The Histochemical Demonstration of a Specific Phosphatase (5-Nucleotidase)

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Animal tissues contain a specific phosphatase named 5-nucleotidase, which attacks only nucleotides with the phosphate group attached to C-5 of a pentose (Reis, 1937, 1940, 1951; Heppel & Hilmoe, 1951). The object of the present study was to adapt Gomori's method for histochemical demonstration of alkaline phosphatase (Gomori, 1939, 1946), to the demonstration of 5-nucleotidase.

Gomori (1949) and Newman, Feigin, Wolf & Kabat (1950) investigated the specificity of phosphatases using Gomori's histochemical method. This consists of the incubation of tissue sections fixed in cold acetone in a buffered solution containing a phosphoric ester and a calcium salt. The orthophosphate produced by enzymic hydrolysis forms insoluble calcium phosphate, which is precipitated on the slide and visualized by one of the methods adopted for staining calcium in the tissues. Many of the results of the above authors differed greatly from those which we have obtained by chemical estimations. Newman *et al.* (1950) failed to find any histochemical evidence of 5-nucleotidase activity in thyroid and testicle, although extracts of both these tissues have a high 5-nucleotidase activity (Reis, 1951), and Gomori was unable to demonstrate 5-nucleotidase in adrenal gland or placenta, both of which were found by Reis (1937) to contain this enzyme in moderate amounts. These differences may be explained partially by the inactivating action of the histological fixation, but it seems probable also that the pH (9.2) used by Gomori and by Newman *et al.* was not suitable for demonstrating 5-nucleotidase activity, as this enzyme has its optimum near pH 8.0.

Gomori, and Newman *et al.* estimated the presence of 5-nucleotidase by comparing two slides incubated at pH 9.2, one with adenylic acid, the other with β -glycerophosphate. The difference between the two was taken as corresponding to the activity of 5-nucleotidase, since adenylic acid is hydrolysed by both 5-nucleotidase and by alkaline phosphatase, whereas β -glycerophosphate is hydrolysed by the alkaline phosphatase only. Such results may be clear enough when 5-nucleotidase is present in the examined tissue in the absence of alkaline phosphatase. If both enzymes are present, however, it is difficult to judge whether the heavier precipitates

with adenylic acid are really due to 5-nucleotidase action. Not only is the degree of precipitation not exactly proportional to enzyme activity, but the visual comparison is also subject to error.

The different activity of alkaline phosphatase against various phosphoric esters has also to be taken into account. Taking, for an example, the phosphatase activities of an extract from rabbit lung (Fig. 1), at pH 9.0 it is obviously difficult to say whether the difference between the adenylic acid and β -glycerophosphate hydrolysis is due to the 5-nucleotidase activity, or whether it is due to a different activity of alkaline phosphatase on adenylic acid. This varying activity of alkaline phosphatase is illustrated by the hydrolysis of phenylphosphate which, as was shown by King & Delory (1939), at pH 9.0 is about four times as fast as that of β -glycerophosphate, while that of adenylic acid is between the two.

Fig. 1. Enzymic hydrolysis of phosphoric esters by an extract of rabbit lung. 5 mg. of tissue (dialysed extract), 20 min., temp. 38°, substrate concentration 0.0008 M. Details of technique as described by Reis (1951).

The chemical estimation of 5-nucleotidase activity was carried out at pH 7.5, since this is near to the optimal pH of this enzyme (pH 7.8 in human tissues), and also because at this pH the rates of hydrolysis of phenylphosphate and β -glycerophosphate are both very low at 7.5, and the hydrolysis of adenylic acid by non-specific phosphatases has a

similar low value. In subtracting such a small value from the total hydrolysis of adenylic acid, we probably make a much smaller error than in subtracting the far larger values for non-specific hydrolysis obtained at pH 9.2. It therefore seemed worth while to try this pH for the histochemical method also, in spite of the increase in the solubility of calcium phosphate and the consequent expectation of an increase in diffusion of this product. The additional advantage of estimations at pH 7.5 is that they give a picture of enzyme activities at the physiological pH.

METHOD

Thin blocks of tissue were fixed, according to Gomori's (1946) method, in two or three changes of cold absolute acetone at 4° for 24 hr. They were then transferred progressively to absolute ethanol, absolute ethanol-ether with one or two changes, and thence to 1% celloidin. After draining off any excess of the latter the blocks were hardened in CHCl_3 , cleared in benzene and embedded in paraffin wax. Sections were cut at 5 μ . and mounted on albuminized slides. Heating on the hot stage was avoided by drying the slides for 3 hr. in a 37° incubator. Slides and blocks were then stored at 4° until required for incubation. This latter process was carried out by removing the wax from the slides by immersion in light petroleum and smearing a line of vaseline round the sections to retain the buffer-calcium-substrate solutions. When these had been added the slides were put into Petri dishes or into Perspex boxes, with a few drops of distilled water nearby to minimize evaporation. They were incubated at 37° for varying times. The necessary development of calcium phosphate precipitates required from 3 to 24 hr., according to the degree of enzyme activity.

To demonstrate the 5-nucleotidase activity three slides were incubated, as a rule, at pH 7.5, one as a blank test with water instead of substrate, one with phenylphosphate and one with adenylic acid. The difference between the sections incubated with adenylic acid and phenylphosphate corresponds to the action of 5-nucleotidase. Generally, at pH 7.5, phenylphosphate incubation gave no Ca precipitate. To demonstrate alkaline phosphatase activity an extra slide was usually incubated with phenylphosphate at pH 9.

Solutions

Buffer for 5-nucleotidase (pH 7.5). Sodium diethylbarbiturate, 0.1 M, 3 vol.; HCl, 0.1 N, 2 vol.; distilled water, 1 vol.

Buffer for alkaline phosphatase (pH about 9). Sodium diethylbarbiturate 0.1 M, 1 vol.; distilled water, 1 vol.

Substrates. Sodium phenylphosphate, sodium β -glycerophosphate, or adenylic acid, 0.04 M.

The pH values of the substrates and the Ca and Mg solutions were adjusted to 7.5 (with 0.1 N-HCl or NaOH).

The incubating fluids were prepared by mixing 5 vol. of a buffer solution with 1 vol. of 12% (w/v) $\text{Ca}(\text{NO}_3)_2$, 1 vol. of 2% (w/v) MgCl_2 and 1 vol. of substrate solutions.

After incubation the slides were washed with 2% $\text{Ca}(\text{NO}_3)_2$ of pH about 8.0, covered with 1% AgNO_3 and exposed to daylight for about 1 hr. The Ag solution was then removed with distilled water and the slides covered with 5% $\text{Na}_2\text{S}_2\text{O}_3$ for 10 min. They were then washed, dehydrated

in ethanol, cleared in xylene and mounted in neutral Canada balsam or in a synthetic medium such as D.P.X. (Kirkpatrick & Lendrum, 1941). The latter causes rapid fading of the precipitates if the cobalt method is used, but only slight deterioration of Ag precipitates.

The AgNO_3 method of visualization of calcium phosphate was preferred to the $\text{Co}(\text{NO}_3)_2$ -yellow $(\text{NH}_4)_2\text{S}$ method, since it gave darker pictures and less non-specific staining.

RESULTS

Fig. 2 shows the action of alkaline phosphatase in rabbit lung at pH 9.0 (substrate: phenylphosphate, 3 hr. incubation). The sites of maximal alkaline phosphatase activity are the bronchial epithelium and the endothelia of arteries and veins. The staining of the nuclei may be an active enzymic process, but is almost certainly due to the diffusion of calcium phosphate. The control slides incubated without substrate and with phenylphosphate or β -glycerophosphate at pH 7.5, showed after 3 hr. no precipitate in either case. Fig. 3 illustrates adenylic acid hydrolysis at pH 7.5 (3 hr.) in a serial section to that of Fig. 2. The precipitate corresponds entirely to 5-nucleotidase activity. Here the medial coat of the artery shows the most intense staining, and only some adjacent nuclei are also stained. Further away from the maximum sites of activity the nuclei are unstained, indicating that this effect is due to diffusion. When adenylic acid was used at pH 9, both medial coat and endothelia were stained.

In tissues where chemical estimations indicated substantial 5-nucleotidase activity this was confirmed by the histochemical method. In human thyroid, aorta or nerve we obtained pronounced staining after 18 hr. incubation with adenylic acid at pH 7.5, whereas these tissues showed only traces of alkaline phosphatase activity, when phenylphosphate or β -glycerophosphate at pH 9 were used for the same period.

Figs. 4 and 5 show the alkaline phosphatase and 5-nucleotidase activity of the human pituitary gland. The substantial 5-nucleotidase activity of the posterior pituitary was first demonstrated histochemically, and afterwards confirmed by chemical estimations (Reis, 1951). Incubation for 3 hr. was quite sufficient to get a good staining with this tissue; in no other human tissue was a comparable 5-nucleotidase activity found.

DISCUSSION

The use of adenylic acid at pH 7.5 allows the histochemical demonstration of 5-nucleotidase activity, which at this physiological pH is in many tissues much greater than that of the non-specific phosphatases. The staining method which we have evolved is therefore interesting in that it reflects

physiological conditions, but it suffers from the disadvantage that the solubility of calcium phosphate is much greater at pH 7.5 than at 9, so that diffusion phenomena are greater. It is therefore impossible, with the method described, to localize 5-nucleotidase within a single cell; only the activity of a group of cells or a whole tissue may be demonstrated. Finer differences in calcium phosphate deposition are due to diffusion.

Gomori (1950) makes the objection that the interpretation of phosphatase activity by his method, when performed between pH 7.4 and 8.4, is very doubtful, especially when long incubation times are used (more than 48 hr.). Our incubations never exceeded 24 hr., and in the case of rabbit lung and human pituitary gland they lasted 3 hr. only. In view of the diffusion of calcium phosphate at pH 7.5, with the 5-nucleotidase method incubation should be restricted to periods of 6 hr. or less and not prolonged to 24 hr. or more, as in the case of alkaline phosphatase. In Fig. 3 the effect of diffusion may be evaluated by the staining of the nuclei, and it can be observed to affect a limited area only. The histochemical findings confirm the chemical estimations, in other tissues as well as in artery wall and posterior pituitary.

We are well aware that by the method described only the sites of maximal 5-nucleotidase activity are demonstrated. The sites of lower 5-nucleotidase activity, as well as the sites of alkaline phosphatase activity, are usually not visualized at pH 7.5, but the positive reaction with adenylic acid at pH 7.5 cannot be considered as an artifact since it corresponds to the chemical estimations and is to a large extent proportional to the time.

SUMMARY

1. A method has been evolved, based on Gomori's alkaline phosphatase method, for histochemical demonstration of specific phosphatase (5-nucleotidase) activity, by incubation with adenosine-5'-phosphate at pH 7.5.

2. Examples of 5-nucleotidase staining are given, and the limits of localization by this method are discussed.

3. In human tissues, the strongest 5-nucleotidase staining was found in the posterior pituitary gland.

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EXPLANATION OF PLATE 4

Fig. 2. Rabbit lung. Stained for alkaline phosphatase (phenylphosphate, pH 9) which is present (top) in the endothelium of a large artery, and (bottom) in the epithelial lining of a bronchus. ($\times 100$.)

Fig. 3. Rabbit lung (serial section to Fig. 2). Stained for 5-nucleotidase (adenylic acid pH 7.5) which is concentrated in the medial coat of the artery and absent from the endothelium. ($\times 100$.)

Fig. 4. Human pituitary gland. Posterior part at the top, anterior below. Stained for alkaline phosphatase (phenylphosphate, pH 9). ($\times 18$.)

Fig. 5. Human pituitary gland (serial section to Fig. 4). Stained for 5-nucleotidase (adenylic acid, pH 7.5). ($\times 18$.)

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.