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Multiple Forms of Acid Phosphatases in the Lysosomes of Rat Liver

by

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SUMMARY Rat liver homogenate was separated into a 15,000 *g* lysosomal fraction and a 15,000 *g* supernatant fraction by differential centrifugation. The 15,000 *g* lysosomal fraction treated with Triton X-100, the supernatant and a solution of lysed rat red blood cells were chromatographed separately on a DEAE-cellulose anion exchange column using a stepwise concentration gradient. The 15,000 *g* lysosomal fraction was separated into three forms of acid phosphatases all of which were inhibited by tartrate but not by formaldehyde. They had very similar pH optima and K_m values; their V_{max} values were different. The acid phosphatases in the 15,000 *g* lysosomal fraction were activated by cysteine but not after their separation on a DEAE-cellulose-column.

A fourth form of acid phosphatase present in the 15,000 *g* supernatant was found to be similar to erythrocytic acid phosphatase in its behaviour on the DEAE-cellulose column and towards the inhibitors formaldehyde and tartrate. The pH optimum and K_m value of this acid phosphatase are very different from those of the lysosomal acid phosphatases.

INTRODUCTION

The existence of enzymes in multiple forms appears now to be the rule rather than the exception. Multiple enzyme forms have been shown to exist in the same species as for example prostatic and erythrocytic type of acid phosphatases in human serum (Abul-Fadl and King, 1949) or in the same organ as in the case of lactate dehydrogenases (Meister, 1950; Neilands, 1952). Eichel and Bukovsky, (1961) have shown the presence of different forms of glutamic oxaloacetic transaminases in the mitochondria and in the supernatant of rat liver cells.

The presence of multiple forms of malate dehydrogenase in mitochondria of pig heart (Thorne, Grossman and Kaplan, 1963) and of chicken (Kitto, Wasserman and Kaplan, 1966) has also been demonstrated. In this paper we are presenting evidence for the existence of three forms of acid phosphatases in the 15,000 *g* lysosomal fraction of rat liver. A brief account of this work has been reported earlier (Balasubramaniam, Reginald and Wijesundera, 1967).

MATERIALS AND METHODS

Preparation of lysosomal acid phosphatase

Rats weighing about 100 g were chosen for these experiments. The livers were removed immediately after the animals were killed, and washed with ice cold 0.25 M sucrose to remove the blood. The liver was minced, washed briefly with 0.25 M sucrose solution

and then homogenized in 0.25 M sucrose (1 g in 10 ml) using a Potter homogenizer. The tissue was kept at 5°C throughout the entire procedure. The homogenate was centrifuged in a refrigerated International centrifuge at 800 *g* for 10 min to remove the cell debris and nuclei. The 800 *g* supernatant was then centrifuged at 15,000 *g* for 20 min. The 15,000 *g* pellet containing lysosomes and mitochondria was washed twice with 0.25 M sucrose and suspended in 0.25 M sucrose or water as described in the text. The 15,000 *g* supernatant was used without any further treatment (Balasubramaniam and Deiss, 1965).

Preparation of erythrocytic acid phosphatase

Oxalated rat blood was diluted with 0.25 M sucrose and the erythrocytes obtained by centrifugation were washed twice with 0.25 M sucrose. One ml of the packed erythrocytes was lysed in 9.0 ml of water.

Separation of isoenzymes

Acid phosphatases from the 15,000 *g* particulate fraction suspended in water were released by the addition of Triton X-100. A 5 ml aliquot of this solution (equivalent to 0.5 g of tissue) was applied to the DEAE-cellulose anion exchange column (1.4 x 40 cm) which had previously been equilibrated with 0.001 M phosphate buffer, pH 7.0. Five millilitre aliquots of the lysed rat erythrocyte preparation and of the 15,000 *g* supernatant were also chromatographed as described above.

Determination of protein

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951) using crystalline bovine albumin as standard. Some assays of protein were also carried out by measuring extinction at 280 nm.

Assay of enzyme activity

The acid phosphatase activity was measured by the method described by King and Jegathesan (1959). The lysosomal preparation was treated with 0.1% w/v Triton X-100 to release the enzyme before assay. All determinations of enzyme activity were carried out in duplicate at 37° C. for 1 h.

RESULTS

Effect of inhibitors and activators on 15,000 g lysosomal acid phosphatases

The results in Table 1 show that the acid phosphatases in the 15,000 *g* lysosomal fraction are about 88% formaldehyde stable and about 21% tartrate stable (Balasubramaniam *et al.*, 1967) whilst the acid phosphatase in the 15,000 *g* supernatant is 83% tartrate stable and 33% formaldehyde stable.

TABLE 1

Effect of formaldehyde and tartrate on the 15,000 *g* lysosmal fraction and the 15,000 *g* supernatant of rat liver.

Experiment number	Lysosomes		Supernatant	
	% Tartrate stable	% Formaldehyde stable	% Tartrate stable	% Formaldehyde stable
1	11	85	88	34
2	30	84	79	31
3	21	94	—	—

The incubation mixture contained 1 ml of 0.2 M citrate buffer pH 4.9, 1 ml of 0.01 M disodium phenyl phosphate, 0.2 ml of enzyme (lysosome or supernatant corresponding to 1 g tissue in 40 ml) and 0.1 ml of water or of 0.5 M DL–tartate or of 4.8 M formaldehyde.

Acid phosphatase activity with water as control was taken as 100%

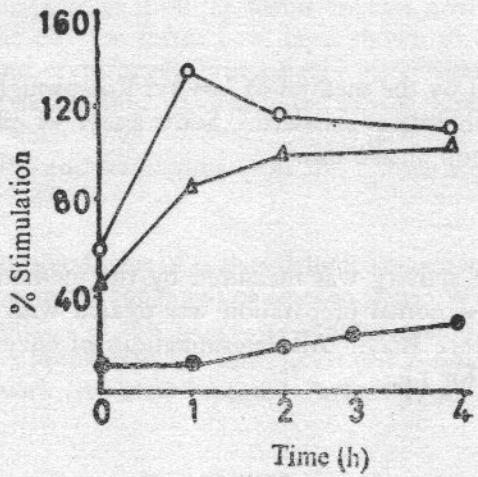

Fig. 1.

Effect of pre-incubating the 15,000 *g* lysosomal acid phosphatase for different periods of time with 0.1 M, (open circles) , 0.01 M, (open triangle) and 0.001 M, (closed circles) concentrations cysteine.

The 15,000 *g* lysosomal fraction suspended in water (1 *g* original tissue in 10 ml) was treated with 0.1%w/v Triton X-100 Two ml portions were made up separately to 10 ml with citrate buffer, pH 6.3 (final conc 0.01 M) and incubated at 37°C with and without cysteine. Samples (0.2 ml) were withdrawn at hourly intervals and assayed for acid phosphatases using 1 ml 0.2 M citrate buffer (pH 4.9) and 1 ml of 0.01 M disodium phenyl phosphate.

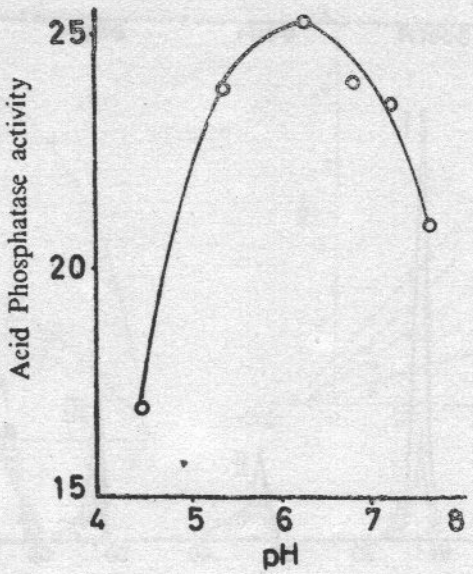

Fig. 2.

Effect of pre-incubating the 15,000 *g* lysosomal acid phosphatase with 0.01 M cysteine in buffers of varying pH for 1 h at 37°C.

The experimental procedures were similar to those described in Fig 1. Acid phosphatase activity is expressed as μg phenol liberated / 0.2 ml sample/h.

The results in Fig. 1 show that the acid phosphatases in the 15,000 *g* lysosomal fraction are activated by cysteine. This effect is maximal when the enzyme is preincubated with cysteine, 10^{-2} M or 10^{-1} M, for 1 h. No further activation was observed even when the preincubation period was increased beyond 2 h. in the presence of cysteine at the above concentrations. At a concentration of 10^{-3} M the stimulatory effect was lower and was seen to increase with time even after 2 h preincubation.

The effect of pH on the activation of lysosomal acid phosphatases during preincubation with 10^{-2} M cysteine is shown in Fig. 2. A bell-shaped activity curve was obtained with optimum activation at about pH 6.3.

Isolation and characterization of acid phosphatase isoenzymes

The activity of the acid phosphatases in the 15,000 *g* lysosomal fraction of rat liver could be separated into three peaks on DEAE-cellulose (Fig. 3). Peak I, the predominant form of the enzyme contains about 50% of the total lysosomal acid phosphatase activity, while peaks II and III contain about 20% and 30% of the activity respectively. Most of the proteins of the 15,000 *g* particulate fraction were eluted along with the acid phosphatase in peak I. The protein concentration determined on the pooled samples of acid phosphatases in peaks, I, II and III were 0.374, 0.060 and 0.054 mg/ml respectively. The 15,000 *g* supernatant chromatographed on DEAE-cellulose has an additional peak (peak IV) and this contained about 80% of the supernatant acid phosphatases. The protein concentration of the pooled sample of peak IV was 0.044 mg/ml. Peak IV is absent in the lysosomes.

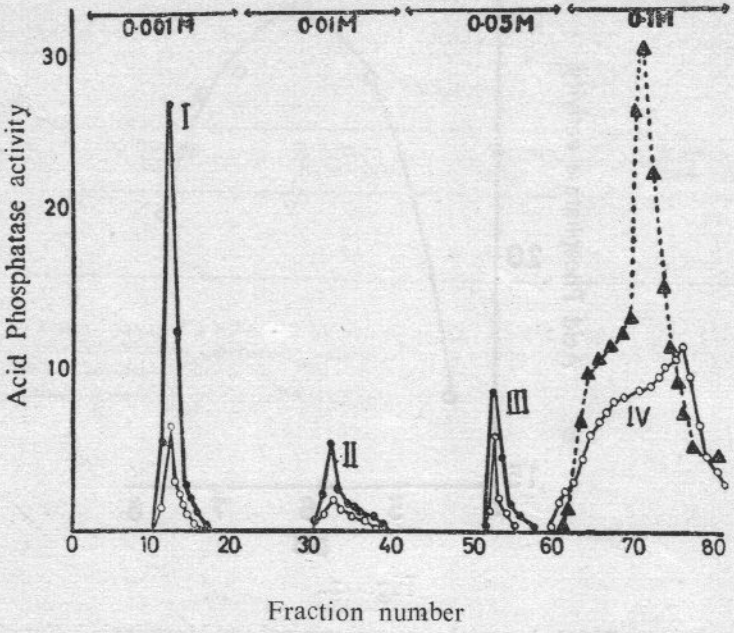

Fig. 3.

Separation of acid phosphatases of rat liver preparations and of rat erythrocytes on a DEAE-cellulose column lysosomal preparation, (closed circles); supernatant, (open circles); and rat erythrocyte preparation, (closed triangles)

Samples were eluted at 4°C by a stepwise concentration gradient as shown in diagram using phosphate buffer pH 7.0 at a flow rate of 100 ml per h. The elute was collected in 6 ml fractions. Aliquots (0.5 ml) of the elute were used for assay of acid phosphatase activity as described in Fig. 1. Acid phosphatase activity is expressed as μg phenol liberated/0.5 ml sample/h.

The acid phosphatase present in the erythrocytic preparation when chromatographed similarly on DEAE-cellulose gave only one activity peak and this corresponded to that of peak IV of the 15,000 *g* supernatant of rat liver.

The pH profiles of the four forms of the acid phosphatases present in liver separated on DEAE-cellulose are shown in Fig. 4. The pH optima of the three forms of lysosomal acid phosphatases in peaks I, II and III were approximately pH 5.0 while the pH optimum of the acid phosphatase in peak IV from the 15,000 *g* supernatant was about pH 5.5 (Balasubramaniam *et al*, 1967). The K_m and V_{max} values were calculated from the Lineweaver-Burke plot (Fig. 5). The three forms of lysosomal acid phosphatases have very similar K_m values of about 25 mM disodium phenyl phosphate, while their V_{max} values were 11, 28 and 40 mg phenol/mg protein/min. The acid phosphatase in peak IV of the 15,000 *g* supernatant has a K_m value of 10 mM disodium phenyl phosphate and a V_{max} of 13.6 mg phenol/mg protein/min.

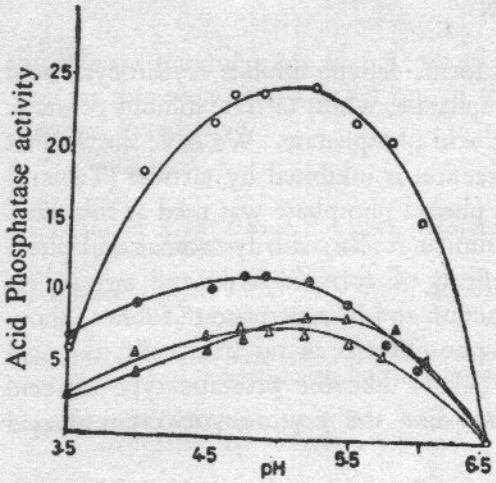

Fig. 4.

pH activity curves of the acid phosphatase isoenzymes of peak I, (open circles); peak II, (open triangles); peak III, (closed circles); and peak IV, (closed triangles).

Enzymes from each peak were pooled separately. Enzymes I, II & III represent those obtained from peaks I, II & III of the lysosomal preparation; enzyme IV was that obtained from peak IV of the supernatant preparation. Acid phosphatase activity was assayed using 0.5 ml aliquots in different buffers. Other conditions were as in Fig. 1. Acid phosphatase activity is expressed as μg phennol liberated / 0.5 ml sample / h.

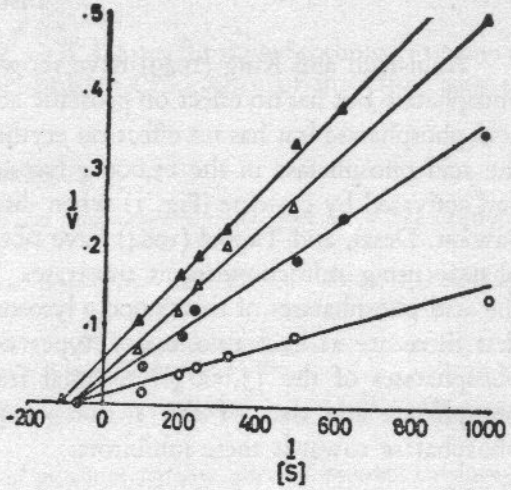

Fig. 5.

Lineweaver-Burke plot for acid phosphatase isoenzymes of peak I, (open circles); peak II, (open triangles); peak III, (open circles); and peak IV (closed triangles).

Enzymes were the pooled fractions described in Fig. 4. Six concentrations of disodium phenyl phosphate ranging from 10^{-3} M to 10^{-2} M were used. Velocity is expressed as μg phenol formed / 0.5 ml pooled sample/h.

The results in Table 2 show that the lysosomal acid phosphatases in peaks I, II and III are very similar in that they are inhibited by tartrate and fluoride but not by formaldehyde. The supernatant acid phosphatase in peak IV is quite different from the three forms of lysosomal acid phosphatases in that it is inhibited by formaldehyde but not by tartrate or fluoride.

TABLE 2

Effect of inhibitors on the acid phosphatases separated on DEAE—cellulose

Inhibitors	Peak Numbers			
	I	II	III	IV
None	100	100	100	100
Formaldehyde	78	68	73	8
Tartrate	5	11	15	91
Fluoride	8	15	20	89

The isoenzymes were pooled as described in Fig. 4. The incubation medium contained 1 ml of 0.01 M disodium phenyl phosphate, 1 ml of 0.2 M citrate buffer pH 4.9, 0.1 ml of water or of 0.5 M DL tartrate or of 4.8 M formaldehyde and 0.5 ml of the pooled enzyme. Acid phosphatase activity with water as control was taken as 100.

DISCUSSION

Abul-Fadl and King (1949) have shown that formaldehyde inhibits erythrocytic acid phosphatase but has no effect on prostatic acid phosphatase, whilst tartrate inhibits prostatic acid phosphatase but has no effect on erythrocytic acid phosphatase. We have shown that the acid phosphatase in the 15,000 *g* lysosomal fraction is inhibited by tartrate (Table 1) and activated by cysteine (Fig. 1) when disodium phenyl phosphate was used as substrate. Sawant, Desai, and Tappel (1964) have obtained similar results with lysosomal acid phosphatase using mitochondria as substrate. The effects of formaldehyde and tartrate on the acid phosphatases of the 15,000 *g* lysosomal fraction and its supernatant (Table 1) show that there are at least two major types of acid phosphatases in rat liver cells; the acid phosphatases of the 15,000 *g* lysosomal fraction behave like the prostatic type of acid phosphatase and that of the 15,000 *g* supernatant like the erythrocytic type of acid phosphatase towards these inhibitors.

Moore and Angeletti (1961) have shown the presence of four acid phosphatases in rat liver homogenate by DEAE-cellulose column chromatography using a continuous concentration gradient. However, the acid phosphatases of peaks III and IV were not well separated by their method. Brightwell and Tappel (1968) have shown the presence of two acid phosphatases in rat liver lysosomes by DEAE-cellulose chromatography using a continuous concentration gradient starting with a 0.01 M buffer. By chromatographing the 15,000 *g* lysosomal fraction on DEAE-cellulose starting with an 0.001 M buffer (Fig. 3), we have been able to show that lysosomes contain three forms of acid phosphatases. In experiments where columns were equilibrated and eluted with 0.01 M buffer as carried out by Brightwell and Tappel (1968) we have also observed only two lysosomal acid phosphatases. This is probably due to the fact that both acid phosphatases appearing in our peaks I & II are eluted as one peak in Brightwell and Tappel's experiment. The three lysosomal acid phosphatases are inhibited by tartrate and fluoride but not by formaldehyde. These three acid phosphatase isoenzymes have similar pH optima and K_m values (Figs. 4 & 5). The fact that they possess different V_{max} values indicates that they probably represent different forms of acid phosphatases and are not artifacts. The activation of the 15,000 g lysosomal acid phosphatases was greater when the enzymes were preincubated with cysteine than when cysteine was added to the assay mixture. When the three forms of acid phosphatases separated on DEAE-cellulose were preincubated with cysteine either individually or in different combinations, no activation was observed.

Moore and Angeletti were unable to separate completely the acid phosphatases in their last two peaks by eluting with a continuous gradient. We have been able to effect a better separation of these by use of a discontinuous concentration gradient.

The acid phosphatase activity peak of red blood cells coincided with peak IV of the supernatant. It is possible that this latter peak might be due partly at least to contamination by the rat blood.

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