Studies on Acid Hydrolases in Adult and Foetal Tissues

2. ACID PHENYL PHOSPHOMONOESTERASES OF ADULT MOUSE LIVER*

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In a previous paper (Neil & Horner, 1964) some of the difficulties involved in the interpretation of tissue acid-phosphomonoesterase determinations were discussed. Attention was drawn to the confusion in interpretation of acid-phosphomonoesterase activities determined with different substrates. In a single tissue this confusion can be the direct result of the presence of a number of enzymes that exhibit, *in vitro* at least, phosphomonoesterase activity on a variety of substrates, and that also exhibit markedly different affinities for these substrates.

Macdonald (1961) investigated acid-phenylphosphomonoesterase (PhPase)† activity at pH 5·9 in whole aqueous homogenates of mouse liver. Much of the enzymic activity he observed was labile at 37°, but in the presence of malonate (0·15M) no lability was observed. The interpretation of the experimental observations was very largely in terms of a labile lysosomal acid phosphatase that was protected from destruction during incubation by malonate. Neil (1961) suggested that the PhPase activity of mouse-liver homogenates comprised at least two components: a thermostable activity resembling that of rat-liver lysosomal acid phosphatase and a thermolabile activity resembling that of glucose 6-phosphatase (G-6-Pase). Neil (1961) also confirmed the effect of malonate observed by Macdonald (1961) but showed that malonate had very little influence on the lability of the PhPase activity of a particle agglutination precipitate containing most of the G-6-Pase activity originally present in mouse-liver cytoplasmic extract (Fig. 1). Macdonald (1962), comparing the hydrolysis of phosphomonoesters by homogenates of rat liver and rat spleen, demonstrated marked differences in enzymic activity in the two tissues, and in the response of these activities to preincubation, with phenyl phosphate, β-glycerophosphate and glucose 6-phosphate as substrates.

* Part 1: Neil & Horner (1964).

† Abbreviations: PhPase, phenyl phosphomonoesterase; G-6-Pase, glucose 6-phosphatase; *p*-NPPase, *p*-nitrophenyl phosphomonoesterase.

A multiplicity of phosphomonesterase activities in mouse-liver preparations was also indicated by Golberg, Martin & Leigh (1962), who observed the markedly different effects of several metabolites and enzyme affector substances on the enzymic hydrolysis of phenyl phosphate, β-glycerophosphate, casein and ATP. The pattern of these effects was altered by intramuscular injections of an iron–dextran complex.

Neil & Horner (1964) have demonstrated the presence in liver, in a variety of animal species, of a thermostable alloxan-inhibited *p*-nitrophenyl phosphomonoesterase (*p*-NPPase) component that is confined almost exclusively to the supernatant fraction obtained by conventional differential-centrifugation techniques. The activity of this component was distinguished from the *p*-NPPase activities of both lysosomal acid phosphatase and G-6-Pase. There are therefore at least three components to the acid *p*-NPPase enzyme system in liver tissue. As shown below, a similar situation exists for the acid PhPase enzyme system in mouse liver, and the suggestion is made that there is a fourth component.

MATERIALS AND METHODS

Animals. Albino mice (I.C.I. strain) were kindly supplied by Dr M. H. Salaman and Miss J. J. Harvey from the colony maintained in the Department of Cancer Research (British Empire Cancer Campaign) of The London Hospital.

Liver preparations. In general, liver homogenates were prepared in 0·25M-sucrose solution and fractionated as described by Neil & Horner (1964). Water homogenates were simply freed from nuclei and cell debris by centrifugation and washing.

Cytoplasmic extracts and resuspended particulate fractions were treated at 0° in a M.S.E. homogenizer at full speed for 10–15 min. before enzyme assays were carried out.

For the experiment in Fig. 1 a water cytoplasmic extract was separated into a washed agglutination precipitate (pH 5·0) and a soluble fraction according to the method of de Duve, Berthet, Hers & Dupret (1949).

Substrates. Laboratory-reagent grade phenyl disodium orthophosphate was purified before use. β-Glycerophosphate was laboratory-reagent grade (British Drug Houses Ltd.) containing less than 3% of the α-isomer. Glucose

6-phosphate (disodium salt) was obtained from British Drug Houses Ltd.

Enzyme assays. Acid-phenyl-phosphomonoesterase assays were carried out for 5–15 min. at pH 5·0 or pH 5·9 with 0·05 M-phenyl phosphate in the presence of 0·10 M- or 0·05 M-acetate buffer in a total volume of 2 ml. The reaction was stopped by the addition of 2 ml. of Folin–Ciocalteu reagent (diluted 1:3) followed by centrifugation. Then 2 ml. of Na_2CO_3 solution (1·5 M) was added to 3 ml. of the supernatant solution and incubated for 15 min. at 37°. Extinctions were read at 660 mμ. Satisfactory blank values were obtained by addition of two extinctions: that observed by incubating enzyme in the absence of substrate followed by the addition of the Folin–Ciocalteu reagent and that observed to be due to the non-enzymic hydrolysis of the substrate in the conditions of the assays. Enzymic hydrolysis of phenyl phosphate proceeded linearly with regard to both time and enzyme concentration in all assay conditions.

Acid β-glycerophosphatase, glucose 6-phosphatase and *p*-nitrophenyl phosphatase were assayed as described by Neil & Horner (1964).

Where the effects of malonate on enzyme activity were studied this compound was present in 0·15 M concentration.

All enzyme activities are expressed as μmoles of substrate hydrolysed/min./g. of fresh tissue in the conditions of the assays, unless otherwise stated.

RESULTS

Intracellular localization of the principal thermolabile acid-phenyl-phosphatase component in mouse liver. The results reported by Neil (1961) suggested that G-6-Pase could account for most, if not all, of the thermolabile phenyl-phosphatase activity in mouse-liver extracts. Localization of this activity in the microsomal fraction would strongly support this hypothesis. Livers were therefore homogenized in ice-cold 0·25 M-sucrose, and granule fractions were prepared by conventional differential centrifugation with a centrifugal force–time integral of about 100000*g*-min., which produced a granule fraction containing over 70 % of the sedimentable acid β-glycerophosphatase with the smallest percentage contamination with G-6-Pase. The PhPase activities at pH 5·0 of liver fractions and of a reconstituted cytoplasmic extract were determined before and after preincubation at 37° and pH 5·0, conditions in which G-6-Pase is rapidly inactivated. The thermolabile PhPase activity was predominantly in the microsomal fraction (Table 1). Similar results are obtained with more prolonged preincubation at pH 5·9. The differential effects of preincubation on the fractions in Table 1 were not due to separation artifacts since activities were additive on recombination of the fractions.

Thermostable acid phenyl phosphatase, inhibited by alloxan, in the supernatant fraction from mouse-liver homogenates. Neil & Horner (1962, 1964) reported the presence in the supernatant fraction from liver tissue of a thermostable acid *p*-NPPase that is inhibited by alloxan. An acid PhPase, having similar properties, has now been demonstrated in supernatant fractions from guinea-pig and mouse livers (Table 2).

Large granule (M) and supernatant (S) fractions were prepared together with, for mouse liver, a reconstituted fraction (MS). PhPase activities in the three fractions were determined after preincubation

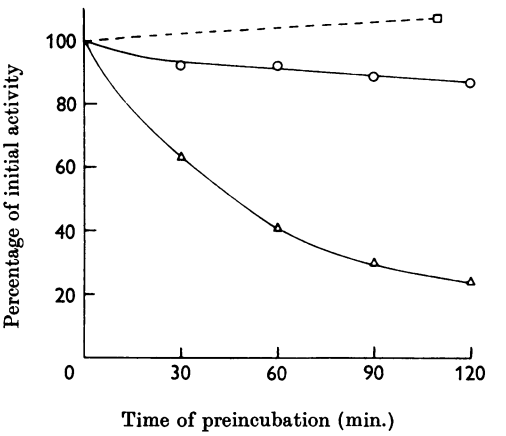

Fig. 1. Variation of phenyl-phosphatase activity in mouse-liver preparations with preincubation in the presence of malonate (0·15 M) at pH 5·9 and 37°. Details of preparations and assay are given in the Materials and Methods section. □, Cytoplasmic extract; ○, soluble fraction; △, agglutination precipitate.

Table 1. *Localization of thermolabile acid-phenyl-phosphatase activity in mouse-liver cytoplasmic extract*

Preincubation was for 20 min. at 37° in 0·1 M-acetate buffer, pH 5·0. Assays were carried out with 0·05 M-phenyl phosphate in 0·1 M-acetate buffer, pH 5·0, at 37° for either 5 min. (no preincubation) or 15 min. (after preincubation).

	Acid-phenyl-phosphatase activity				
	Large-granule fraction (10^5*g*-min.)	Microsomal fraction ($3·5 \times 10^6$*g*-min.)	Supernatant fraction	Reconstituted cytoplasmic extract	Recovery (%)
No preincubation (*a*)	1·99	3·66	2·82	8·50	99·7
Preincubation (*b*)	1·07	1·45	2·29	5·00	96·2
Fall in activity (*a* − *b*)	0·92	2·21	0·53	3·50	104·5

Table 2. *Effect of alloxan on acid-phenyl-phosphatase activities of guinea-pig-liver and mouse-liver fractions*

Preincubation was for 20 min. at 37° in 0·1 M-acetate buffer, pH 5·0. Assays were carried out with 0·05 M-phenyl phosphate in 0·1 M-acetate buffer, pH 5·0, at 37° for 15 min.

	Acid-phenyl-phosphatase activity			
	Large-granule fraction (M)	Supernatant fraction (S)	MS	$\frac{M+S}{MS}$
Guinea-pig liver				
Without alloxan	2·14	1·54	—	—
With alloxan (0·05 M)	2·14	0·30	—	—
Inhibition by alloxan (%)	0	80·5	—	—
Mouse liver				
Without alloxan	0·81	2·34	3·31	0·953
With alloxan (0·05 M)	0·67	0·16	0·84	0·988
Inhibition by alloxan (%)	17·2	93·2	74·6	—

for 20 min. at pH 5·0 and 37°, with and without alloxan (0·05 M). The results (Table 2) show that, though the residual PhPase activities (after preincubation) of the large-granule fractions from livers of both species are inhibited less than 20 % by alloxan, those of the supernatant fractions are inhibited by over 80 %.

Effects of malonate on the acid-phenyl-phosphatase activities of mouse-liver subcellular fractions. Macdonald (1961) demonstrated that 0·15 M-malonate prevented the fall in PhPase activity of mouse-liver homogenates caused by preincubation at pH 5·9. This effect was confirmed for whole cytoplasmic extracts by Neil (1961) and by Golberg *et al.* (1962). If the lability of mouse-liver PhPase in the absence of malonate is due principally to the inactivation of G-6-Pase, then malonate must exert its effect either by protecting this enzyme from heat denaturation in a whole cytoplasmic extract or by activating another PhPase component. Alternatively, both mechanisms may operate. The protection of G-6-Pase would be dependent on the presence of a soluble factor that is removed during the preparation of particulate fractions containing the enzyme, since these preparations are not protected by malonate (Fig. 1). The possible activation effect of malonate would, in the conditions of the experiment at pH 5·9, almost exactly balance the (? independent) fall in G-6-Pase activity.

The results summarized in Table 3 indicate that the 'protective' effect of malonate is independent of whether the cytoplasmic extract is prepared in a water or sucrose (0·25 M) medium. Preincubation at pH 5·9 with malonate slightly increased the PhPase activity of the water extract and slightly decreased that of the sucrose extract. At pH 5·0, however, malonate produced an increase of over 80 % in the activity of the sucrose extract (Table 3). Further results (Table 4) demonstrate that preincubation with malonate does not affect the decrease in G-6-Pase activity. When the extract was preincubated before the addition of malonate, the latter produced only a very small increase in PhPase activity over that observed after preincubation and assay without the addition of malonate (Table 4); this suggests that the PhPase component that is activated by malonate is unstable at pH 5·0 and 37°.

Table 3. *Acid-phenyl-phosphatase activities at pH 5·0 of water and sucrose extracts of mouse liver before and after preincubation at 37°*

Details of the preincubation conditions are given in the Table. Assays were carried out with 0·05 M-phenyl phosphate in 0·05 M-acetate–0·15 M-malonate buffer, pH 5·0 at 37° for 5 min.

	Acid-phenyl-phosphatase activity	
	Enzyme activity	Percentage of initial activity
Preincubation of water cytoplasmic extract:		
None	9·04	100
100 min., pH 5·9	5·10	56·4
100 min., pH 5·9, +0·15 M-malonate	9·76	108·0
Preincubation of sucrose cytoplasmic extract:		
None	9·11	100
100 min., pH 5·9	4·84	53·2
100 min., pH 5·9, +0·15 M-malonate	8·80	96·6
20 min., pH 5·0	6·41	70·4
20 min., pH 5·0, +0·15 M-malonate	16·70	183·2

Investigation of the effects of malonate on large-granule and supernatant fractions (Table 5) shows that, though the PhPase activity of the supernatant fraction falls by about 30 % on preincuba-

tion with malonate for 20 min. at 37° and pH 5·0, that of the large-granule fraction increases by 200 %. The β-glycerophosphatase activity at pH 5·0 of the large-granule fraction is virtually unaffected by preincubation either with or without malonate. Similar results to those obtained with phenyl phosphate are obtained with *p*-nitrophenyl phosphate as substrate (Table 5), though the percentage enhancement of large-granule *p*-NPPase activity at pH 5·0 by malonate (0·15M) is only about one-half of that of the corresponding PhPase activity. These results resemble those obtained by Golberg *et al.* (1962) at pH 5·8.

DISCUSSION

The results described above consolidate the suggestion of Neil (1961) that the PhPase activity of mouse-liver extracts comprises several enzymic components, and indicate that a similar situation is observed with *p*-nitrophenyl phosphate as substrate. In view of its frequent omission from discussions of acid-PhPase or *p*-NPPase activities in liver tissue (e.g. Macdonald, 1961; Barka, 1961; Golberg *et al.* 1962), it is worth re-emphasizing the high activity of G-6-Pase (or of an enzyme very similar both in properties and intracellular location) on phenolic phosphate esters. This activity was clearly established by Beaufay & de Duve (1954). In addition to lysosomal β-glycerophosphatase, G-6-Pase and the soluble alloxan-inhibitable acid *p*-NPPase (Neil & Horner, 1964), all of which hydrolyse phenyl phosphate, there appears to be a fourth component to the acid-PhPase complex of mouse-liver sucrose homogenates which is sedimented with the large-granule fraction. The activity of this component, unlike that of lysosomal β-glycerophosphatase, is enhanced several-fold by preincubation with malonate at pH 5·0. Enhancement of the PhPase activity of large-granule fractions from mouse liver at pH 5·8 by a variety of di- and tri-carboxylic acids has been demonstrated by Golberg *et al.* (1962). From the results described above the 'protective' action of malonate at pH 5·9 described by Macdonald (1961) is explicable, at least in part, in terms of a decrease in PhPase activity owing to G-6-Pase destruction, which is offset by the enhancement of a separate large-granule PhPase component by malonate.

The question whether or not acid β-glycerophosphatase is the same enzyme as acid PhPase in liver tissue is beside the point. It is more useful to consider that a number of proteins in this tissue exhibit acid-phosphomonoesterase activity, and that each of these catalyses the hydrolysis of β-glycerophosphate and phenyl phosphate to different extents. The ratio of β-glycerophosphatase to PhPase activity observed will then depend on (*a*) the proportions of the enzyme components

Table 4. *Effects of preincubation of mouse-liver cytoplasmic extract at* pH 5·0 *in the absence and presence of malonate*

Preincubation was for 20 min. at 37° in 0·05M-acetate buffer, pH 5·0. Phenyl-phosphatase activity was determined at pH 5·0 as described in the Materials and Methods section, and glucose 6-phosphatase activity at pH 6·5 as described by Neil & Horner (1964). Enzyme activities after preincubation are expressed as percentages of the initial activities.

	Phenyl-phosphatase activity	Glucose 6-phosphatase activity
Preincubation of sucrose cytoplasmic extract:		
None	100	100
With no additions	57·7	5·4
With malonate (0·15M)	180	5·0
With no additions followed by further preincubation (20 min.) with malonate (0·15M)	67·2	5·8

Table 5. *Effects of preincubation with malonate on the phenyl-phosphatase activities at* pH 5·0 *of large-granule and supernatant fractions, and on the β-glycerophosphatase and* p*-nitrophenyl-phosphatase activities at* pH 5·0 *of large-granule fractions from mouse liver*

Preincubation was for 20 min. at 37° in 0·05M-acetate buffer, pH 5·0. Phenyl-phosphatase activity was determined as described in the Materials and Methods section, and the β-glycerophosphatase and *p*-nitrophenyl-phosphatase activities as described by Neil & Horner (1964) (the three substrates represent three separate experiments).

	Phenyl-phosphatase activity			*p*-Nitrophenyl-phosphatase activity	β-Glycero-phosphatase activity
	Large-granule fraction ($10^5 g$-min.)	Supernatant fraction ($3 \times 10^6 g$-min.)	Cytoplasmic extract	Large-granule fraction ($10^5 g$-min.)	Large-granule fraction ($10^5 g$-min.)
No preincubation (*a*)	2·47	3·01	7·78	1·73	0·38
Preincubation + malonate (0·15M) (*b*)	7·41	2·14	14·6	3·35	0·36
$(b/a) \times 100$	300	71·2	188	194	94·7

present, (*b*) the relative affinities of these components for the two substrates and (*c*) the different influences of the assay conditions on each component.

The dominant β-glycerophosphatase component in liver tissue is located in the lysosomes and, at pH 5·0 in acetate buffer, hydrolysis of β-glycerophosphate by large-granule fractions appears to measure this component almost exclusively. Hydrolysis of β-glycerophosphate by whole cytoplasmic extracts in the same conditions, however, is not subject to such a simple interpretation. In rat liver the amount of lysosomal enzyme is so large, relative to the other acid-phosphomonoesterase components, that little error is involved in equating cytoplasmic β-glycerophosphatase with lysosomal acid phosphatase. In liver tissue from several other species the lysosomal acid-phosphatase activity is relatively low and the contribution of the alloxan-inhibitable soluble component to the total β-glycerophosphatase activity of a cytoplasmic extract is appreciable (Neil & Horner, 1964).

Observations of the hydrolysis of the phenolic phosphate esters by liver cytoplasmic extracts indicate that there is no consistently dominant component, and exceptional caution is required in interpreting the results obtained.

SUMMARY

1. Evidence is presented indicating the existence of four acid-phenyl-phosphatase components in mouse-liver cytoplasmic extracts.

2. Three of these components are located principally in the lysosomal, microsomal and soluble fractions of the liver respectively.

3. The fourth component is associated with the large-granule fraction, and its activity is greatly enhanced by preincubation with malonate.

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