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Histochemical Specificity of Phosphatases.*

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The question of the unity or plurality of phosphatases (Ph) has been a moot point for over 20 years, with numerous champions on both sides. There can be no doubt that alkaline and acid Ph are distinctly different enzymes; also the individuality of adenosinetriPh of muscle,[1] of pyroPh[2] and of hexosediPh[3] appears to be firmly established. However, a number of workers maintain that, besides these generally recognized differences, Phs differ among themselves in 3 more respects: 1 substrate specificity; 2, organ specificity and 3, specific activation and inhibition effects. The latter 2 groups often overlap.

As far as substrates specificity is concerned, Forrai[4] thinks that there is a specific sucrosePh and other sugarPhs; according to King,[5] lecithin is dephosphorylated by an enzyme not identical with the nonspecific Ph; Roche and Latreille[6] maintain that the kidney contains, besides glyceroPh, a phenylPh; Reis[7,8] claims the existence of a 5-nucleotidase; Bowers and coworkers[9] believe that aminoethylphosphate is hydrolyzed by a special enzyme; Waldschmidt-Leitz and Koehler,[10] Ichihara,[11] and Bredereck and Geyer[12] maintain that there is a special "phosphamidase" hydrolyzing the P-N bond.

There are data available to the effect that alkaline Phs of different organs may be actually different enzymes as shown by their slightly different resistance to inhibitors or by different pH optima. Belfanti and coworkers,[13] and Hommerberg[14] believe that bone Ph is different from renal or hepatic Ph. Bodansky[15] thinks that intestinal Ph can be distinguished from bone or renal enzyme. Masayama and Shuto[16] found a Ph in hepatomas, different from that of the normal liver.

Cloetens[17,18] distinguishes two alkaline Phs on the basis of their different degrees of activation by Mg. Drill, Annegers and Ivy[19] find that in jaundice a Ph appears in the plasma, different from the normal enzyme in respect to inactivation by cyanide.

Whether the differences reported should be considered as indications of the existence of several truly different enzymes, or as results of the admixture of various activators and inhibitors or of differences in technic cannot be decided on the basis of data available. The question is: Can histochemical technic be

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16 Masayama, T., and Shuto, M., *Gann*, 1940, **34**, 176.

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utilized as an approach to the solution of the problem? A number of authors answer the question in the affirmative.[20-25]

Since many of the findings of the above mentioned authors could not be confirmed in this laboratory it was decided to reexamine the problem of the histochemical specificity of phosphatases on a broader basis.

Experimental. The preparation of tissue sections of mouse, rat, guinea pig, dog and human material was essentially the same as reported previously.[26] Six to 20 different tissues of material fixed in chilled alcohol or acetone were embedded in a single paraffin block. In view of the large molecular size of some of the substrates the slides were not coated with collodion. They were incubated for 1 to 24 hr.; phosphate precipitates were visualized by the sulfide technique. The following 19 substrates were used: Metaphosphate; pyrophosphate; methyl-, glycero- and aminoethylphosphate; phenyl, o-chlorophenyl, α-naphthyl-, resorcyl and phenolphthalein-phosphate; glucose-1-phosphate, hexosediphosphate, adenosinetriphosphate, yeast nucleate and thymonucleate; phosphorylcholine and lecithine:octanoylphosphate; p-chloranilidophosphonate. Phosphoric esters not available on the market were synthesized by esterification with $POCl_3$;[27] p-chloranilidophosphonate was prepared by the method of Otto;[28] octanoylphosphate was the gift of Dr. A. Lehninger of the Department of Biochemistry. The concentrations of the substrates ranged from 0.02 to 0.005M or, in the case of nucleates and lecithin, from 0.1 to 0.5%. Experiments were performed at pH5 (acetate buffer), pH7 (tris(hydroxymethyl)-aminomethane-maleate buffer)[29] and pH9 (2-amino-2-methyl-1,3-propanediol buffer).[30] The cation used to trap the phosphate ions was Ca at pH9 and Pb at pH5 and 7. In most cases the concentration of Ca was 0.01M; that of Pb, 0.003M. In some cases, owing to special conditions of solubility, the concentration of the cation had to be changed. For instance, the highly insoluble meta- and pyrophosphates of Ca and Pb will go into solution in the presence of an excess of PO_3^- or $P_2O_7^{\equiv}$ ions. Therefore, a dilute solution of $CaCl_2$ or of Pb $(NO_3)_2$, respectively, was added drop by drop, with constant stirring, to the buffered substrate, until a slight permanent turbidity resulted. This was centrifuged off, and the clear supernatant was used. In other cases, as with phenyl-, o-chlorophenyl- and naphthylphosphate, phosphorylcholine, adenosinetriphosphate, nucleic acids and lecithin, the solubility of the Pb salts (in the case of lecithin and nucleic acids, even that of the Ca salts) proved to be so low that only a very small amount of cation was tolerated without precipitation. It is felt that some of the unsatisfactory results obtained with this latter group of substrates at pH5 and 7 were due to the very poor solubility of the Pb salts of the esters.

Some substrates (octanoylphosphate, adenosinetriphosphate, chloranilidophosphonate) have a slow rate of spontaneous hydrolysis under the conditions of the histochemical experiment. To avoid indiscriminate precipitation of phosphate in the tissues, the Coplin jars were supported in an inclined position, and the slides were placed in them with the sections facing downward. In this way the precipitate collected on the back surface of the slides.

Results. Pyrophosphate did not give positive results at any pH. With metaphosphate a minimal reaction was obtained on the sur-

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face of intestinal villi at pH9, otherwise the results were negative. With both substrates spurious reactions consisting of random staining of various tissue elements were seen in all pH ranges. The non-enzymatic nature of this staining could be proven by its presence in inactivated sections, treated for 5 min. with Lugol's solution prior to incubation. Otherwise at pH9 the pictures obtained with all substrates were identical as far as the distribution of the enzyme in the tissues and the relative intensity of the staining at various sites is concerned. Occasionally slides incubated with nucleates for over 5h showed a somewhat more pronounced staining of nuclei than that seen with glycerophosphate as a substrate. However, this difference was neither constant nor conspicuous. Methylphosphate, p-chloranilidophosphonate and especially lecithin were hydrolyzed very much slower than the rest of the substrates; pictures obtained in 24h with the three substrates mentioned were comparable in intensity to those obtained in 1h with the rest. Since no reaction different from that seen with glycerophosphate was observed in the liver with hexosediphosphate or in the muscle with adenosinetriphosphate, it must be assumed that hexosediPh and adenosinetriPh do not survive the embedding procedure.

At pH5 the results were variable. Satisfactory and constant pictures were obtained only with glycerophosphate and resorcinolphosphate, and only after acetone fixation. Alcohol fixation often resulted in patchy, uneven staining. With all other substrates the intensity of the staining was very much lower and, in addition, the results were capricious, even consecutive serial sections showing differences in staining. In the case of nucleate or lecithin as substrates there was often a nonspecific impregnation of mucin, random groups of nuclei, nerve and muscle fibers. Otherwise, the distribution of the enzyme was identical with all substrates and in all tissues, as far as it could be judged from the often unsatisfactory slides. The only exception was p-chloranilidophosphonate. This substrate produced pictures entirely different from the rest.

At pH7, as could be expected, various combination pictures of the typical distribution of acid and alkaline Phs were obtained. The picture normally seen at pH5 showed up as a fairly strong component only when glycerophosphate or resorcinolphosphate were used as substrates, and only after acetone fixation. With the other substrates and/or alcohol fixation the distribution of the enzyme in all tissues was identical with that of alkaline Ph, although the intensity of the staining was much lower. Artifacts consisting of non-enzymatic impregnation of various structures were even more marked than at pH5. Otherwise the results were the same with all substrates. Specifically, the differences due to variation of the substrate, reported by Dempsey and Wislocki,[23] could not be confirmed in either human or guinea pig material.

Since there are reports on the specific inhibitory effects of taurocholate[15] and of cyanide,[24] a number of slides were incubated at pH9 in the presence of these substances, glycerophosphate being used as a substrate. Taurocholate in concentrations from 0.002 to 0.01M produced no visible effect in any of the tissues. Cyanide in concentration from 0.001 to 0.01M caused a marked inhibition of enzymatic activity in all organs. In some cases pictures comparable to those published by Emmel[24] were obtained; in other cases an intense staining persisted in the intestine in the presence of 0.01M NaCN while at the same time the reaction in the kidney was wiped out completely. All possible transitions between these two extremes could be observed in slides carrying a number of kidneys and pieces of small intestine from several animals.

Comment. The dependence of enzymatic behavior on the presence of various colloid substances is well known.[31,32] Even purified enzymes may show widely varying pH optima with different substrates.[33] Owing to these and other factors, the decision on the identity or nonidentity of enzymes may be an exceed-

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TABLE I.

Comparison of Results Obtained with Various Substrates.

Six to 20 different tissues of humans, dogs, rats, mice, and guinea pigs mounted on each slide. Pattern of distribution of enzymatic activity in any given organ is constant and independent of the substrate used. All-over intensity of reaction denoted by plus signs. In the column of pH7, *5* indicates the normal distribution as seen at pH5, *9* that seen at pH9, glycerophosphate being used as a substrate. ? stands for unsatisfactory results.

Substrate	pH5	pH9	pH7
Glycerophosphate	+++	+++	*9* + to ++; *5* + to ++
Pyrophosphate	—	—	—
Metaphosphate	—	—	—
Methylphosphate	—	+	—
Aminoethylphosphate	—	++ to +++	— to *9* +
Phenylphosphate			
o-Chlorophenylphosphate	— to +	++ to +++	*9* + ?
α-Naphthylphosphate	?		
Phenolphthaleinphosphate			
Resorcylphosphate	+++	+++	*9* + to ++; *5* + to ++
Glucose-1-phosphate	—	+++	—
Hexosediphosphate	— to + ?	+++	— to *9* +
Adenosinetriphosphate	—	+++	—
Yeast nucleate	+ to ++	++ to +++	*9* + to ++; *5* +
Thymonucleate	— to + ?	++	*9* +
Phosphorylcholine	—	++	not done
Lecithine	—	+	,, ,,
Octanoylphosphate	not done	++	,, ,,
p-Chloranilidophosphonate	special	+	*9* +

ingly difficult one, even if quantitative chemical procedures are used. The histochemical approach to the problem is beset with the danger of additional sources of error among which only a few will be mentioned.

1. The two main factors regulating the deposition of poorly soluble phosphates in tissue sections are the rates of phosphate production and of diffusion. Varying ratios between these two factors may result in an "all or none" effect, depending on whether the rate of phosphate production will or will not overtake that of diffusion to a point where the solubility product of the phosphate in question is exceeded locally. Any kind of inhibition of enzymatic action (partial inactivation of the enzyme by unsuitable fixation; non-optimal pH or substrate; the presence of various inhibitors) is likely to obliterate areas below a critical level of activity, while areas of much higher activity may remain apparently uninfluenced or only slightly weakened.

2. In the case of substrates of large molecular size the different rates of diffusion into various tissue elements may be a decisive factor.

3. The nonenzymatic impregnation of tissue structures by Pb is well known.[34] The danger of such impregnation is especially great when, on account of long periods of incubation, spontaneous hydrolysis of the substrate may lead to supersaturation in respect to Pb (possibly also of Ca) phosphate. Such artifacts may be recognized by simultaneously incubating inactivated tissues as controls.

Since some of the complications of the histochemical technic cannot be avoided, great caution is warranted in the evaluation of its results, especially when non-optimal conditions are used. Only marked and uniform differences in the picture of enzymatic distribution, obtained by changing conditions such as substrate, pH or inhibitors and activators, can be accepted as a presumptive evidence for the presence of different enzymes. Results of this type have been reported in the case of acid Ph,[35] of acid adenosinetriPh of grain,[20] and of yeast Phs.[25] The importance of such minor differences as reported by Dempsey and Singer,[21] Dempsey and Deane,[22] Dempsey and Wislocki[23] and by Emmel[24] is questionable.

Summary. Nineteen different substrates

[34] Lassek, A. M., *Stain Techn.*, 1947, **22**, 133.

[35] Gomori, G., *Arch. Path.*, 1941, **32**, 189.

were used in this study on the histochemical specificity of phosphatases. With 18 substrates no indication of the presence in paraffin-embedded mammalian tissues of phosphatases other than the common non-specific alkaline and acid variety was found. With one of the substrates, p-chloranilidophosphonate, a strikingly different picture of enzymatic distribution was observed in the acid range, but otherwise the pattern of distribution of enzymatic activity in any given organ was constant and independent of the substrate used.

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Effect of Tetraethylammonium on Venous and Arterial Pressure in Congestive Heart Failure.

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That widespread vasoconstriction occurs during congestive failure was first proposed many years ago by Starling[1] and by Bolton.[2] Recently, it has been suggested that reflex vasoconstriction contributes to the maintenance of the usually normal arterial pressure[3,4] and the elevated venous pressure[3,5,6] in heart failure. Conversely, the fall in calculated peripheral resistance accompanying the improved output of the digitalized failing heart[7,8] speaks for a release of vasoconstriction. Renin has been found in the renal veins of patients in congestive failure,[3] and the reduction of hepatic and renal blood flows in such cases is further evidence of increased peripheral resistance attributable to vasoconstriction.

Tetraethylammonium chloride is a quaternary ammonium salt which transiently blocks the transmission of impulses through peripheral autonomic ganglia.[9] Hayward[10] has recently observed that tetraethylammonium bromide decreases the venous and arterial pressures in hypertensive patients with compensated or failing circulations. Since peripheral resistance is greatly increased in essential hypertension, it was of interest to observe the effect of this drug on the venous and arterial pressures of normotensive patients with and without failure, in an attempt to delineate the role of vasoconstriction in congestive failure *per se*.

Methods. Venous pressure was measured directly with a No. 18 gauge needle in an antecubital vein, using the sternal angle of the supine patient as a reference point. Arterial blood pressure was measured with a sphygmomanometer and the mean pressure was arbitrarily taken to be the average of the systolic and diastolic pressures. The heart rate was counted at the apex for 30 seconds. After repeated observations of heart rate and pressures had established a constant baseline, tetraethylammonium chloride (TEAC)* in doses of 2 to 6 mg/kg of body

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* Etamon Chloride, Parke-Davis and Company.