

A new approach to the histochemical problem is the formation of an insoluble dye by utilization of the organic instead of the phosphate moiety of a phosphate ester, other than glycerophosphate, following splitting by the phosphatase. The essentials of this procedure are the hydrolysis of a monoaryl, instead of an alkyl organic phosphate, and immediately following the release of the aryl molecule its direct coupling with a diazotized amine to precipitate an insoluble dye at the site of the enzyme activity.

The formation of such an insoluble dye with the released β-naphthol of calcium β-naphthol phosphate is described in the following pages. The occurrence of the bulk of this dye in the proximal convoluted tubules of the kidney agrees generally with the renal distribution of alkaline phosphatase reported with other techniques.

Preliminary Procedures

The chemical details of the dye test were developed by one of us (M. H. G.). The procedure required the selection of a suitable phosphate ester, as well as an acceptable diazotized amine, in order that the resultant azo dye compound would be rapidly formed, insoluble, and of a deep easily recognized color. A suitable amine was readily available but, before a satisfactory enzyme substrate was obtained, several phosphate esters, mentioned below, were tested. Phenyl phosphates were first tried. Commercial disodium monophenyl phosphate was readily available for preliminary testing, but proved unsatisfactory because, although a dye was formed in the test-tube by reaction of the phosphatase-liberated phenol with any of the following diazotized amines, namely *p*-nitroaniline, toluidine, or α-naphthylamine, the dye formed in kidney sections was not sufficiently insoluble and was not retained *in situ* in the tissues. Phenyl phosphates were accordingly eliminated.

Members of the naphthol phosphate series were next tried. Trial coupling reactions performed with β-naphthol and each of the following diazotized amines, namely *p*-nitroaniline, *p*-aminoacetophenone, and α-naphthylamine, yielded insoluble dyes of different colors and varying low solubility. α-Naphthylamine was selected because of the deep red to purple color and extreme insolubility of the coupled product. β-Naphthol phosphate was not available commercially and it or its salt had to be prepared. Barium β-naphthol phosphate, the first compound prepared, was insoluble in water. An attempt was next made to prepare β-naphthol-phosphoric acid ester from the barium salt. The two samples of the acid ester which were prepared were small in amount and contained traces of barium which apparently slowed the hydrolytic activity of the phosphatase in tissue sections. Although paraffin sections of kidney, treated at a suitable pH with the acid ester prepared by sulfuric acid, showed the precipitated purple dye to be located at the expected site in the kidney tissue, the test was almost negative at the end of 2 hours and a period of 20 to 24 hours was needed for the dye to acquire its maximum intensity. During this time secondary black dust-like deposits formed over the sections. Calcium β-naphthol phosphate was the ester which finally gave satisfactory dye precipitates, ranging in color from a red to a deep red-purple.

Methods

Preparation of Reagents

Preparation of Monoaryl Phosphate Ester. Barium β-Naphthol Phosphate—Barium β-naphthol phosphate was prepared by a modification of King and Nicholson's method (5) for the preparation of phenylphosphoric

acid esters. To 10 ml. of phosphorus oxychloride, a solution of 15.8 gm. of β-naphthol in 50 ml. of dry pyridine was added with stirring. The reaction was allowed to proceed for 20 minutes and the resulting β-naphthol phosphoryl chloride was decomposed with 5 ml. of water. A saturated aqueous solution of barium hydroxide was added until the mixture turned pink to phenolphthalein. The barium β-naphthol phosphate precipitated almost completely and, after the mixture was chilled in the refrigerator, it was filtered with suction, washed with 50 per cent and then with absolute alcohol, and air-dried. An attempt to convert the insoluble barium compound into the disodium β-naphthol phosphate failed because of the insolubility of the former.

Preparation of β-Naphtholphosphoric Acid Ester—The barium β-naphthol phosphate was suspended in an equivalent amount of 4 N hydrochloric acid and the acid solution extracted with ether. A small amount of acid ester crystallized out of the ether, but the ester was so difficultly soluble in ether that the yield was poor. An unsuccessful attempt was then made to obtain the acid ester in greater yield by taking up the barium salt in dilute sulfuric acid, thereby precipitating barium sulfate and removing the precipitate by centrifugation. This acid ester was also contaminated with traces of barium and furthermore much of the ester was hydrolyzed in preparation.

Preparation of Calcium β-Naphthol Phosphate—Preparation of this ester was exactly like that described above for barium β-naphthol phosphate up to decomposition of the phosphoryl chloride. At this point saturated aqueous calcium hydroxide was added until the reaction mixture became pink to phenolphthalein (approximately 18 gm. in amount). The precipitate was filtered after being chilled and washed with 50 per cent alcohol, then with absolute alcohol, and air-dried. The calcium salt of this ester was more soluble than the barium salt and gave a large yield. An additional crop of calcium naphthol phosphate was obtained by adding alcohol to the combined filtrate and washings. The calcium β-naphthol phosphate was readily hydrolyzed in the test-tube by the phosphatase contained in a thin slice of fresh kidney. A strong coupling reaction obtained with diazotized naphthylamine yielded a red-purple dye in trial tests *in vitro* within as short a time as 10 minutes. With the application of this chemical procedure to mounted sections of alcohol-fixed kidney the dye was visible to the naked eye in a section 6 μ in thickness in a few minutes.

Diazotization of α-Naphthylamine

A slight excess, namely 2.5 instead of 2 equivalents, of 2 N hydrochloric acid was added to 0.2 gm. (0.0014 mole) of α-naphthylamine suspended in

a little water. The use of freshly sublimed α-naphthylamine is advised in order to eliminate any degradation products of oxidation. Saunders ((6) p. 4) recommends the use of excess acid in order to insure complete diazotization. The mixture was shaken until the base was completely dissolved and the solution diluted to 50 ml. The solution was chilled to 10–12°, and to each 50 ml. of amine 5 ml. (1 equivalent) of a freshly prepared solution of sodium nitrite (0.9884 gm. dissolved in 50 ml. of water) were added. The diazotization solution was tested with starch iodide indicator and "balanced" by adding more nitrite or more naphthylamine hydrochloride as required until the end-point was reached (Saunders (6) p. 5). Diazotization was complete after the mixture had reacted for 15 minutes at 10°. The diazotized solution should be used immediately following completion of diazotization.

Preparation of Tissue

Young adult white rats of the Wistar strain and white mice, both with fasting for 24 hours and without, were killed by a blow on the head and the kidneys immediately removed and fixed. About 60 rats were used. Necropsy tissues from a few human kidneys were also studied. Slices of kidney 3 to 4 mm. in thickness were fixed in 95 per cent alcohol or in cold acetone. Alcohol-fixed tissues were run through two changes of absolute alcohol to xylol. Acetone-fixed tissues were run through three changes of special pure acetone for 48 hours in the refrigerator and followed by benzene for 45 minutes. Both sets of tissues were then treated with two changes of paraffin, each of 2 hours duration in the thermostat, and embedded. Paraffin sections 6 μ in thickness, mounted on slides, were run through the xylol and alcohols to water just previous to immersion in the "dye" solution. Acetone fixation appeared to afford somewhat greater retention of phosphatase in the tissue, but was accompanied by a considerable amount of shrinking and distortion of the tissues owing to rapid dehydration.

Technique of Phosphatase Test

20 cc. each of a substrate suspension of calcium β-naphthol phosphate (roughly 0.3 gm. of dried ester in 250 cc. of distilled water) and diazotized α-naphthylamine were mixed and the reaction immediately adjusted by a Beckman pH meter to the required pH by the addition of 6 N NaOH. Tests were always made at the optimal reaction of pH 9.4 and frequently at pH 9.0, 8.0, and 7.4 as well. Following adjustment of pH, 20 cc. of buffer solution of the HCl-veronal buffer mixture of Michaelis (7) were added to obtain the desired pH. Upon the addition of the alkali a brown precipitate formed which was removed by rapid filtration through a Buch-

ner funnel and quickly cooled to approximately 10°. The mounted tissue sections were immediately placed in a staining dish filled with this filtrate and slides were removed after incubation of 15 and 30 minutes and 1 and 2 hours in a refrigerator at 6°, washed in water, counterstained with light green if desired, and mounted in 50 per cent glycerol. The positive phosphatase reaction was manifested by the formation of a deep red to purple dye.

The intensity and speed of the reaction bear a direct relationship to the H ion concentration and to the temperature at which the enzymic hydrolysis is carried out. The optimal H ion concentration is at pH 9.0 to 9.4, where a maximum precipitate is formed. Definite though weaker reactions requiring a longer time to give a maximum precipitate occur at pH 8.0 and even at pH 7.4 with the kidney sections of most animals. The coupling of diazo compounds with the β-naphthol to form an insoluble azo dye occurs only in alkaline solution and this fact may be in part responsible for the less intense reactions observed at pH 7.4. Under the conditions of the experiment a slight fall in pH may occur during the course of the test. In the optimal 18 minutes incubation period this change in pH was not enough to affect the results noticeably and at the end of this period the concentration of active diazo compounds in the substrate mixture was still sufficient to give a heavy dark red precipitate with β-naphthol.

The "dye-coupling" test is characterized by an extreme rapidity of reaction. A strong deposition of the dye could be observed as early as 5 minutes after exposure of the phosphatase-containing tissues to the substrate preparations at the optimum pH. In 15 minutes at ice box temperature (4–6°) a very intense reaction could be obtained. Further incubation only moderately increased the precipitate. Tests performed at 37° showed a correspondingly enhanced reaction, but incubation at 4–6° was preferable in order to retard possible disintegration of the diazotized amine compound and formation of secondary products which are accelerated at higher temperatures upon prolonged incubation.

The dye which is precipitated in the tissues is not the actual phosphatase but a compound formed with the naphthol freed by the hydrolytic action of the enzyme. The dye reaction is complex and in addition to the specific red-purple precipitate, resulting from enzymic action in the tissue, secondary products may be formed during the diazotization which behave as simple diffusible stains. These stains diffuse through the tissue, giving a generalized yellowish background tissue color. This diffuse color does not alter the location or visibility of the phosphatase precipitate. To minimize or prevent such ancillary reactions, a high pH (9.0 to 9.3) should be established and the performance of the test not unduly prolonged.

Control Procedures

Certain preliminary control measures were observed in carrying out the test. Control tests performed upon the β-naphthol ester solution showed it to contain no free naphthol as determined by the color test for phenolic compounds with the Folin-Ciocalteu reagent. A further test carried out by exposure of this ester solution to serum known to contain phosphatase resulted in the freeing of sufficient β-naphthol by hydrolysis of the phosphate ester to produce with the Folin-Ciocalteu reagent the appearance of a deep blue color which was absent in phosphatase-free serum controls. It was demonstrated, therefore, that the ester preparation employed contained no free β-naphthol but could be hydrolyzed by substances containing phosphatase with the resultant liberation of β-naphthol.

A second series of control tests, performed upon the diazotized α-naphthylamine solutions used, showed that the addition of the β-naphthol ester to properly buffered solutions produced no red dye precipitates in the absence of enzymic substances. If, however, a small quantity of β-naphthol was added to the alkaline diazo solution, an immediate red dye precipitate was formed.

Control histochemical tests in which prepared tissue sections were incubated with alkaline buffered diazo compounds, in the manner of the regular procedure but with the omission of β-naphthol phosphate substrate, exhibited no red dye precipitate.

Control reactions gave considerable evidence of the inhibiting effect of heat. Immersion of rat tissue sections, immediately before testing for phosphatase, in a water bath at a temperature of between 75–80° for 10 minutes resulted in complete inactivation of the phosphatase, as shown by absence of any color reaction.

In view of the general instability of all diazo compounds it is necessary to carry out the diazotization of the α-naphthylamine immediately prior to use in the test and to allow as little time as possible to elapse between completion of diazotization, mixing the ester substrate, pH adjustment and addition of buffer, and the immersion of the tissues for testing. Even under apparently optimal conditions with a short incubation period (15 to 18 minutes) at 4–6°, yellow-brown disintegration products may form in the solution. It is possible that the test may be further refined by procedures aimed at stabilizing the diazo compound. In its present form the test is intricate and probably is not adapted to routine use without modification.

Results

The histochemical dye technique for alkaline phosphatase has been extensively used on tissue from mouse, rat, and human kidneys. It may be briefly stated here that our results in general confirm those reported with

the Gomori and Takamatsu techniques. The phosphatase occurred mainly in the cortex, where it was observed in definite locations. The medulla was for the most part free of the enzyme. Species variations and minor differences due to physiologic conditions or pathologic changes will be published elsewhere. Our results premise that the principle of azo dye formation by means of coupling with products mediated by tissue phosphatase may be applicable to the histochemical demonstrations of other enzymes in tissues.

SUMMARY

1. A histochemical test for alkaline phosphatase based on the precipitation of an insoluble azo dye formed by coupling of β-naphthol, derived from hydrolysis of calcium β-naphthol, and diazotized α-naphthylamine has been described.

2. With the dye test, the location of the phosphatase in the kidney agrees substantially with that obtained by the silver technique of other investigators.

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