COLORIMETRIC ESTIMATION AND CYTOCHEMICAL LOCALIZATION OF ALKALINE RIBONUCLEASES WITH α-NAPHTHYL URIDINE-3'-PHOSPHATE

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A procedure has already been described for the cytochemical localization of kidney phosphodiesterase, PDase I, based on the use of a synthetic specific substrate, α-naphthyl thymidine-5'-phosphate (Sierakowska & Shugar, 1963; Sierakowska, Szemplińska & Shugar, 1963), and coupling of the enzymatically liberated naphthol with a suitable diazotate according to the standard azo-dye coupling technique.

It was pointed out then that analogous α-naphthyl purine and pyrimidine riboside-2'(3')-phosphates might well prove to be suitable substrates for the localization of ribonuclease type enzymes, subject to limitation of the diffusion of the latter from fixed tissue sections (Sierakowska & Shugar, 1960). A suitable substrate along these lines for pancreatic ribonuclease, RNase I, has now been prepared. Furthermore, during the course of preliminary tests with this compound, it was found, somewhat to our surprise, that earlier reports on the inactivation of tissue RNase as a result of formalin fixation (e.g. Jonsson & Lagerstedt, 1959) are at least partially in error; and that the tendency of RNase I to diffuse out of formalin-fixed sections during incubation is relatively low. The necessary conditions for the specific cytochemical localization of RNase I consequently appear to exist.

Of five variants employed for the preparation of α-naphthyl uridine-3'-phosphate, the two most promising involve the use of tetrahydropyran as a selective blocking agent for the carbohydrate hydroxyls. In the first of these, uridine-3'-phosphate was treated with dihydropyran to give 2',5'-di-O-tetrahydropyranyluridine-3'-phosphate (Rammler & Khorana, 1962). The phosphate group was then removed with the aid of nonspecific phosphomonoesterase, and the resulting 2',5'-di-O-tetrahydropyranyluridine condensed with the pyridinium salt of naphthyl phosphoric acid in the presence of dicyclohexylcarbodiimide to give α-naphthyl 2',5'-di-O-tetrahydropyranyluridine-3'-phosphate. Removal of the blocking groups in 80% acetic acid at room temperature gave the required α-naphthyl uridine-3'-phosphate.

An alternative procedure involved the treatment of uridine with dihydropyran for 2 hours at a temperature not exceeding 28-30° to give a mixture of starting compound (30%), 5'-O-tetrahydropyranyluridine (40%) and 2'(3'),5'-di-O-tetrahydropyranyluridine (30%). The R_f values in ascending chromatography with Whatman paper 1 and water-saturated butanol as solvent were, respectively, 0.18, 0.61, 0.80. The 5'-O-tetrahydropyranyluridine was isolated from the reaction mixture by chromatography on thick sheets of paper, and treated with naphthyl phosphoric acid as above to give α-naphthyl 5'-O-tetrahydropyranyluridine-2'(3')- phosphate. Removal of the blocking agent with 80% acetic acid gave α-naphthyl uridine-2'(3')-phosphate, which can be used as a general substrate for nuclease enzymes. The two isomers may be separated by column chromatography, or the 2' isomer isolated following enzymatic hydrolysis of the 3' isomer.

α-naphthyl uridine-3'-phosphate (and presumably also the 2' isomer) is slightly labile, decomposition leading to liberation of free naphthol and uridine-2':3'-cyclic phosphate.

The free acid, stored over P_2O_5 under vaccum, was more stable than the sodium salt. Immediately prior to use, the free acid is converted to the sodium salt by neutralization with NaOH. In neutral or slightly alkaline medium only trace amounts of naphthol are liberated over a period of several hours, and these are readily removed simply by extraction with ether.

α-naphthyl uridine-3'-phosphate is resistant to DNases and PDases I and II and is completely hydrolyzed by RNase I to naphthol and uridine-2':3'-cyclic phosphate, the latter in turn undergoing a slow conversion to uridine-3'-phosphate. The rate of enzymatic hydrolysis of α-naphthyl uridine-3'-phosphate is at least 100-fold greater than that for uridine-2':3'-cyclic phosphate, in agreement with the qualitative findings of Ehinger & Lagerstedt (1959) for the relative rates of hydrolysis of benzyl cytidine-3'-phosphate and cytidine-2':3'-cyclic phosphate, but in sharp contrast to the results of Witzel & Barnard (1962) on methyl and benzyl cytidine-3'-phosphates. This relatively rapid rate of hydrolysis of the naphthyl ester is naturally advantageous for the colorimetric estimation of RNase I, and as a cytochemical substrate.

For the colorimetric estimation of crystalline RNase activity, or the RNase content of tissue homogenates, 0.01M substrate in 0.05M tris-HCl buffer pH 7.2 containing 0.003M $MgCl_2$ was incubated with either 0.05 - 0.20 μg RNase or 20 μl 0.1% pancreatic tissue homogenate in a total volume of 180 μl. The reaction was terminated by addition of 100 μl of a solution containing 4 mg./ml. Fast Red TR and 80 μl of 0.2M tris buffer pH 9. The resulting azo-dye color was then photometered at 500 mμ against an enzyme-free control.

Rat organs were employed for cytochemical localization

of alkaline RNase. The animals were sacrificed under ether anaesthesia and the various tissues examined as 20 μ frozen sections of formalin-fixed material and 10 μ paraffin sections of acetone-fixed tissue. Some tests have also been run on 10 μ fresh frozen sections.

The incubation medium contained 2 mg./ml. substrate and 4 mg./ml. Fast Red TR in 0.1M tris-HCl buffer pH 9, which provided optimal conditions for dye formation. This pH is somewhat higher than that optimal for alkaline RNase activity, but the decrease in enzyme activity was more than compensated for by the increased rate of insoluble dye formation. Incubations were at room temperature for periods ranging from 2 to 30 minutes. Control sections, prepared by heat inactivation in water at pH 10, were always negative.

Sections fixed in Baker's formol-calcium exhibited no evidence of diffusion of the enzyme. Up to several hours rinsing in water of formalin-fixed pancreas sections was without effect on the intensity of the reaction or the nature of the localization. By contrast, and in agreement with previous data (Sierakowska & Shugar, 1960), sections fixed either in acetone or alcohol exhibited considerable loss in enzyme activity following even short rinsing periods in water. Immersion of such sections in the incubation media was almost instantly succeeded by the appearance in the solution of the azo-dye product, which subsequently deposited non-specifically over the entire section and on the surface of the slide adjacent to the section.

Formol-calcium fixed pancreas sections exhibited intense enzymatic activity in the apical portion of the acinar cells adjacent to the lumen; the remaining cytoplasmic area of the acinar cells was less active. No positive reaction was observed in the nuclei, the islets of Langerhans or the blood vessels.

Some activity was visible in the excretory ducts and the epithelium of the excretory duct adjoining the lumen. By contrast acetone-fixed sections showed no activity in the acinar cells, diffusion of the enzyme being noted throughout the course of incubation; only the blood vessel walls showed activity, with no signs of enzyme diffusion.

In formol-calcium fixed kidney sections there was appreciable activity in the brush border of the epithelial cells of the proximal tubules, with no visible activity in the nuclei. Other parts of the nephron and the Malpighi corpuscles were negative. No activity was observed in the medulla. Results were the same following acetone fixation, with no indication of enzyme diffusion as for the pancreatic acini enzyme.

Full details for preparation of substrate, data on the effects of various fixatives, and photographs of localization patterns in different tissues, will be published elsewhere (Zan-Kowalczewska, Sierakowska & Shugar, 1965). Attempts are also under way to extend the above procedure to the localization of acid RNase and ribonucleases active against the 2' isomer, and to the synthesis of α-naphthyl purine ribonucleoside-2'(3')-phosphates for other types of ribonucleases.

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