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The Acid Phosphatases of Rat Liver

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It was shown by Norberg (1949) that the shape of the pH–activity curve of rat-liver acid phosphatase varied during the process of regeneration after partial hepatectomy. It has also been reported that rat-liver acid phosphatase activity, assayed at pH 5·5, showed a 50% increase in animals maintained on a low protein, low choline diet, although the activity assayed at pH 3·75 showed no change (Goodlad, Mills & Smith, 1951).

It thus appears possible that at least two non-specific acid phosphatases may be present in rat liver. The present work was undertaken to obtain more information on the nature of these enzymes. The effects of various factors on the pH–activity curve of unfractionated rat-liver homogenates have been examined. An attempt has been made to fractionate the enzymes by acetone precipitation in the presence of Zn^{2+} ions, and the intracellular distribution of rat-liver acid phosphatase activity has been studied by differential centrifuging. A preliminary account of this work has been published (Goodlad & Mills, 1956).

MATERIALS AND METHODS

p-Nitrophenyl phosphate was prepared by the method of Bessey & Love (1952). Glucose 6-phosphate was prepared enzymically from glucose 1-phosphate, a partially purified preparation of phosphoglucomutase (Najjar, 1948) being used, followed by purification by the method of McCready & Hassid (1944). The glucose 1-phosphate was prepared by the method of Hanes (1940) as modified by McCready & Hassid (1944). Phenyl phosphate was obtained from British Drug Houses Ltd. Adenosine 3′-phosphate and adenosine 5′-phosphate were supplied by L. Light and Co. Ltd. β-Glycerophosphate was obtained from The General Chemical and Pharmaceutical Co. Ltd.

Assay of phosphatase activity. A general method, applicable to all substrates, was based on the β-glycerophosphate method of Bodansky (1933) where the inorganic phosphate liberated was separated by the method of Delory (1938), and subsequently determined by the method of Allen (1940). Buffer solution (0·5 ml.), 0·1 ml. of substrate solution, 0·1 ml. of water (or a solution of activator or inhibitor) and 0·3 ml. of enzyme solution, usually a 1/50 tissue homogenate, were incubated at 38° for a specified time, usually 1 hr., after which the reaction was stopped by the addition of 2 ml. of 5% (w/v) trichloroacetic acid. Precipitated protein was removed by centrifuging and 2 ml. samples were made alkaline to phenolphthalein with aq. NH_3 soln. (sp.gr. 0·880); 1 ml. of 2·5% (w/v) $CaCl_2$ was then added, followed by 1 ml. of 0·5% $MgCO_3$ (light) suspension. The tubes were shaken at intervals for 30 min., centrifuged and the precipitates washed once with 1 ml. of 5% (w/v) aq. NH_3 soln. The tubes were drained for 2–3 min. in an inverted position, the precipitate was dissolved in 1 ml. of 2·5 N-H_2SO_4 and colour developed by the addition of 0·4 ml. of 1% (w/v) amidol (2:4-diaminophenol hydrochloride) in 20% (w/v) sodium metabisulphite, 0·2 ml. of 8·3% (w/v) ammonium molybdate and 3·4 ml. of water. The intensities of the blue colour were estimated on the Hilger Spekker absorptiometer (Ilford 608, red filter) between 10 and 30 min. after addition of reagents. Phosphate was determined from a calibration curve for the range 5–40 μg. of P. Controls were employed to determine the inorganic phosphate in the tissue and that formed by autolysis during incubation. In these, the substrate was added after the addition of the trichloroacetic acid. One unit of activity = 1 μg. of P liberated/hr.

When phenyl phosphate was used as substrate, the method of assay was essentially that of King (1946). Enzyme preparation (0·1 ml.), usually a 1/100 tissue homogenate, was used in a 1 ml. incubation volume. Tissue blanks were determined by addition of substrate after the phenol reagent. No measurable breakdown of substrate occurred in the absence of the enzyme at any pH value used. One unit of activity = 1 μg. of phenol liberated/hr.

A modification of the procedure of Bessey, Lowry & Brock (1946) was employed when *p*-nitrophenyl phosphate was used as substrate. Enzyme preparation (0·3 ml.), usually a 1/400 tissue homogenate, was used in a 1 ml. incubation volume with a final substrate concentration of 0·0025 M. One unit of activity = 1 μg. of *p*-nitrophenol liberated/hr.

Preparation of tissue extracts. Male albino rats weighing 175–300 g. were killed by stunning and exsanguination. The livers were removed, washed with water to remove blood,

and excess of moisture was absorbed with filter paper. Homogenates were prepared in an all-glass homogenizer (Potter & Elvehjem, 1936). Unless otherwise stated the suspending fluid was water.

Protein nitrogen. This was determined by the method of Ma & Zuazaga (1942) with the Markham (1942) distillation apparatus.

Deoxyribonucleic acid phosphorus (DNAP) and ribonucleic acid phosphorus (RNAP). These were determined by the method of Schmidt & Thannhauser (1946).

Electrophoretic examinations. These were carried out by the method of Tiselius (1937) with the Philpot–Svensson (Svennson, 1939) optical system (Adam Hilger and Co. London).

Filter-paper electrophoresis. This was carried out by the method of Mills & Smith (1951), which is based on the method of Durrum (1950). All electrophoretic runs were carried out at 5°. Duplicate strips were sectioned at 5 mm. intervals and assayed for enzyme activity with phenyl phosphate or for protein content with bromophenol blue.

Differential centrifuging of intracellular fractions. This was carried out by the technique of Schneider (1948) or that of Hogeboom, Schneider & Striebich (1952). All fractions were frozen in a mixture of solid CO_2 and acetone, and rapidly thawed to facilitate enzyme release from the particles (cf. Berthet, Berthet, Applemans & de Duve, 1951).

RESULTS

Variation in shape of pH–*activity curve of unfractionated rat-liver homogenates with different substrates*

pH–activity curves were determined with phenyl phosphate, *p*-nitrophenyl phosphate, β-glycerophosphate and adenosine 3′-phosphate. The effect of 0·01 M-$MgSO_4$ was also studied in each. The results are recorded in Fig. 1.

Inhibition at low pH values was found to be caused by Mg^{2+} ions with all substrates, suggesting

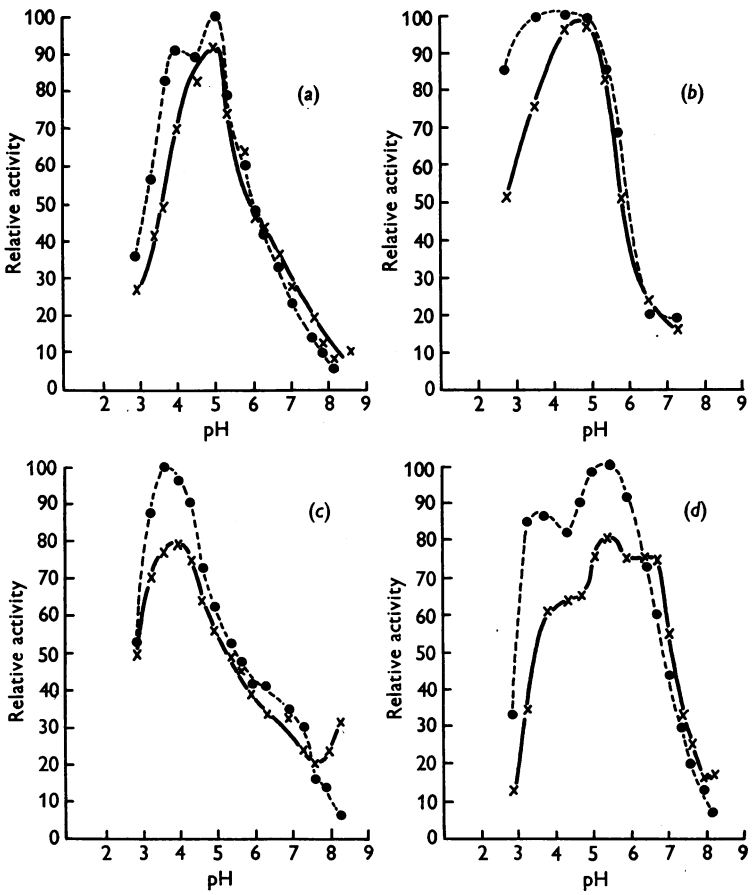

Fig. 1. Effect of pH, nature of substrate and Mg^{2+} ions on phosphomonoesterase activity of rat liver at 38°. Veronal–acetate buffers were used. ×, 0·01 M-Mg^{2+} ions present; ●, Mg^{2+} ions absent. Substrates: (*a*) 0·005 M-phenyl phosphate; (*b*) 0·0025 M-*p*-nitrophenyl phosphate; (*c*) 0·01 M-β-glycerophosphate; (*d*) 0·001 M-adenosine 3′-phosphate. Relative activity is the percentage of maximum activity.

the presence in rat liver of a Mg^{2+} ion-sensitive enzyme with a low optimum pH. This inhibitory effect was absent, or present to only a small extent at pH values above 5·0.

The shape of the curves obtained with β-glycerophosphate differed from those obtained with phenyl- and *p*-nitrophenyl-phosphates at higher pH values, suggesting that there may be an enzyme, with optimum activity between pH 6·0 and 7·0, which acts preferentially on β-glycerophosphate. With adenosine 3′-phosphate two peaks in the pH–activity curve were observed, although in the presence of added Mg^{2+} ions the shape of the curve suggested the presence of a third enzyme with optimum activity above pH 6·0.

Effect of various substances on the acid phosphatase activity of rat-liver homogenates

In preliminary experiments it was found that the shape of the pH–activity curves obtained with acetate buffers differed from those obtained when veronal–acetate buffers were used. This effect was therefore investigated more fully with acetate and veronal–acetate buffers of the same ionic strength. The results of these experiments with phenyl phosphate as substrate are shown in Fig. 2. In all cases higher activities at the more acid pH values were obtained with veronal–acetate than with acetate buffers.

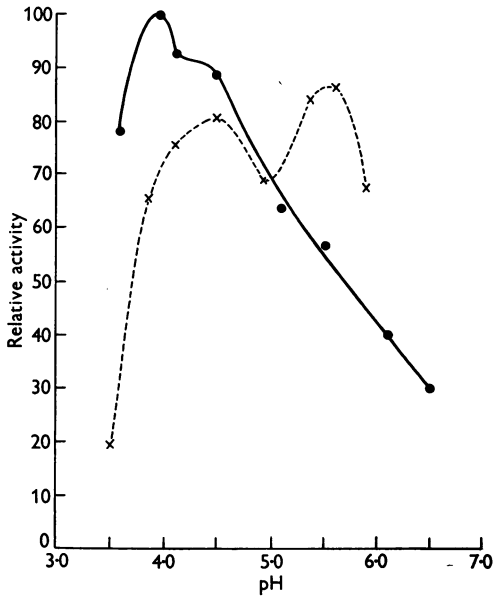

Fig. 2. Effect of type of buffer on pH–activity curve of acid phosphatase activity of rat-liver homogenate. ●, Veronal–acetate buffer; ×, acetate buffer. *I* 0·172 in both cases. 0·005 M-Phenyl phosphate was present as substrate. Temp. of incubations, 38°.

A series of substances which had been shown by Abul-Fadl & King (1949) to affect erythrocyte and prostate acid phosphatase activities were tested on the phosphatase activity of rat-liver homogenates. The results obtained with *p*-nitrophenyl phosphate are shown in Figs. 3 and 4.

The results found here are typical of those found with all substrates which have been used in the present work. Copper ions, even at a concentration of 2×10^{-4} M, in contrast with Mg^{2+} ions caused a very marked inhibition at pH values above 4·0, although they brought about little change in activity at lower pH values. Considerable inhibition was caused by 0·01 M-tartrate below pH 4·0, although the effect was less marked at higher pH values. Greatest inhibition with 0·5% formaldehyde was observed between pH 3·4 and 4·9, whereas 0·01 M-fluoride ion caused considerable inhibition at all the pH values studied. There is some indication of two maxima in the pH–activity curve when fluoride is present, one at about pH 4·0 and the other at pH 5·5. Citrate caused a marked inhibition below pH 4·5 but had no effect above this pH. None of these substances, in the concentrations used here, interfered with the determination of *p*-nitrophenol.

It was found that 0·01 M-cyanide had little or no effect on the acid phosphatase activity of rat-liver homogenates.

The effects of Zn^{2+}, Ba^{2+} and Cu^{2+} ions on acid phosphatase activity were studied at pH values 3·75

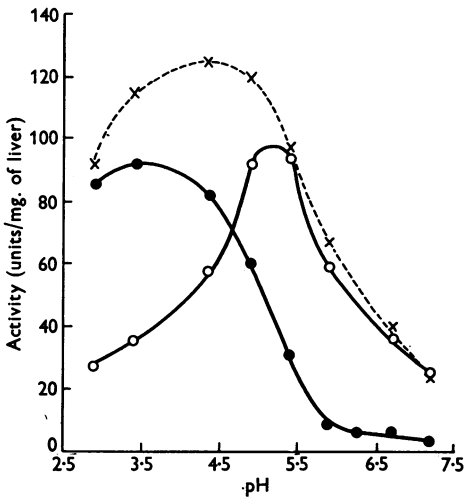

Fig. 3. Effect of Cu^{2+} ions compared with that of Mg^{2+} ions on pH–activity curve of acid phosphatase activity of rat-liver homogenates. ×, No addition; ●, 0·002 M-Cu^{2+} ions present; ○, 0·01 M-Mg^{2+} ions present. 0·0025 M-*p*-Nitrophenyl phosphate was present as substrate. Veronal–acetate buffers were used throughout. Temp. of incubations, 38°.

and 5·6 in acetate buffers, and at pH 6·5 in a veronal–acetate buffer. Phenyl phosphate was used as substrate, and in this experiment the liberated inorganic phosphate was determined to avoid any interference, by the metal ions, with the method of estimating phenol. The results are summarized in Table 1, where it is seen that Zn^{2+} ions inhibit strongly at all pH values, whereas Ba^{2+} ions cause a slight activation at the two lower pH values.

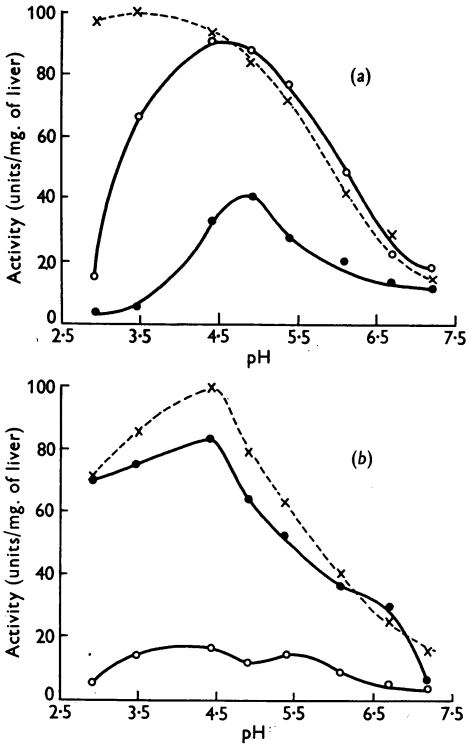

Fig. 4. Effect of DL-tartrate, citrate, fluoride and formaldehyde on the pH–activity curve of acid phosphatase activity of rat-liver homogenates. Additions: (*a*) ×, none; ○, 0·01 M-sodium citrate; ●, 0·01 M-sodium DL-tartrate. (*b*) ×, none; ○, 0·01 M-NaF; ●, 0·5% formaldehyde. 0·005 M-Phenyl phosphate was present as substrate. Veronal–acetate buffers were used throughout. Temp. of incubations, 38°.

Table 1. *Effect of* Cu^{2+}, Zn^{2+} *and* Ba^{2+} *ions on hydrolysis of phenyl phosphate by rat-liver homogenates*

Enzyme digests were buffered at pH 3·75 and 5·6, with acetate buffers, and at pH 6·5 with veronal–acetate buffers. Substrate concn. 5 mM.

Metal added	pH	Activation (+) or inhibition (−) (%)
0·0002 M-Cu^{2+}	3·75	−2
	5·60	−60
	6·50	−92
0·01 M-Zn^{2+}	3·75	−50
	5·60	−86
	6·50	−87
0·01 M-Ba^{2+}	3·75	+20
	5·60	+16
	6·50	−2

Fractionation of the acid phosphatases of rat liver

The results reported above suggested the existence of at least two acid phosphatases in rat liver. Attempts were therefore made to fractionate the enzymes in order to obtain more evidence of the multiple nature of rat-liver acid phosphatase. All assays were carried out in 0·1 M-acetate buffer, 0·005 M-phenyl phosphate being used as substrate.

Stage 1. Rat liver (91 g.) was homogenized in a Waring Blendor and extracted with 300 ml. of 0·2 M-acetate buffer, pH 5·0, for 24 hr. at 0°. This yielded, after centrifuging, 290 ml. of a red opalescent solution containing 41% of the original activity (Table 2). This solution (*A*) was dialysed against distilled water at 0° for 21 hr., during which time a precipitate settled, leaving a clear brown supernatant, solution (*B*). The precipitate and supernatant were separated by centrifuging at 0°. It will be seen from Table 2 that a 50% loss in activity resulted from this step, which does not justify the retention of this procedure in any future purifications.

Stage 2. Most of the acid phosphatase activity present in the supernatant after dialysis was precipitated by the addition of $(NH_4)_2SO_4$ to 75% saturation and equilibration at 0° for 8 hr. The precipitate was separated by centrifuging at 0°, dissolved in 50 ml. of water and dialysed for 14 hr. at 0° against 2 l. of distilled water. A precipitate which appeared was removed by centrifuging. The supernatant, solution (*C*), contained all the activity of solution (*B*).

Stage 3. Further fractionation was carried out with acetone in the presence of Zn^{2+} ions (Cohn *et al.* 1950). Little activity was precipitated at acetone concentrations of less than 15%, whereas at concentrations over 40% considerable inactivation took place. It was necessary to remove Zn^{2+} ions from the preparations before the phosphatase assays and this was achieved by suspending the precipitates in 0·1 M-citrate buffer at pH 5·0 (Cohn *et al.* 1950), and dialysing overnight against distilled water at 0°. Preliminary experiments indicated that it was best to fractionate the main bulk of the preparation between 15 and 40% (v/v) acetone at a Zn^{2+} ion concentration of $1·5 \times 10^{-2}$ M.

Zinc acetate dihydrate (0·218 g.) was added to 50 ml. portions of solution (*C*) and the inactive precipitate removed at 0°. The solution was cooled to −7° and 80% (v/v) acetone at −10° added to a final concentration of 15% (v/v). After equilibration at −7° for 15 min. the precipitate was centrifuged at −7°, suspended in 8 ml. of 0·1 M-citrate buffer, pH 5·0, and dialysed against 1 l. of distilled water overnight at 0° (solution *D*). The supernatant was further precipitated at −7° by the addition of Zn^{2+} ions and 80% (v/v) acetone to final concentrations of 0·015 M and 40% (v/v) respectively. After equilibration at −7° for 15 min. the precipitate was removed by centrifuging and treated as for the 15% acetone precipitate (solution *E*).

The pH–activity curves for these preparations are shown in Fig. 5. Solution (*D*) showed maximum activity at pH 5·6, and the shape of the curve suggested the presence of another

enzyme acting at a much lower pH. A single peak of activity with a maximum at pH 4·4 was given by solution (*E*). The pH–activity curve of solution (*D*) was very similar to that of the original solution. From these results it appeared likely that two enzymes were indeed present in the original solution and that solution (*E*) contained a larger amount of the enzyme with optimum activity at a lower pH.

Stage 4. The possibility of attaining further separation by electrophoresis was investigated. Fig. 6*a* shows the electrophoretic pattern of the phosphatase activity and protein concentration of solution (*E*) as determined by paper electrophoresis in acetate buffer, pH 4·5, *I* 0·02, with assays for phosphatase activity carried out at pH 4·4. The significance of the peak of phosphatase activity and protein occurring at the origin is difficult to interpret since it is probable that some of the protein is adsorbed at the origin. Nevertheless, a peak of very high phosphatase activity was seen to be associated with the fastest-moving protein component. On repeating the experiment with phosphatase assays at pH 3·5 and 5·6 a similar distribution of phosphatase activity was observed in both, although the leading peak of phosphatase activity was much more pronounced when the strips were assayed at the lower pH (Fig. 6*b*).

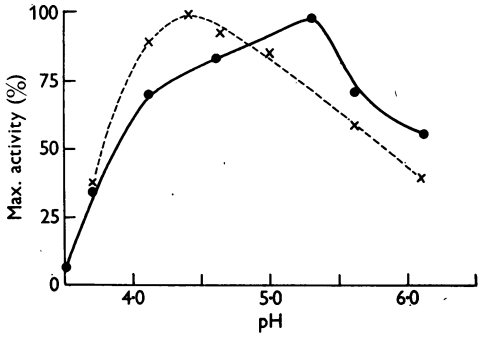

Fig. 5. pH–activity curves of solutions (*D*) and (*E*) obtained in fractionation studies. Solution (*D*), ●; solution (*E*), ×. Assays were carried out at 38° in 0·2M-acetate buffer with 0·005M-phenyl phosphate as substrate.

The fact that a large proportion of the phosphatase activity was associated with the leading protein component suggested that it might be possible to separate a protein fraction, rich in acid phosphatase, by electrophoresis in the Tiselius apparatus. Solution (*E*) was therefore lyophilized and dissolved in 5 ml. of acetate buffer, pH 4·25, *I* 0·02. The solution was dialysed overnight against the buffer and the small precipitate which formed was removed by centrifuging. The schlieren diagram (ascending limb) obtained is presented in Fig. 7. Owing to the low ionic strength of the buffer a large boundary anomaly was obtained. The protein fractions associated with the leading boundary and the slower-moving components were separated and collected at the completion of the run.

pH–activity curves were determined for these fractions and for the original solution, and the results are shown in Fig. 8. The fast fraction showed a well-defined optimum at pH 4·1 with a secondary peak of activity at pH 5·0. The slower-moving fraction showed a broad maximum of activity between pH 4·4 and 5·0. It was concluded that a phosphatase with optimum activity at about pH 4·0 is concentrated largely in the faster-moving fraction, whereas another enzyme, with optimum activity at about pH 5·0, appeared to be associated mainly with the slower-moving fraction. The form of the pH–activity curve of the original solution appeared to be a summation of these two curves. The activity of the fast fraction assayed at pH 4·1 was 3680 units/mg. of protein, which represents a 14·3-fold purification over the starting material.

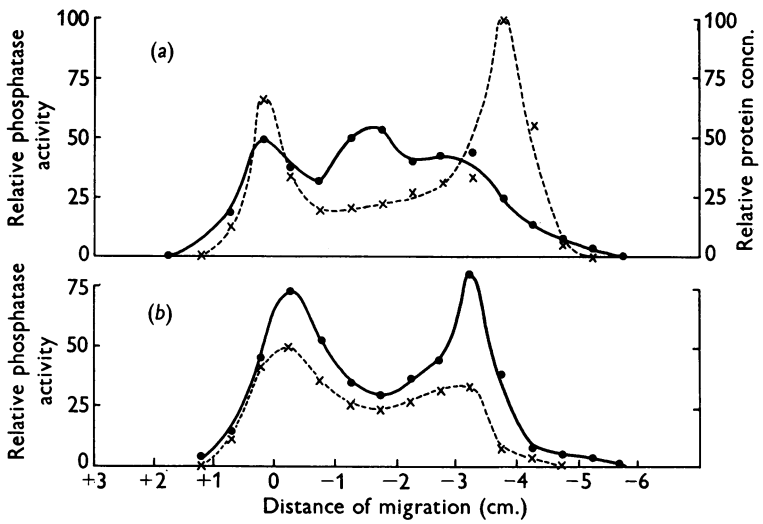

Fig. 6. Filter-paper electrophoresis of acid phosphatase activity of solution (*E*). Electrophoresis was carried out at 5° on Whatman no. 3MM filter paper, 3 cm. wide, for 14 hr. (*a*), and for 17 hr. (*b*), at a voltage of 4·5v/cm. length, in acetate buffer, pH 4·5, *I* 0·02. Assays were at 38°. (*a*) ×, Relative phosphatase activity assayed at pH 4·4; ●, relative protein concn. (*b*) ×, Relative phosphatase activity assayed at pH 3·5; ●, relative phosphatase activity assayed at pH 5·6. +, Migration towards anode; −, migration towards cathode.

The enzymic activity was very unstable in these purified preparations, all activity disappearing on storing overnight at −15°.

Intracellular distribution of the acid phosphatase activity of rat liver

A study of the intracellular distribution in rat liver of acid phosphatase activity at pH 3·8, 5·5 and 6·5 was undertaken to obtain more information on the nature of the enzyme complex present. Alkaline phosphatase, glucose 6-phosphatase and adenosine 5′-phosphatase activities were also studied in the same fractions to compare the distribution of these activities with that of acid phosphatase.

In preliminary experiments it was found that the time of homogenization of the tissue in 0·25 M-sucrose was very critical. A time of 4–5 min. was required to ensure a preparation free from a significant number of unbroken cells, but unfortunately homogenization for this period brought about serious breakdown of cytoplasmic particles, as evidenced by increasing activities in the non-particular cytoplasm with time of homogenizing, accompanied by a corresponding decrease in the activities of the mitochondrial or microsomal fractions or both. In an attempt to overcome this difficulty the distribution of activities between nuclei and total cytoplasm was investigated by the method of Hogeboom *et al.* (1952), and further studies on the distribution of activities in the cytoplasmic fractions were carried out with the original Schneider (1948) technique. In the latter series of experiments homogenizing in 0·25 M-sucrose was restricted to 2 min. and the nuclear fraction was discarded.

The results of a typical distribution between nuclei and cytoplasm are shown in Table 3. Negligible amounts of acid phosphatase activity were found in the nuclear fraction. From the distribution of DNAP it appeared that the nuclear fraction contained 75 % of the nuclei originally present. The low RNAP content of this fraction was an indication that it was free from significant contamination by unbroken cells and cytoplasmic particles, and this was also confirmed by microscopic examination.

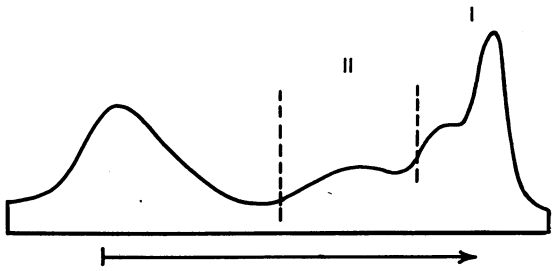

Fig. 7. Ascending-limb diagram from electrophoretic analysis of rat-liver acid phosphatase preparation (solution *E*). Acetate buffer, pH 4·25, *I* 0·02. Fractions I and II separated as indicated after 60 min., fraction I from ascending limb and fraction II from descending limb.

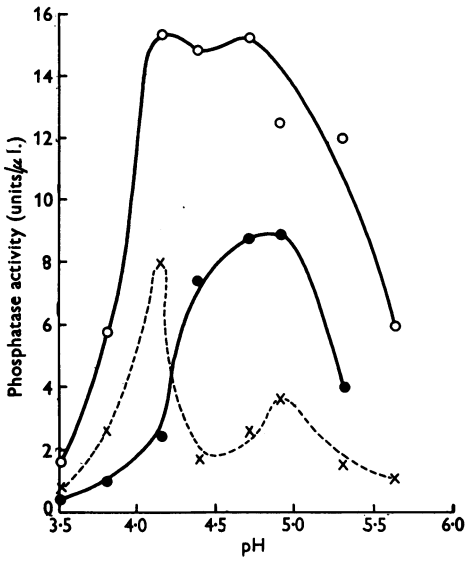

Fig. 8. pH–activity curves of fractions separated by electrophoresis of solution *E* in the Tiselius apparatus. ○, Original solution; ×, fraction I; ●, fraction II. Assays were carried out in 0·2 M-acetate buffers with 0·005 M-phenyl phosphate as substrate at 38°.

Table 2. *Fractionation of rat-liver acid phosphatase*

Assays carried out in 0·2 M-acetate buffer, pH 5·3, with 0·005 M-phenyl phosphate as substrate. Purity of fractions are expressed in terms of activity/mg. of protein (6·25 × protein N). For unit of enzyme activity see Methods.

	Activity (units/ml.)	Total volume (ml.)	10^3 × total units	Purity	Recovery (%)
Liver homogenate	584	—	5305	334	100
Soln. (*A*)	7600	290	2200	—	41·4
Soln. (*B*)	3624	309	1120	—	21·1
Precipitate removed from *B*	—	—	57	—	1·0
Soln. (*C*)	4525	250	1131	—	21·3
Soln. (*D*)	3400	57·5	196	395	3·7
Soln. (*E*)	9500	75	711	2892	13·5

Table 3. *Relative phosphatase activities of rat-liver nuclei and cytoplasm*

Separations by the method of Hogeboom *et al.* (1952). Assays at pH 3·80 and 5·55 were carried out in 0·2 M-acetate buffers, those at pH 6·50 in veronal–acetate buffers, I 0·172. Temp. of incubation, 38°. 0·01 M-β-Glycerophosphate was used as substrate. Activity of unfractionated homogenate was taken as 100 in each case.

Fraction	Relative activity pH of assay 3·80	5·55	6·5	DNAP (μg./100 mg. of liver)	RNAP (μg./100 mg. of liver)
Whole homogenate	100	100	100	20·2	82·0
Nuclear	3	6	3	15·0	6·8
Cytoplasmic	84	98	83	5·2	65·6
Recovery	87	104	86	20·2	72·4

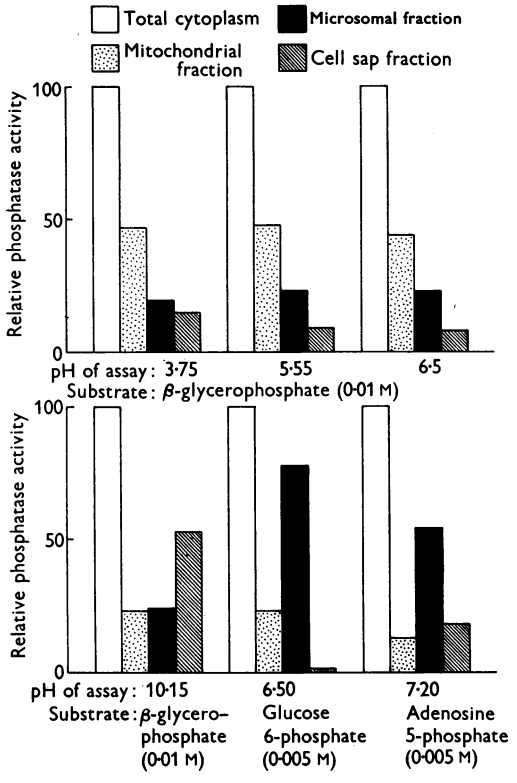

Fig. 9. Relative phosphatase activities of various fractions of rat-liver cytoplasm. Fractions were separated in 0·25 M-sucrose by the method of Schneider (1948). Assays at pH 3·75 and 5·55 were carried out in acetate buffers, those at pH 6·5 and 7·2 in veronal–acetate buffers and those at pH 10·15 in boric acid–KCl–NaOH buffer. Temp. of incubation, 38°. Activity of whole cytoplasm was taken as 100 in each case.

Fig. 9 shows a typical experiment on the relative activities of the various cytoplasmic fractions. Acid phosphatase activity (45–60 %) was recovered in the mitochondrial fraction, whereas the microsome fraction contained 20–38 % of this activity. Glucose 6-phosphatase activity was concentrated chiefly in the microsomal fraction and to a lesser extent in the mitochondrial fraction, the amount present in the non-particulate cytoplasm being negligible. 5′-Nucleotidase also appeared to be concentrated in the microsomal fraction. Over 50 % of the cytoplasmic alkaline phosphatase activity was recovered in the non-particulate fraction with variable amounts in the other two fractions. These results are in agreement with those of Hers, Berthet, Berthet & de Duve (1951); Berthet & de Duve (1951) and Novikoff, Podber, Ryan & Noe (1953).

The distribution of acid phosphatase activity was essentially the same at the three different pH values of assay.

DISCUSSION

The work of Folley & Kay (1936) indicated the existence of three types of acid phosphatase differentiated by their pH optima, sensitivity to Mg^{2+} ions and relative activity towards α- and β-glycerophosphates. Since this early work evidence has assumulated which suggests that more than one non-specific acid phosphatase is present in liver (Roche, 1950).

The results obtained in the present work suggest the presence in freshly prepared rat-liver homogenates of at least two acid phosphatases, one having a pH optimum between 3·5 and 4·0, and another with optimum activity at pH 5·0–5·5. The former appears to be activated by veronal, whereas the latter appears to be inhibited by this substance. Various metallic ions differed markedly in their effect on the two enzymes (Fig. 3 and Table 1). Citrate and tartrate inhibited the enzyme which had optimum activity at low pH values but had little effect on the other (Fig. 4). Fluoride caused considerable inhibition of both enzymes, whereas cyanide had little effect on either.

Abul-Fadl & King (1949) carried out a similar study on the acid phosphatases of erythrocytes and prostate, and Gordon (1952) examined the effects of some of these substances on the enzyme in extracts of adrenal cortex. Table 4 compares some of these data with the results obtained in the present work.

This comparison indicates a variation in behaviour of these various substances towards the activities of preparations from different tissues. Whether these differences can be accounted for by

Table 4. *Comparison of the effects of various substances on the acid phosphatase activity at* pH 5·0–5·5 *in different tissues*

Results with erythrocyte, prostate and liver enzyme were obtained with 0·005 M-phenyl phosphate in acetate buffers; those with adrenal cortical enzyme were obtained with β-glycerophosphate in acetate buffers. Negative values represent inhibitions; positive values represent activations.

Substance added	Source of enzyme: Liver (%)	Prostate* (%)	Erythrocytes* (%)	Adrenal cortex† (%)
0·01 M-Mg^{2+}	Nil	Slight inhib.	Slight inhib.	Nil
0·0002 M-Cu^{2+}	−85	Slight inhib.	−95	−86
0·01 M-Cyanide	Nil	+12	+8	—
0·01 M-DL-Tartrate	−41	−95	Nil	−91
0·01 M-Fluoride	−53	−96	−8	—
0·5% Formaldehyde	−68	Nil	−100	−30

* Results quoted from Abul-Fadl & King (1949). † Results quoted from Gordon (1952).

variation in protein content of the preparations or whether the enzymes themselves are different is still a matter of conjecture. This question can only be settled by studies on more highly purified enzyme preparations. In this connexion it is interesting to note that Anagnostopolous (1953) found that L-tartrate caused a 95% inhibition of purified prostate phosphatase.

Abul-Fadl & King (1949) found that human-liver acid phosphatase was almost completely inhibited by 0·01 M-L-tartrate over the pH range 4·0–6·0. This suggests the possibility of a species difference in acid phosphatase activity.

The results obtained from fractionation studies, although providing evidence for the existence of at least two phosphatases, did not show a very high over-all purification. Obviously further studies are necessary in this direction.

The acid phosphatase activities of rat liver would appear to have a similar intracellular distribution. The distribution of glycerophosphatase activity, assayed at pH 6·5, was of interest in view of the finding of Swanson (1950) that purified glucose 6-phosphatase preparations from liver had glycerophosphatase activity with an optimum at pH 6·5, and also in view of the possible presence of a third acid phosphatase, with optimum activity about pH 6·0–7·0, in crude homogenates with β-glycerophosphate as substrate. It will be seen from Fig. 9 that the ratio of distribution of glucose 6-phosphatase activity between mitochondrial and microsomal fractions is different from the ratio of distribution of β-glycerophosphatase activity assayed at pH 6·5. This suggests that the two activities are distinct.

SUMMARY

1. pH–activity curves have been determined for the acid phosphatase activity of rat-liver homogenates with β-glycerophosphate, phenyl phosphate, *p*-nitrophenyl phosphate and adenosine 3′-phosphate as substrates. These curves suggested the presence in rat liver of two non-specific acid phosphatases, with the possibility of a third showing some specificity towards β-glycerophosphate and adenosine 3′-phosphate.

2. The effect of various substances on the phosphatase activity of rat-liver homogenates was studied over a wide pH range and the results confirmed the presence of at least two acid phosphatases.

3. Further evidence for the existence of two acid phosphatases was obtained from fractionation studies involving acetone precipitation in the presence of Zn^{2+} ions and electrophoresis in the Tiselius apparatus.

4. The intracellular distribution in rat liver of acid phosphatase activity was studied by differential centrifuging. No difference in the location of the different activities was apparent.

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The Hydrolysis of Glucose 1-Phosphate by Rat-Liver Extracts

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The existence in rat liver of a phosphomonoesterase specific for the hydrolysis of glucose 6-phosphate is now well established, after the isolation of a partially purified preparation of this enzyme by Swanson (1950). It had been previously suggested by Broh-Kahn & Mirsky (1948) that no specific glucose 1-phosphatase activity exists in liver and that glucose 1-phosphate is converted into glucose 6-phosphate before hydrolysis. Faulkner (1955), on the other hand, has demonstrated the presence in silkworm blood of a phosphatase specific for glucose 1-phosphate. The work of Broh-Kahn & Mirsky (1948) did not entirely eliminate the possibility of hydrolysis of glucose 6-phosphate by other routes, and the present work was undertaken to examine the hypothesis of these workers. A preliminary account of this work has already appeared (Goodlad & Mills, 1956).

EXPERIMENTAL

Substrates. Glucose 1-phosphate and glucose 6-phosphate were prepared as previously described (Goodlad & Mills, 1957).

Phosphatase activity. This was assayed by the general method described by Goodlad & Mills (1957).

Phosphoglucomutase activity. This was measured by a method based on that of Najjar (1948): 0·1 ml. of 0·05 M-glucose 1-phosphate (potassium salt, in solution containing 0·1 M-cysteine), 0·5 ml. of buffer solution, 0·1 ml. of 0·01 M-$MgSO_4$ and 0·3 ml. of enzyme solution, all preheated to 38°, were incubated together for 15 min. at 38°. The reaction was stopped by the addition of 1 ml. of 5 N-H_2SO_4 followed by 3 ml. of water, and precipitated protein was removed by centrifuging. A portion (0·5 ml.) of the supernatant was heated at 100° for 10 min. and the total inorganic phosphate was estimated by the method of Allen (1940). The initial acid-labile phosphorus was determined in the same manner except that the substrate was added after the H_2SO_4. The decrease in acid-labile phosphorus (glucose 1-phosphate) is equivalent to the amount of acid-stable glucose 6-phosphate formed.

One unit of phosphoglucomutase activity $\equiv 1\ \mu g.$ of acid-stable phosphorus formed/hr. under the above conditions.

Separation of cytoplasmic particles. This was carried out by the method of Schneider (1948), which involves differential centrifuging in 0·25 M-sucrose solution. Separations were carried out at 4° with an MSE Major refrigerated centrifuge, a force of 600 *g* for 10 min. being used to separate nuclei and unbroken cells from the original homogenate. Centrifuging the supernatant at 8500–10 000 *g* for 10 min. separated the mitochondrial fraction (MT), and centrifuging the supernatant and washings from the MT fraction at 18 000 *g* for 1 hr. separated the microsomal fraction (MS) and the supernatant cell sap (S). Each fraction was frozen in a solid CO_2–acetone mixture and thawed rapidly, this process being repeated twice more to ensure liberation of enzymic activity from the particles (cf. Berthet, Berthet, Applemans & de Duve, 1951).

RESULTS

Hydrolysis of glucose 1-phosphate (G 1-P) and glucose 6-phosphate (G 6-P) by unfractionated rat-liver homogenates. The hydrolysis of G 1-P by rat-liver homogenates differs in a number of respects from the hydrolysis of G 6-P. The pH optimum for the hydrolysis of G 1-P was found to lie between