frozen sectioning techniques. Certain other manipulations, to be described in later papers, are also facilitated.

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Histochemical Demonstration of 5-Nucleotidase Activity in Cell Nuclei[1]

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Evidence has been brought forward recently which seriously questions the value of histochemical methods for the localization of alkaline (*1*) and acid (*2*) phosphatase within cellular structures. In the case of alkaline phosphatase, nuclear staining is seen in many tissues with the Gomori (*3*)–Takamatsu (*4*) technique, but not with the azo-dye method (*5, 6*). All or part of this nuclear activity may, however, be due to unspecific staining, since cell nuclei absorb phosphatase, which readily diffuses from tissue components with high activity, as well as calcium phosphate which has been split off the substrate in the Gomori–Takamatsu method (*1, 7–9*).

In the present communication, evidence is presented that an enzyme attacking adenosine-5-phosphoric acid (muscle adenylic acid), first found by Reis (*10, 11*) in various tissue extracts, can be localized not only in the cytoplasm but also in the nuclei of some tissues. Such a 5-nucleotidase, differing from the unspecific phospomonoesterase, has been previously demonstrated histochemically (*12, 13*).

Thin slices of tissue were fixed in ice-cold acetone for 24 hr, then dehydrated for 24 hr in absolute alcohol or cedar wood oil and cleared in 2 changes of xylol for 1 hr each. Paraffin sections were cut at 5 μ and incubated in the substrate at 37° C. The substrate contained 1.44×10^{-3} *M* adenosine-5-phosphoric acid,[2] 8.0×10^{-2} *M* calcium chloride, 1×10^{-2} *M* magnesium sulfate, and 2×10^{-1} *M* 2-amino-2-methyl-1,3 propanediol buffer at pH 8 (*14*). The sites of formed calcium phosphate were visualized by treatment with cobalt nitrate followed by diluted ammonium sulfide. The adenosine-5-phosphoric acid was added to the substrate mixture as dry powder (50 mg/100 cc). In preliminary experiments this amount was found to be the smallest that gave satisfactory results. The concentration of calcium chloride was within the range suggested recently by Gomori (*12, 15*). Gomori pointed out that a high concentration of calcium would substantially decrease the solubility of the formed calcium phosphate and thus prevent unspecific staining caused by secondary absorption of the dissolved calcium phosphate from the substrate.

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[2] The adenosine-5-phosphoric acid was obtained from Sigma Chemical Co., St. Louis, Mo., and from Schwarz Laboratories, Inc., New York. Identical results were obtained with both preparations.

Myometrium of fresh surgically removed uteri and aorta of recently killed rabbits was used. In both tissues the usual technique for demonstrating alkaline phosphatase with glycerophosphate as substrate buffered at either pH 8 or pH 9 revealed only staining of capillaries in the myometrium and of the endothelial layer in the aorta. No other structures showed activity even if the incubation time was extended to 12 hr.

5-Nucleotidase activity was demonstrable in the cytoplasm of smooth muscle fibers, interstitial fibroblasts, and the cells composing the wall of the myometrial arteries after 3 min incubation and increased markedly in the following minutes. Staining was always diffuse and even. The coating of sections with celloidin did not alter the results. For comparison, frozen sections cut at 10–15 μ were prepared from fresh, unfixed uteri, as well as from material which had been fixed for 24 hr in ice-cold acetone. In these preparations enzymatic activity in cytoplasm and nuclei could be recognized after 2–3 min and was quite distinct after 4 min. The distribution of histochemically demonstrable enzymatic activity was identical with that seen in paraffin sections. Excellent preparations for microscopic study were obtained in paraffin sections after an incubation period of 10–60 min. Within the nuclei the nuclear membrane, nucleoli and chromatin particles were stained. Within the cytoplasm of both the smooth muscle cells and the fibroblasts fine dark staining fibrils could be recognized (Fig. 1). If the incubation period was extended over several hours, all cellular details were obliterated by the heavy deposit of calcium phosphate after its visualization as dark cobalt sulfide.

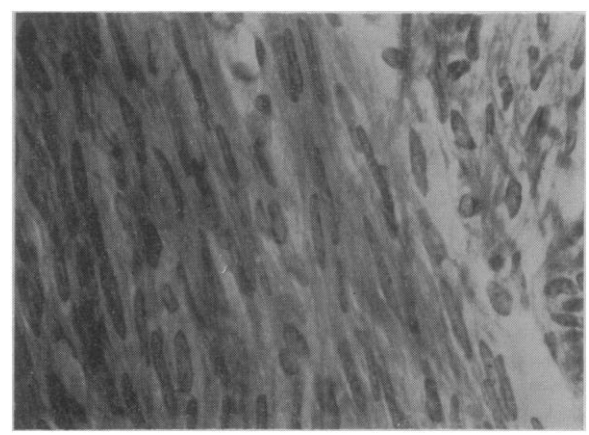

FIG. 1. Myometrium from human uterus. Paraffin section incubated for 10 min. Note prominent nuclear staining and fibrillar structure of cytoplasm. × 500.

With the rabbit aorta activity was demonstrable in 6 min and was quite distinct after 10 min incubation in paraffin sections (Fig. 2). Nuclear staining in smooth muscle fibers and fibroblasts was as prominent as in the myometrium. The cytoplasm of these cells showed a comparable amount of activity, whereas the elastic membranes remained completely unstained.

In order to establish the optimal pH range for the

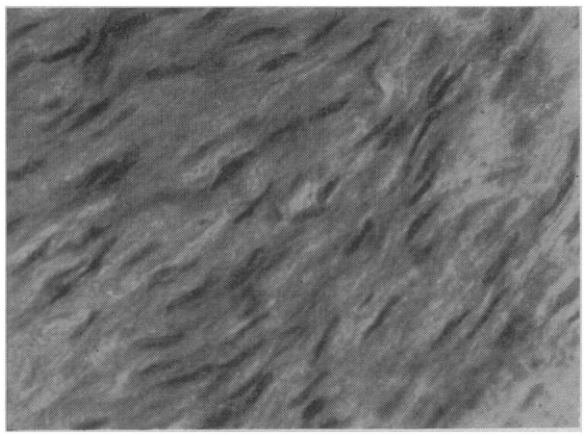

FIG. 2. Rabbit aorta. Paraffin section incubated for 10 min. × 500.

histochemical demonstration of 5-nucleotidase activity, sections of uterus and aorta were incubated for 1 hr at 37° C in a substrate mixture of which the pH was varied between 7 and 9.5. This was accomplished with a 2×10^{-1} *M* tris (hydroxymethyl) aminomethane-maleate buffer (*14*) for a pH of 7 to 7.8 and with a 2×10^{-1} *M* 2-amino-2-methyl-1,3 propanediol buffer (*14*) for a pH of 8 to 9.5. The results with both tissues were identical. Incomplete staining was seen with a pH of 7 to 7.4. Optimal intensity was observed between a pH 7.5 and 8.2. Between pH 8.4 and 9.5 the histochemical staining reaction was decreased in intensity. This decrease was noticed both in the cytoplasm and in the nuclei.

The incorporation of magnesium in the incubation mixture is not essential. Comparison of adjacent sections of aorta and uterus incubated with and without magnesium sulfate revealed that magnesium caused a moderate increase of enzymatic staining for both organs up to 1 hr incubation. With incubation times of 2 hr or longer staining became so intense as to obliterate any difference.

If magnesium was omitted from the incubation mixture, activity was completely inhibited by addition of 1×10^{-2} *M* potassium cyanide in sections incubated up to 2 hr. If, however, magnesium was present in the substrate mixture, the inhibiting effect of potassium cyanide was not complete, and residual enzymatic activity was present in sections incubated up to 2 hr. Cytoplasmic activity was more inhibited than nuclear staining.

In order to examine the occurrence of unspecific nuclear absorption staining, the following tests were carried out with paraffin sections from myometrium which had been inactivated by immersion in 0.25% nitric acid for 5 min. (1) Active praffin sections of uterus were mounted over inactivated sections, covering part of the latter (*7, 15, 16*). No diffusion staining was noted up to 2 hr incubation. After 4 hr some nuclei of the inactivated sections showed slight staining adjacent to the active sections. Only when the incubation was further extended did diffusion staining become marked, although it was limited to a very narrow area adjacent to the by now markedly overstained active sections. (2) When an active and an inactive slide were put face to face and slides were separated by a strip of celluloid film and fastened together with a rubber band (*15*), identical results were obtained as described above. Up to 2 hr there was no diffusion staining in the nuclei of the opposite inactivated sections. There was faint nuclear staining after 4 hr, which became more distinct if the incubation time was extended beyond 6 hr. (3) Inactivated sections were incubated in the substrate to which dilute H_2O_2 (5 ml of a freshly prepared 3% solution for 100 cc of substrate) had been added. This produces a slow precipitation of calcium phosphate in the substrate mixture (*8, 16*). No nuclear staining occurred up to 12 hr. If, however, the inactivated sections were incubated in the usual glycerophosphate substrate at either pH 8 or pH 9, absorption staining became quite marked after 6 hr incubation.

In contrast to recent reports in which a predominantly nuclear localization of acid phosphatase was found in paraffin sections and cytoplasmic localization in frozen sections (*17, 18*) identical localization of 5-nucleotidase activity occurring in nuclei and cytoplasm was noted in both frozen sections of unfixed and acetone-fixed material and paraffin sections. This activity was demonstrable within a few minutes, whereas no nuclear staining whatsoever was seen if glycerophosphate was used as a substrate even after many hours of incubation.

None of the artifacts that are due to nuclear absorption staining either by diffused enzyme or liberated calcium phosphate occurred at the short time interval in which optimal staining was observed (*19*). Furthermore, fractionation studies have shown that although acid (*2, 20*) and alkaline (*20*) phosphatase, at least in the rat liver, are predominantly localized in the cytoplasm, the enzyme-splitting adenosine-5-phosphoric acid is concentrated to a large portion in the nucleus (*20*).

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