The Specificity of Phosphomonoesterases in Human Tissues

By J. L. REIS
Postgraduate Medical School, London, W. 12

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Phosphates are present in many human and animal tissues, and the activity of these enzymes may easily be estimated, but it is very difficult to draw conclusions about their physiological role. In discussing the significance of the phosphatases, we must consider not only the enzyme activity but also the substrate; first, whether it has access to the enzyme and, secondly, how the enzyme acts upon it. This second question leads to the problem of the specificity of phosphatases.

The alkaline phosphatases of ossifying cartilage, kidney or intestine, and the acid phosphatases of prostate and red blood cells are not highly specific. King & Delory (1939), considering the relative rates of hydrolysis, pointed out that the rate depends on the acidity of the phosphoric ester, the more acid esters being more easily hydrolysed by the phosphatases.

Besides these non-specific phosphatases there exists a highly specific phosphatase which acts only on nucleotides with the phosphate group attached to the fifth carbon of the pentose, i.e. adenosine-5-monophosphoric acid (muscle adenylic acid; this nucleotide, for brevity, will be referred to as adenylic acid) and inosine-5-monophosphoric acid (inosinic acid). It does not act on the closely related yeast adenylic acid (adenosine-3-monophosphoric acid), or on adenosinetriphosphoric acid. This enzyme was first described in Parnas's laboratory by Reis (1934) in heart muscle and has been named 5-nucleotidase. Later it was found in nervous tissues (Reis, 1937*a*), and in many other tissues, especially lungs, testicles, foetal membranes, retina and choroid (Reis, 1937*b*, 1940). Animals may be classified as 'rich' or 'poor' in 5-nucleotidase; in the rat it is present in almost every tissue, while in the pigeon it was found only in the lungs. The presence of this enzyme in brain was confirmed by Fleischhacker (1938). Gulland & Jackson (1938) found it very active in snake venom, and Mann (1945) demonstrated a still greater activity in bull seminal plasma. Reis (1950) studied the distribution of 5-nucleotidase in human tissues and found the activity was highest in the thyroid, testicle and aorta walls. At the physiological pH in all human tissues, perhaps with the exception of prostate gland and intestinal mucosa, adenylic acid was much more easily hydrolysed than phenylphosphoric acid or β-glycerophosphoric acid.

The object of the present work was to compare the activities of 5-nucleotidase and non-specific phosphatases at different pH values. Estimations at a physiological pH are sufficient to demonstrate 5-nucleotidase and non-specific phosphatase activity, and may indicate the physiological role of these enzymes; but in order to indicate which non-specific phosphatase (the 'acid' or the 'alkaline') is present, estimations at alkaline and acid pH are necessary, and complete pH-activity curves may be traced with advantage.

METHODS

Since both the 5-nucleotidase and the non-specific phosphatase attack adenylic acid, the activity of 5-nucleotidase in a tissue extract can be deduced by measuring the activity against adenylic acid and subtracting that measured against some other phosphoric ester. For this purpose phenylphosphoric acid seems very suitable (King & Armstrong, 1934) as it has a higher rate of hydrolysis than many other phosphoric esters.

Tissue extracts. The tissues were usually taken 24 hr. after death. They were homogenized in a blender with distilled water in the proportion 1:20. The tissue homogenates, after the addition of $CHCl_3$ or thymol, were left for 2 days at room temperature for autolysis to occur. After that time the activity of the extracts was usually found to have increased, except in hot summer weather when it decreased, and hence it was thought better to keep the homogenates in a refrigerator. After 2 days the whole was centrifuged, and the supernatant liquid used for estimations of enzyme activity. The extracts kept in a refrigerator with thymol remained active for months. In order to diminish the phosphate content the extracts were dialysed in collodion tubes. This reduced the phosphate to about one-tenth of the original value without significant change in the activity of phosphatases.

Enzymic activities. These were tested by adding to 0·4 ml. of a buffer solution (0·05 M-biphthalate, 0·05 M-diethylbarbiturate, or Delory & King's (1945) carbonate-bicarbonate buffer, according to the desired pH) 0·1 ml. of 0·005 M substrate solution (adenylic acid, phenylphosphate or β-glycerophosphate, pH adjusted to 7·5). The mixture was placed in a water bath at 38°, and after 5 min. 0·1 ml. of the tissue extract, diluted if necessary, was added. After the desired incubation time, usually 0·5 hr., 1·0 ml. of 5% (w/v) trichloroacetic acid was added and the sample centrifuged. The clear supernatant was decanted into another tube and used for the phosphate estimation, which was based on the Fiske & Subbarow (1925) method. To develop the colour, 0·1 ml. of 10% ammonium molybdate in 30% (v/v) H_2SO_4, and 0·05 ml. of 0·2% aminonaphthol-

sulphonic acid in a solution of 12% (w/v) sodium metabisulphite and 2·4% sodium sulphite (crystalline), were added. In order to avoid inhibitory effects the dilution of tissue extracts and the incubation times were chosen so that not more than 50% of the substrate was hydrolysed.

RESULTS

The results are presented in Table 1. Comparing the activities of the enzymes near their respective optimal pH's (that is for 5-nucleotidase at pH 7·5, and for the non-specific phosphatases at pH 5·5 and 9·0), human tissues fall into groups showing the following types of phosphomonoesterase activity:

(1) *5-Nucleotidase nearly free from non-specific phosphatase*: thyroid, testicle, aorta and other arterial walls.

(2) *5-Nucleotidase much more active than non-specific phosphatases*: nervous tissues and posterior lobe of pituitary; the latter has the highest activity of 5-nucleotidase of all human tissues.

(3) *Both enzymes of an approximately equal activity*: liver, lung, kidney medulla, anterior lobe of pituitary.

(4) *Alkaline phosphatase much more active than 5-nucleotidase*: the highest alkaline phosphatase activities were found in ossifying cartilage, choroid plexus and choroid membrane (of the eye); the alkaline phosphatase activity in kidney cortex was comparatively very low.

(5) *Non-specific phosphatase, but no or very little 5-nucleotidase*: prostate, highly active acid phosphatase, which is still considerable at the physiological pH; intestinal mucosa (duodenum, jejunum), a comparatively low activity of alkaline phosphatase.

At the physiological pH, in all tissues examined, except prostate and intestinal mucosa, the activity of 5-nucleotidase was several times greater than that of the non-specific phosphatases.

A more detailed study of phosphomonoesterase activity in human tissues is demonstrated in Figs. 1–3. Fig. 1 shows the influence of pH on the 5-nucleotidase activity of aorta. The hydrolysis of phenylphosphoric acid (non-specific phosphatases) is almost negligible with this tissue, and therefore the hydrolysis of adenylic acid is due almost entirely to 5-nucleotidase. The optimal pH is 7·8, very near to

Table 1. *The activity of phosphomonoesterases in human tissues*

(The enzyme activity is expressed as μg. P hydrolysed/hr./mg. wet wt. tissue corresponding to volume of extract used.)

	Enzyme activity			
	Phenylphosphoric acid			Adenylic acid
Tissue	pH 5·5	pH 7·5	pH 9·0	pH 7·5
Group 1				
Thyroid	0·22	0·06	0·22	4·8
Thyroid	—	0·05	0·30	5·1
Testicle	0·30	0·10	0·40	3·8
Aorta wall (media)	0·09	<0·03	0·06	2·2
Aorta wall (media)	—	<0·03	—	2·9
Group 2				
Brain (cortex)	0·5	0·2	0·5	1·3
Nerve (optic)	0·3	<0·1	0·9	2·6
Retina	0·1	<0·1	1·0	2·2
Pituitary posterior lobe	0·6	0·6	7·0	21·2
Group 3				
Pituitary anterior lobe	0·7	0·2	3·4	4·2
Liver	0·3	0·1	1·3	0·9
Lung	0·4	0·2	2·0	1·2
Lung	—	0·5	4·6	2·1
Kidney medulla	0·9	0·3	1·9	1·2
Group 4				
Kidney cortex	1·9	0·7	4·1	1·5
Kidney cortex	0·9	0·3	2·1	0·9
Choroid plexus	0·1	0·6	14·0	3·6
Choroid plexus	—	0·6	13·0	2·8
Choroid membrane	0·2	0·3	8·3	1·8
Ossifying cartilage	0·2	0·9	21·0	4·3
Ossifying cartilage	—	1·6	56·0	3·2
Group 5				
Duodenal mucosa	0·1	0·2	2·6	0·2
Jejunal mucosa	0·4	0·4	3·7	0·6
Prostate	1030	395	0·5	80

the physiological pH. Similar hydrolysis curves were obtained for thyroid gland and for testicle.

In many tissues 5-nucleotidase is found together with non-specific phosphatases. Fig. 2 shows the action of an extract of human brain. In agreement with the work of Giri & Datta (1936), two optima

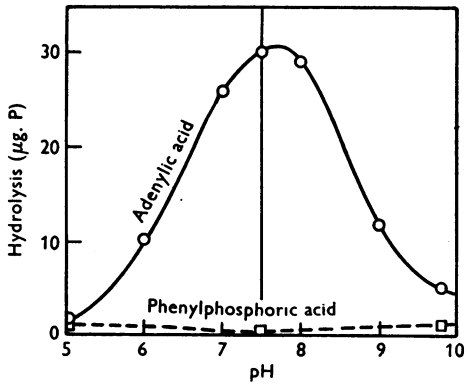

Fig. 1. Hydrolysis of adenylic acid and phenylphosphoric acid by human aorta wall. Dialysed extract corresponding to 25 mg. of tissue, 0·5 hr. at 38°.

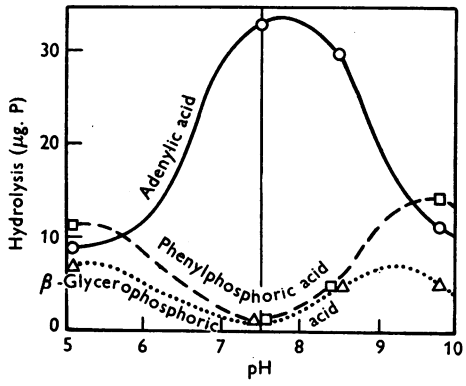

Fig. 2. Hydrolysis of adenylic acid, phenylphosphoric acid and β-glycerophosphoric acid by human brain. Dialysed extract corresponding to 25 mg. of tissue, 1 hr. at 38°.

were found for the non-specific phosphatases, an acid and an alkaline. These are demonstrated by the hydrolysis of phenylphosphate and β-glycerophosphate. As found by King & Delory (1939) the phenylphosphate is hydrolysed at a much greater rate than the β-glycerophosphate, and the pH optimum for phenylphosphate is more alkaline than that for β-glycerophosphate. The rate of hydrolysis of adenylic acid in acid and alkaline solutions is intermediate between the two other esters, but near the physiological pH it is very much greater. Adenylic acid is hydrolysed optimally near pH 7·5 at a point where the hydrolysis of phenylphosphate and β-glycerophosphate is at a minimum. Similar hydrolysis curves were found for human lungs and liver.

Fig. 3 represents the action of an extract of human ossifying cartilage. This tissue is the classical site of alkaline phosphatase. As in Fig. 2 the extent of hydrolysis of phenylphosphate is much greater than that of β-glycerophosphate; both curves show that the alkaline phosphatase action is very low at physiological pH, at pH 7·5 the hydrolysis of phenylphosphate being only about 5 % of the hydrolysis at

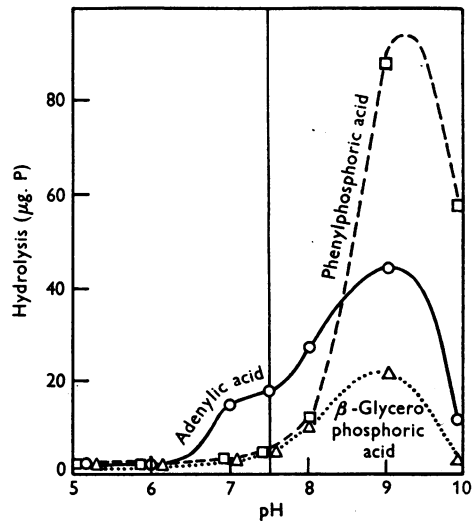

Fig. 3. Hydrolysis of adenylic acid, phenylphosphoric acid and β-glycerophosphoric acid by human ossifying cartilage. Extract corresponding to 8 mg. of tissue, 0·5 hr. at 38°. (Results at pH 9 and 10 for phenylphosphoric acid obtained from 15 min. hydrolysis, ×2.)

the optimal pH. At pH 7·5 the rate of hydrolysis of adenylic acid is about four times as great as the hydrolysis of the others esters. This indicates the presence of 5-nucleotidase, which, although comparatively weaker than the non-specific phosphatase at the optimal pH, is about four times more active than the latter at physiological pH. The action of extracts of kidney cortex and choroid plexus is similar to the above.

DISCUSSION

Newman, Feigin, Wolf & Kabat (1950) described the use of several phosphoric esters for a histological method of demonstrating phosphatase activity. They found that the hydrolysis of muscle adenylic acid in tissues is sometimes very different from that of the others phosphoric esters (e.g. in artery wall), and they rightly ascribed it to the presence of 5-nucleotidase. These authors failed to find any

5-nucleotidase activity in such human tissues as the thyroid gland, testicle or lungs. In the present experiments 5-nucleotidase was always very abundant in these tissues. The failure of Newman *et al.* may be explained by inactivation during histological fixation, and, which seems more important, by the fact that their work was carried out at pH 9·2, whereas 5-nucleotidase is optimally active near pH 7·8.

The physiological role of 5-nucleotidase is not known at present, but the existence of such an enzyme, specific for a substance so important and widely distributed as adenylic acid, seems to indicate a mechanism able to regulate the concentration of both phosphate and adenylic acid, which are so important for glycogenesis.

Since Robison's (1932) work it has been generally considered probable that the alkaline phosphatase plays a part in calcification of bones. As the 5-nucleotidase acts optimally near the physiological pH, where the alkaline phosphatase has a very low activity, it seems possible also that 5-nucleotidase may play a role in calcifications. It is of interest that certain tissues, e.g. aorta wall or thyroid gland, which are apt to undergo pathological calcifications, contain this enzyme, but not the alkaline phosphatase.

SUMMARY

1. At the physiological pH in all human tissues examined, with the exception of intestinal mucosa, the activity of the specific phosphatase, 5-nucleotidase, is much more pronounced than the activity of the non-specific alkaline phosphatase. This includes the tissues notably rich in alkaline phosphatase, e.g. ossifying cartilage, choroid plexus and kidney cortex.

2. The optimal pH for 5-nucleotidase from human tissues is pH 7·8.

3. The highest 5-nucleotidase activity in human tissues was found in the posterior lobe of the pituitary.

4. In adults the highest alkaline phosphatase activity (pH 9) was found in choroid plexus. The alkaline phosphatase activity of kidney cortex and intestinal mucosa was comparatively much lower.

5. The wide distribution of 5-nucleotidase seems to indicate a mechanism regulating phosphate and adenylic acid concentrations, which may perhaps play a part in the calcification of tissues.

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Observations on the Use of *Escherichia coli* for the Reduction and Estimation of Dehydroascorbic Acid

By L. W. MAPSON and M. INGRAM
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research

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Gunsalus & Hand (1941), working with suspensions of *Escherichia coli*, reported that the bacteria were capable of reducing dehydroascorbic acid to ascorbic acid in milk, wine and fruit juices, but seemed incapable of effecting the reduction with vegetable extracts. In these latter juices the *Esch. coli* acted as an oxidation catalyst and oxidized the ascorbic acid present.

Stewart & Sharp (1945), working with suspensions of *Esch. coli* and *Staphylococcus albus* and with a wide range of animal and plant extracts, were able to obtain a rapid and quantitative reduction of dehydroascorbic acid in such materials. Only after the complete removal of oxygen from these solutions, or under aerobic conditions if sodium cyanide was added, was there any reduction of dehydroascorbic