CYTOCHEMICAL LOCALIZATION OF PHOSPHODIESTERASE BY THE AZO DYE SIMULTANEOUS COUPLING METHOD

Halina Sierakowska & D. Shugar

Institute of Biochemistry & Biophysics, Academy of Sciences; and Dept. of Biochemistry, State Institute of Hygiene, Warsaw.

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Two major difficulties have previously been encountered in attempts at the cytochemical localization of ribonuclease and deoxyribonuclease enzymes (Sierakowska & Shugar, 1960, 1961): (a) the lack of a sufficient number of substrates of adequate specificity, and (b) the readiness with which both classes of enzymes diffuse out of fresh frozen or fixed tissue sections during incubation. The film-substrate technique (Daoust, 1957; Daoust & Amano, 1960), upon which a limited degree of specificity may be conferred by the use of suitable oligo- and polynucleotide substrates (Sierakowska & Shugar, 1961), is rather restricted in its application since it provides at best only gross histochemical localization.

The preparation by Razzell & Khorana (1959) and Turner & Khorana (1959) of p-nitrophenyl thymidine-5'-phosphate and p-nitrophenyl thymidine-3'-phosphate, which exhibit different specificities towards kidney and spleen phosphodiesterases, PDase I and PDase II respectively according to the nomenclature of Razzell (1961), prompted us to prepare the analogous α-naphthyl phosphodiesters. It was expected that the latter might serve as substrates for the two phosphodiesterases in the standard cytochemical azo dye method in which the

enzymatically liberated naphthol is coupled with appropriate diazotates to form an insoluble dye at the sites of enzymatic activity (Pearse, 1960).

Tp-naphthyl (α-naphthyl thymidine-3'-phosphate) proved, in fact, to be relatively resistant to PDase II; supplementary experiments with other substrates such as the dinucleotide TpT have shown that this is due to steric hindrance by the aryl substituent. On the other hand naphthyl-pT (α-naphthyl thymidine-5'-phosphate) was rapidly hydrolyzed by PDase I to thymidine-5'-phosphate; since, as expected, it was found to be completely resistant to both pancreatic DNase I and spleen DNase II, it appeared to be a promising cytochemical substrate for PDase I. This was further confirmed when it was found that PDase I showed little or no tendency to diffuse out of fixed tissue sections.

Tp-naphthyl and naphthyl-pT were prepared by the treatment of 5'-trityl thymidine and 3'-acetyl thymidine, respectively, with α-naphthylphosphoryl dichloride (Friedman & Seligman, 1950) essentially as described by Turner & Khorana (1959) for the corresponding nitrophenyl 3' analogue. Final purification of the products required, however, the use of either chromatography or exhaustive digestion with phosphomonoesterase in order to eliminate traces of contaminating α-naphthyl phosphate, which would be expected to interfere in the cytochemical tests. In ascending chromatography on Whatman paper No. 1 with water-saturated butanol as solvent, the R_f values of thymidine, Tp-naphthyl and naphthyl-pT were 0.52, 0.36 and 0.32 respectively.

Albino rats were sacrificed under ether anaesthesia and thin slices of tissue were fixed overnight in formol-

calcium at 4°C. Sections were cut at a thickness of 15 μ on a freezing microtome, mounted on slides, and allowed to dry for about an hour prior to incubation with substrate. Storing of mounted sections for 1-2 days in the refrigerator did not significantly affect enzyme activity.

The incubation medium contained naphthyl-pT, 2 mg./ml. and Fast Red TR (5-chloro-o-toluidine), 4 mg./ml. in 0.1M tris-HCl buffer pH 9; these were found to be optimal concentrations for dye formation. It is advisable to add the diazonium salt immediately prior to incubation since, if stored in buffer at room temperature for an hour, it tends to form a fine yellow precipitate which partially decreases precipitation of enzymatically liberated naphthol on addition of substrate. The effectiveness of such a medium may be restored by addition of diazonium salt. Alternatively, if a given tissue requires more than 30 minutes incubation, it may be transferred periodically to fresh medium.

In the present study incubation times for formol-calcium fixed sections varied from 1 to a maximum of 30 minutes for different rat organs, at room temperature. Maximum PDase I activity was exhibited by the kidney and pancreas. The enzyme was also found in the spleen, duodenum, tongue, liver, thymus and thyroid, but to only a small extent in the brain. Controls, consisting of either heat-inactivated sections (100° for 10 mins.), or of sections incubated in medium from which substrate was excluded, were always negative. Incubation in media of various pH values showed that the optimum pH for activity was 9.

Fresh frozen kidney and pancreatic sections were employed in several experiments designed to check the validity of the localization patterns obtained with fixed sections. The results were in all instances identical for both. Incubation

times were, however, considerably shorter for the frozen sections, of the order of 15 seconds, but this may be due in part to their greater thickness. The effect of fixation on enzymatic activity is under investigation.

PDase I activity in the kidney was found in the epithelial cells of the proximal tubules of the cortex, although the straight portions of the tubules exhibited more pronounced activity than the convoluted ones. The nuclei were unstained, and cytoplasmic activity was concentrated in the brush border zone. Prolonged incubation led to some staining in the adventitial coat of the blood vessels, as well as in the Malphigi corpuscles.

In the pancreas the enzyme was found centred in the supranuclear portion of the acinar cells, adjacent to the lumen, with lesser activity in the adventitial coat of the blood vessels. The islets of Langerhans were negative.

From cell fractionation studies, Razzell (1961) was led to suspect that PDase I might be present in, or bound to, rat liver and kidney nuclei. The present cytochemical investigation demonstrated the complete absence of this enzyme in the nuclei of all the tissues investigated.

Full details, including photographs of the localization patterns for PDase I activity in various rat tissues, will be published elsewhere (Sierakowska, Szemplińska & Shugar, 1963). Attempts are also in progress to apply the foregoing technique for PDase I to other enzymes involved in nucleic acid metabolism, bearing in mind the importance of these enzymes and the considerable value of obtaining independent results by both cell fractionation and cytochemical techniques.

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