Studies on Acid Hydrolases in Adult and Foetal Tissues

ACID *p*-NITROPHENYL PHOSPHATE PHOSPHOHYDROLASES OF ADULT GUINEA-PIG LIVER

BY M. W. NEIL AND MARIAN W. HORNER

Biochemistry Department, The London Hospital Medical College, London, E. 1

(Received 18 October 1963)

Some tissues contain more than one enzyme exhibiting 'acid-phosphatase' action and the partial separation of such enzymes has been reported, e.g. from rat liver (Goodlad & Mills, 1957; Barka, 1961*a, b*) and from *Escherichia coli* (Rogers & Reithel, 1960). Among the factors that make the interpretation of experimental observations on 'acid-phosphatase activity' difficult are (a) in the earlier work, lack of knowledge of the lysosomal sequestering of acid β -glycerophosphate phosphohydrolase (β -glycerophosphatase) and the conditions in which it is released, (b) the variety of substrates used for assay coupled with a lack of information concerning the identity of the enzyme(s) responsible for the hydrolysis of any given substrate and (c) the wide diversity of properties of 'acid phosphatases', both from different tissues within a species and from similar tissues in different species.

Some of these inherent difficulties are illustrated by the work of Macdonald (1961) on the phenyl phosphate-phosphohydrolase (phenyl phosphatase) activity of mouse-liver homogenates and the subsequent interpretations of the results obtained (Neil, 1961; Macdonald, 1962).

The present work is concerned principally with the differentiation of guinea-pig-liver enzymes that hydrolyse β -glycerophosphate and *p*-nitrophenyl phosphate in acid solution.

Some of the results of this work have been briefly reported (Neil & Horner, 1962).

MATERIALS AND METHODS

Animals. Laboratory-stock adult albino guinea pigs were used throughout. The stock consisted of an inbred colony derived from that maintained at the Research Institute (Animal Virus Diseases), Pirbright, Surrey.

Chemicals. β -Glycerophosphate was laboratory-reagent grade (British Drug Houses Ltd., Poole, Dorset) containing less than 3% of the α -isomer. Phenolphthalein mono- β -glucuronic acid was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Sucrose was Tate and Lyle Co. Ltd. caster sugar. Alloxan was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex. All other chemicals were of AnalaR or laboratory-reagent grade from British Drug Houses Ltd.

Purification of *p*-nitrophenyl phosphoric acid. Initially, acid-*p*-nitrophenyl phosphate-phosphohydrolase (acid-*p*-nitrophenyl phosphatase) activity was determined with

disodium *p*-nitrophenyl phosphate from Hopkin and Williams Ltd., British Drug Houses Ltd. and L. Light and Co. Ltd., Colnbrook, Bucks. These materials gave different assay values for the same enzyme preparation and the reagent blanks were high. The amounts of *p*-nitrophenol and inorganic phosphate (P_i) liberated on acid hydrolysis of these samples were not equimolar, and recrystallization (Axelrod, 1947) produced only marginal improvement.

The following procedure was adapted from that of Hoeftlake (1916) for the preparation of pure anhydrous *p*-nitrophenyl phosphoric acid. Commercial disodium *p*-nitrophenyl phosphate (10 g.) was recrystallized twice by the method of Axelrod (1947). The product was dissolved in ice-cold water (about 30 ml.), and *p*-nitrophenyl phosphoric acid was liberated by the addition of an excess of cold 16*N*-nitric acid. The cold solution was extracted repeatedly with ice-cold diethyl ether and the extracts were evaporated to small volume by using a stream of dry air. Removal of the solvent was continued in a vacuum desiccator until solidification (with decrepitation) occurred. The solid was redissolved in ether (about 30 ml.) and solvent removed as above. The latter process was repeated once more. Final traces of water and nitric acid were removed from the solid in a vacuum desiccator (over P_2O_5 and NaOH) for 24 hr. at 4°. The final anhydrous *p*-nitrophenyl phosphoric acid is an almost white solid, m.p. 154–155° (uncorr.). This product could be stored indefinitely in a desiccator over P_2O_5 in the dark at 4°. A stock solution of *p*-nitrophenyl phosphate was prepared by dissolving *p*-nitrophenyl phosphoric acid in water and neutralizing to approx. pH 6.5 with NaOH, and stored at –14°. The purity of the product was ascertained to be 100±1% by the following analyses.

Acid hydrolysis. (a) Determination of P_i liberated by perchloric acid digestion indicated a purity of 99.6%. (b) Determination of P_i and *p*-nitrophenol after hydrolysis with 1.0*N*-HCl at 105° for 16.5 hr. in sealed tubes indicated purities of 100.0% and 101.0% respectively. (c) P_i and *p*-nitrophenol were liberated in equimolecular proportion at all stages during hydrolysis with 1.0*N*-HCl at 100°.

Electrometric titration. Titration of a solution of *p*-nitrophenyl phosphoric acid with carbonate-free NaOH was followed by using a conventional saturated calomel-glass electrode system. Full neutralization indicated an equiv. wt. for *p*-nitrophenyl phosphoric acid of 109.5–110, corresponding to a mol.wt. of 219–220 assuming a dibasic acid (calc. for $C_6H_5NO_6P$: 219).

Flame-photometric determination. The Na^+ ion concentration in the neutralized solution indicated the presence of 1.96 equiv. of Na^+ ion/mole of neutralized *p*-nitrophenyl phosphoric acid.

Liver preparations. Animals were stunned and the livers rapidly excised and transferred to ice-cold 0.25*M*-sucrose

solution. Samples (2–4 g.) were homogenized in ice-cold 0.25 M-sucrose (6–12 ml.) by slowly passing a loose-fitting Teflon pestle (diam. 2.0 cm., 1500 rev./min., stroke 7.5 cm.) once down and up in a precision-bore glass tube over a period of 15–20 sec. After centrifugation (5000 g.-min.) the pellet was resuspended and resedimented in fresh sucrose. This process was repeated and the supernatants (cytoplasmic extract) combined.

Subcellular fractions were prepared from the cytoplasmic extract in 0.25 M-sucrose solution at 0–2° by differential centrifugation. The cytoplasmic extract was reconstituted from the separated fractions wherever practicable to indicate possible separation artifacts. Centrifugal forces are expressed as time integrals (g.-min.) of the average field (de Duve & Berthet, 1953).

Total release of lysosomal enzymes was achieved by treatment of particulate preparations at 0° in a MSE homogenizer run at full speed for 15 min. Occasionally release was effected by use of Triton X-100 (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955).

Enzyme assays. Determinations of acid β -glycerophosphatase and of β -glucuronidase (EC 3.2.1.31) activities were carried out at pH 5.0 (Gianetto & de Duve, 1955; Appelmans, Wattiaux & de Duve, 1955). Acid-*p*-nitrophenyl phosphatase activity was determined by incubating samples for 10 min. at 37° with acetate buffer, pH 5.0 (0.05 M or 0.10 M), *p*-nitrophenyl phosphate (2.5 mM) and water in a final volume of 2.0 ml. The reaction was started by the addition of substrate and stopped with 10 ml. of 0.04 N-NaOH. The extinction of the liberated *p*-nitrophenol was read at 415 m μ . In the assay conditions above, no phosphotransferase activity was observed. Spontaneous hydrolysis of the substrate was negligible. This degree of purity and stability avoids the principal strictures on the use of *p*-nitrophenyl phosphate (see Fishman & Davidson, 1957). Our experience with this substrate is comparable with that of Jacobsson (1960).

D-Glucose 6-phosphate-phosphohydrolase (glucose 6-phosphatase; EC 3.1.3.9) activity was assayed with glucose 6-phosphate (0.04 M) and 25 mM-acetate–25 mM-cacodylate buffer, pH 6.5, for 10 min. at 37° in a volume of 2.0 ml. The reaction was stopped with 10 ml. of 8.0% (w/v) trichloroacetic acid and the liberated P_i was determined.

Enzyme activities are expressed in units/g. of fresh tissue. A unit is defined as a μ mole of substrate hydrolysed/min. in the conditions of the assay. Unless otherwise specified, the enzyme activities given are total values.

Alloxan was dissolved in ice-cold buffer immediately before use.

RESULTS

Thermolabile acid-p-nitrophenyl phosphatase component in guinea-pig-liver homogenates

The time-course of the acid-*p*-nitrophenyl phosphatase activity of guinea-pig-liver cytoplasmic extracts was not linear, and suggested the presence of a thermolabile enzyme that was destroyed during the first 5 or 10 min. of incubation at pH 5.0 and 37°. When extracts were preincubated for 10 min. under these conditions, the curves were linear from zero time (Fig. 1).

Whereas over 90% of the thermolabile acid-*p*-nitrophenyl phosphatase activity was destroyed in

the first 10 min. at pH 5.0, over 50% remained even after 30 min. at pH 6.4 (Fig. 2). Similar rapid destruction at pH 5.0 of a proportion of the free acid *p*-nitrophenyl phosphatase, without significant activation of lysosomal enzymes, was observed under osmotically protective conditions (Gianetto & de Duve, 1955).

The properties described above are not exhibited by guinea-pig-liver β -glycerophosphatase. The labile acid *p*-nitrophenyl phosphatase was therefore not identical with lysosomal β -glycerophosphatase. This does not exclude the possibility of a separate *p*-nitrophenyl phosphatase in lysosomes, and fractionations of homogenates were undertaken to determine the principal location of the thermolabile enzyme (Table 1). It was clear that the thermolabile component was principally a microsomal enzyme.

In view of these findings and of the results of de Duve, Berthet, Hers & Dupret (1949), Hers & de Duve (1950) and Beaufay & de Duve (1954), it seemed likely that the thermolabile component was glucose 6-phosphatase or an enzyme with similar properties. The rates of destruction at pH 5.0 and 37° of glucose 6-phosphatase and of acid *p*-nitrophenyl phosphatase in microsomal fractions were compared (Fig. 3). After preincubation for 20 min. there was practically no glucose 6-phosphatase

Fig. 1. Progress of release of *p*-nitrophenol from *p*-nitrophenyl phosphate by blender-treated guinea-pig-liver cytoplasmic extract before (○) and after (●) preincubation. Preincubation was for 10 min. at 37° in 0.10 M-acetate buffer, pH 5.0. Assays were carried out with 2.5 mM-*p*-nitrophenyl phosphate in 0.10 M-acetate buffer, pH 5.0, at 37° for the times indicated. The procedure was as described in the Materials and Methods section.

activity, whereas almost one-third of the initial *p*-nitrophenyl phosphatase activity remained. Part at least of this residual component would be lysosomal enzyme present as a contaminant which is very much less active towards glucose 6-phosphate than towards phenolic phosphoric esters.

The rising curve is a fiducial line representing destruction of thermolabile activity with the two substrates. Although the coincidence of the experimental points is not exact, the fit is compatible with the hypothesis that a common enzyme is involved.

Evidence for the existence of two thermostable p-nitrophenyl phosphatase components in guinea-pig-liver homogenates

Free and total enzyme activities (Gianetto & de Duve, 1955) were determined in cytoplasmic extracts with *p*-nitrophenyl phosphate and β -glycerophosphate as substrates after preincubation

of the enzyme solution at pH 5.0 and 37°. However, the free *p*-nitrophenyl phosphatase activities were over 50 % of the total activities, whereas the free β -glycerophosphatase activities were very much lower.

To investigate whether the *p*-nitrophenyl phosphate substrate solution was causing activation of lysosomes during assays of free activity, its effect was tested on free β -glucuronidase activity. In two experiments the ratios of free β -glucuronidase activity in the absence of *p*-nitrophenyl phosphate to that in the presence of *p*-nitrophenyl phosphate (7.5 mM) were 1.015 and 0.990. Since neither *p*-nitrophenol nor *p*-nitrophenyl phosphate interfered with the measurement of phenolphthalein (extinction at 550 m μ at pH 10.4), nor did they affect the activity of β -glucuronidase in these experiments, it is concluded that *p*-nitrophenol and *p*-nitrophenyl phosphate do not rupture or increase the permeability of the lysosomal membrane.

Fig. 2. Thermolability at 37° of *p*-nitrophenyl phosphatase activity in guinea-pig-liver cytoplasmic extract at pH 5.0 and 6.4. The extracts were treated with Triton X-100. O, Extract preincubated for the times indicated at 37° in 0.10M-acetate buffer, pH 5.0; ●, extract preincubated alone for the times indicated at 37° at pH 6.4. Assays were carried out with 7.5 mM-*p*-nitrophenyl phosphate in 0.05M-acetate buffer, pH 5.0, at 37° for 5 min. Complete destruction of the thermolabile component is assumed to have occurred after 30 min. preincubation at 37° at pH 5.0.

Fig. 3. Effect of preincubation at pH 5.0 on glucose 6-phosphatase and *p*-nitrophenyl phosphatase activities of guinea-pig-liver microsomal fractions. ●, Glucose 6-phosphatase activity as a percentage of activity before preincubation; ▲, *p*-nitrophenyl phosphatase activity as a percentage of activity before preincubation; △, percentage decrease in glucose 6-phosphatase activity. Preincubation was for the times indicated at 37° in 5 mM-acetate buffer, pH 5.0. Glucose 6-phosphatase assays were carried out with 0.04M-glucose 6-phosphate in 25 mM-acetate-25 mM-cacodylate buffer, pH 6.5, at 37° for 10 min. *p*-Nitrophenyl phosphatase assays were carried out with 2.5 mM-*p*-nitrophenyl phosphate in 25 mM-acetate-25 mM-cacodylate buffer, pH 5.0, at 37° for 5 min.

Table 1. Localization of thermolabile acid-*p*-nitrophenyl phosphatase activity in guinea-pig-liver cytoplasmic extract

Preincubation was for 20 min. at 37° in 0.10M-acetate buffer, pH 5.0. Assays were carried out with 2.5 mM-*p*-nitrophenyl phosphate in 0.10M-acetate buffer, pH 5.0, at 37° for either 5 min. (for extract not preincubated) or 10 min. (for preincubated extract). Enzyme activities are given as units/g. of fresh tissue.

	<i>p</i> -Nitrophenyl phosphatase activity		
	Large-granule fraction (2.4 × 10 ⁶ g.-min.)	Microsomal fraction (3 × 10 ⁶ g.-min.)	Supernatant fraction
No preincubation (a)	1.49	4.33	1.75
Preincubation (b)	1.46	2.16	1.58
Fall in activity [(a)-(b)]	0.03	2.17	0.17

Table 2. Comparison of *p*-nitrophenyl phosphatase and β -glycerophosphatase activities of blender-treated guinea-pig-liver fractions after preincubation

Preincubation was for 20 min. at 37° in 0.10M-acetate buffer, pH 5.0. Assays were carried out with either 2.5 mM-*p*-nitrophenyl phosphate for 10 min. or 0.05M- β -glycerophosphate for 30 min. in 0.10M-acetate buffer, pH 5.0, at 37. Enzyme activities are given as units/g. of fresh tissue, and are the means of two experiments.

	<i>p</i> -Nitrophenyl phosphatase activity	β -Glycero- phosphatase activity	<i>p</i> -Nitrophenyl phosphatase activity: β -glycero- phosphatase activity ratio
Large-granule fraction (10 ⁵ g.-min.)	1.29	1.12	1.15
Microsomal fraction (3 × 10 ⁶ g.-min.)	2.02	1.15	1.76
Supernatant fraction	1.42	0.27	5.3

To investigate the disparity between the free activities measured with *p*-nitrophenyl phosphate and β -glycerophosphate, acid-*p*-nitrophenyl phosphatase and acid- β -glycerophosphatase activities were compared in fractions from cytoplasmic extracts (Table 2). In view of the ratio of *p*-nitrophenyl phosphatase activity to that of β -glycerophosphatase observed for the large-granule fractions, the high ratio in the supernatant fraction cannot represent the relative activities of lysosomal enzymes on the two substrates. The results strongly suggest that there is, in the supernatant fraction, a second thermostable enzyme that hydrolyses *p*-nitrophenyl phosphate rapidly and β -glycerophosphate slowly or not at all.

During a search for an inhibitor of thermolabile *p*-nitrophenyl phosphatase it was found that, though preincubation alone at pH 5.0 and 37° decreased the acid-*p*-nitrophenyl phosphatase activity of a blender-treated cytoplasmic extract by 10–15 %, the presence of 0.05M-alloxan at pH 5.0 and at 37° decreased the activity by about 50 %. This inhibitory action of alloxan was found to be very largely confined to the supernatant fraction, the activity of which was decreased by up to 80–90 %, depending on the concentration of alloxan (Fig. 4). Maximum inhibition was reached with 0.03M-alloxan. Inhibition of acid-*p*-nitrophenyl phosphatase activity in the supernatant fraction by alloxan was unaffected by perfusion of the liver.

Fig. 5 shows a comparison of the effects of preincubation and addition of alloxan on the acid-*p*-nitrophenyl phosphatase activities of large-granule and supernatant fractions. The inhibitions of thermostable activity produced by alloxan were 18 and 90 % respectively, and reached a maximum within 3 min. of addition in both instances.

In view of this differential inhibitory action of alloxan, β -glycerophosphatase and *p*-nitrophenyl phosphatase activities were compared in the three principal cytoplasmic fractions after preincubation alone and in the presence of alloxan (Table 3). The distributions of β -glycerophosphatase and *p*-nitrophenyl phosphatase activities among the fractions after preincubation are markedly different

Fig. 4. Inhibition of *p*-nitrophenyl phosphatase activity in guinea-pig-liver supernatant fraction (3 × 10⁶ g.-min.) by alloxan. Preincubation was for 10 min. at 37° in 0.3M-acetate buffer, pH 5.0, in the presence of alloxan at the concentrations indicated. Assays were carried out with 2.5 mM-*p*-nitrophenyl phosphate in 0.30M-acetate buffer, pH 5.0, at 37° for 10 min.

(columns 1 and 3). However, after preincubation with alloxan (columns 2 and 4) these distributions, though not identical, are very similar. It seems probable therefore that, after preincubation with alloxan, assays with either β -glycerophosphate or *p*-nitrophenyl phosphate as substrate measure the distribution of the same lysosomal enzyme among the subcellular fractions.

Further, since alloxan inhibition of the β -glycerophosphatase of large granules is 16 % and that of supernatant is 67 %, and since inhibition of a combination of the two fractions is additive, it can be concluded that the supernatant alloxan-inhibitable enzyme has significant activity towards β -glycerophosphate.

Some further properties of the thermostable acid monoester phosphohydrolases of liver cytoplasmic extracts

Effect of pH. Mixtures of cacodylate (which has no effect on *p*-nitrophenyl phosphatase activity) and acetate buffers were used to determine acid-*p*-

nitrophenyl phosphatase and β -glycerophosphatase activities of large-granule and supernatant fractions over the pH range 4.0–7.5 (Fig. 6).

Single broad regions of maximal activity were observed for large-granule *p*-nitrophenyl phosphatase (pH 4.5–5.5), large-granule β -glycerophosphatase (pH 4.6–5.4) and supernatant *p*-nitrophenyl phosphatase (pH 4.9–5.9). The difference in shape of the curves was most pronounced between large-granule and supernatant *p*-nitrophenyl phosphatase activities. There was appreciable residual activity at pH 6.5–7.5 and this was greatest for the supernatant *p*-nitrophenyl phosphatase, the activity at pH 7.0 being over 50% of the maximal activity. There was no evidence for double peaks of activity in the pH range investigated.

Fig. 5. Effect of preincubation, and of alloxan, on large-granule (10^6 g.-min.) (○) and supernatant (3×10^6 g.-min.) (●) fractions of guinea-pig liver. Preincubation was for 15 min. at 37° in 0.10M-acetate buffer, pH 5.0; then (arrow) alloxan in 0.10M-acetate buffer, pH 5.0, was added to give a final concentration of 0.05M, and the incubation continued. Assays were carried out with 2.5 mM-*p*-nitrophenyl phosphate in 0.10M-acetate buffer, pH 5.0, at 37° for 5 min.

Inhibitors. The effects of a number of substances on the large-granule and supernatant thermostable *p*-nitrophenyl phosphatases and on large-granule β -glycerophosphatase were studied (Table 4). On the basis of their effects these compounds are divided into four groups. Group I compounds inhibit considerably large-granule *p*-nitrophenyl phosphatase but have little effect on supernatant *p*-nitrophenyl phosphatase. Group II compounds behave in the reverse fashion. Group III compounds inhibit neither fraction. Group IV compounds inhibit considerably both fractions. Except for cysteine and group IV compounds, most of these compounds affect similarly both β -glycerophosphatase and *p*-nitrophenyl phosphatase of large granules.

Studies on other species

Studies on the activities of large-granule and supernatant β -glycerophosphatase and *p*-nitrophenyl phosphatase, and the effects of alloxan, were extended to other species (Table 5).

These activities vary widely from species to species, but the pattern of alloxan inhibition is

Fig. 6. Effect of pH on the *p*-nitrophenyl phosphatase (▲, ●) and β -glycerophosphatase (△) activities of large-granule (10^6 g.-min.) (△, ▲) and supernatant (3×10^6 g.-min.) (●) fractions of guinea-pig liver. Fractions were adjusted to pH 5.0 with 2N-HCl, and preincubated for 15 min. at 37°. The pH was then readjusted to the values indicated with 0.05M-acetate–0.05M-cacodylate buffers and assays were carried out at 37° with either 2.5 mM-*p*-nitrophenyl phosphate for 10 min. or 0.05M- β -glycerophosphate for 30 min.

Table 3. Effect of alloxan on acid monoester phosphohydrolases of guinea-pig-liver fractions

Preincubation was for 20 min. at 37° in 0.10M-acetate buffer, pH 5.0, in the presence or absence of alloxan (0.05M) as indicated. Assays were carried out with either 2.5 mM-*p*-nitrophenyl phosphate for 10 min. or 0.05M- β -glycerophosphate for 30 min. in 0.10M-acetate buffer, pH 5.0, at 37°. Enzyme activities are given as percentages of the sum of the activities in all fractions, and as units/g. of fresh tissue in parentheses.

	<i>p</i> -Nitrophenyl phosphatase activity		β -Glycerophosphatase activity	
	Preincubation without alloxan	Preincubation with alloxan	Preincubation without alloxan	Preincubation with alloxan
Large-granule fraction (10^6 g.-min.)	25 (1.29)	42 (1.24)	42 (1.09)	46 (0.915)
Microsomal fraction (3×10^6 g.-min.)	44 (2.26)	52 (1.55)	47 (1.24)	50 (1.01)
Supernatant fraction	31 (1.54)	6 (0.165)	11 (0.262)	4 (0.087)

Table 4. *Effect of various compounds on the thermostable p-nitrophenyl phosphatase and β -glycerophosphatase activities of guinea-pig-liver fractions*

Fractions were preincubated for 15 min. at 37° in 0.10M-acetate buffer, pH 5.0, in the presence of the compounds indicated at the concentration shown. Assays were carried out at 37° with either 2.5 mM-*p*-nitrophenyl phosphate for 10 min. or 0.05 M- β -glycerophosphate for 30 min. in 0.10 M-acetate buffer, pH 5.0.

	Percentage inhibition		
	<i>p</i> -Nitrophenyl phosphatase		β -Glycero-phosphatase
	Large-granule fraction (10 ⁵ g.-min.)	Supernatant fraction (3 × 10 ⁶ –5 × 10 ⁶ g.-min.)	Large-granule fraction (10 ⁵ g.-min.)
Group I			
Fluoride (3 mM)	89	11	94
(+)-Tartrate (0.05M)	86	23	93
(-)-Tartrate (0.05M)	55	15	66
<i>meso</i> -Tartrate (0.05M)	84	21	85
Molybdate (0.1 mM with respect to Mo)	95	34	99
Group II			
Alloxan (0.05M)	16	86	13
Formaldehyde (0.17M)	24	86	Approx. 10*
Group III			
Ethanol (5M)	1	4	—
Cysteine (0.01M)	-91†	1	1
Group IV			
<i>p</i> -Chloromercuribenzoate (1 mM)	82	98	59
Hg ²⁺ ions (0.1 mM)	95	94	56

* Approximate only, owing to the difficulties involved in the estimation of P_i in solutions containing formaldehyde.

† i.e. percentage activation.

fundamentally the same. The differential effects of alloxan on the fractions were not due to separation artifacts since activities were additive (except for mouse enzymes) on recombination of the fractions. Tentative estimates were made of the activity ratios of the enzymes towards the two substrates. Though the ratios of the activity of *p*-nitrophenyl phosphatase to that of β -glycerophosphatase for the large-granule fractions vary between 1.4 and 2.2, those for the supernatant fractions (between 6 and 28) indicate the presence in all species of a supernatant enzyme that is very considerably more active at pH 5.0 towards *p*-nitrophenyl phosphate than towards β -glycerophosphate.

DISCUSSION

The pH 5.0-thermolabile *p*-nitrophenyl phosphatase found in the microsomal fraction from guinea-pig liver has properties similar to those of glucose 6-phosphatase and resembles the pH 5.0-thermolabile phenyl phosphatase activity in rat-liver microsomes (Beaufay & de Duve, 1954) and in mouse-liver microsomes (Neil, 1961; Neil & Horner, 1964). In all three instances this microsomal enzyme has low activity on β -glycerophosphate.

The thermostable *p*-nitrophenyl phosphatase found in supernatant fractions may conceivably be identical with an already recognized enzyme. In some respects it resembles the phosphoprotein phosphatase present in supernatant fractions from mouse liver (Paigen & Griffiths, 1959). Hofman

(1958) and Glomset & Porath (1960) have demonstrated that the phosphoprotein phosphatase of ox spleen also hydrolyses *p*-nitrophenyl phosphate, phenyl phosphate and pyrophosphate, but has little action on β -glycerophosphate. The activity of phosphoprotein-phosphatase preparations is characteristically enhanced by the addition of thiols (e.g. Sabel, Glomset & Porath, 1961; Revel & Racker, 1960; Hofman, 1958; Sundararajan & Sarma, 1954). The increase observed, on the addition of cysteine, in the *p*-nitrophenyl phosphatase activity of large-granule fractions from guinea-pig liver (Table 4) may therefore be due to activation of lysosomal phosphoprotein phosphatase. The activity of the supernatant *p*-nitrophenyl phosphatase was, however, unaffected by cysteine.

Comparisons of the action of inhibitory substances on the acid-monoester-phosphohydrolase activity of tissue preparations (e.g. Bell & Lake, 1962; Golberg, Martin & Leigh, 1962; Gordon, 1952; Abul-Fadl & King, 1949) reveal that, particularly for crude tissue preparations, patterns of inhibition and activation are almost invariably unique to the particular enzyme preparation studied. These patterns, however, are an essential part of the evidence for the existence of separate phosphohydrolases within a single tissue, and, further, a sufficiently selective inhibitor may completely suppress one component. Thus, after preincubation of guinea-pig-liver subcellular fractions with alloxan at pH 5.0 and 37°, assays with either *p*-nitrophenyl phosphate or β -glycerophosphate as

Table 5. *p*-Nitrophenyl phosphatase and β -glycerophosphatase activities of liver fractions in various species, and inhibition by alloxan

Preincubation was for 20 min. at 37° in 0.10M-acetate buffer, pH 5.0, in the presence or absence of alloxan (0.05M) as indicated. Assays were carried out with either 2.5 mM-*p*-nitrophenyl phosphate for 10 min. or 0.05M- β -glycerophosphate for 30 min. in 0.10M-acetate buffer, pH 5.0, at 37°. M fraction: large-granule fraction (10⁵g.-min.), washed twice, blender-treated; S fraction: supernatant fraction (3 × 10⁶g.-min.); (M + S) fraction: recombination of M and S fractions. Enzyme activities are given as units/g. of fresh tissue. The numbers of experiments are given in parentheses after the names of the animal species.

Species	Substrate	Phosphohydrolase activity			Percentage inhibition by alloxan (0.05M)		
		M fraction	S fraction	(M + S) fraction	M fraction	S fraction	(M + S) fraction *
				M fraction + S fraction			M fraction + S fraction
Cat (2)	β -Glycero-phosphate	0.492	0.233	1.01	24.2	42.8	1.00
	<i>p</i> -Nitrophenyl phosphate	0.772	1.09	1.08	20.7	72.8	1.03
Guinea pig (2)	β -Glycero-phosphate	1.23	0.292	0.93	15.8	57.9	0.91
	<i>p</i> -Nitrophenyl phosphate	1.58	1.29	1.13	17.4	85.1	1.10
Rabbit (1)	β -Glycero-phosphate	0.168	0.069	0.98	26.8	39.0	0.97
	<i>p</i> -Nitrophenyl phosphate	0.327	1.00	1.08	26.0	85.8	1.13
Rat (2)	β -Glycero-phosphate	3.76	0.449	0.920	17.5	40.8	0.95
	<i>p</i> -Nitrophenyl phosphate	4.26	2.64	1.00	10.2	85.2	1.00
Mouse (2)	β -Glycero-phosphate	0.622	0.306	1.06	14.2	85.5	1.24
	<i>p</i> -Nitrophenyl phosphate	1.360	2.60	1.29	35.6	95.1	2.29

* These values are ratios of activities observed after preincubation of the fractions with alloxan.

substrate appear to measure essentially the same enzymic activities (Table 3). Further experiments have shown that, by using intact particulate fractions in the presence of alloxan, the release of the lysosomal enzymes can be followed with *p*-nitrophenyl phosphate as substrate.

Many experimental studies have indicated the presence of a variety of 'acid-phosphatase' activities in tissue extracts, largely on the basis of differential inhibition and differences in thermal stability and pH optima. Goodlad & Mills (1957) partially separated the acid phosphatases present in rat-liver homogenates, and concluded that the intracellular distributions of these enzymes were essentially the same. This conclusion, however, is only valid for the acid β -glycerophosphatases. In the present work assays with *p*-nitrophenyl phosphate as a substrate for phosphohydrolase activity reveal a very different intracellular distribution. Since *p*-nitrophenyl phosphate should be considered as an enolic phosphate (Oesper, 1950; Ohlmeyer & Shatas, 1952) and has a heat of hydrolysis (about 5000 cal./mole) approximately double that for β -glycerophosphate, the enzymes that hydrolyse these two compounds are likely to exhibit considerably different affinities for them. The differences in the activities of subcellular

fractions towards these two substrates is therefore not surprising.

Barka (1961*b*) and Moore & Angeletti (1961) have fractionated the *p*-nitrophenyl phosphatases of rat-liver extracts on DEAE-cellulose columns. The properties of the fractions obtained in the two Laboratories, however, were different. Neither can any of the fractions separated by Barka (1961*b*) be identified with any of the enzyme activities described in the present paper. The fractions isolated by Moore & Angeletti (1961) are sharply differentiated by their response to fluoride and by their relative activities towards β -glycerophosphate and *p*-nitrophenyl phosphate, observations very similar to those obtained in the present work. The enzymic properties of fractions I plus Ia and of fractions II plus III (Moore & Angeletti, 1961) correlate approximately with those of large-granule and supernatant acid *p*-nitrophenyl phosphatases respectively. The relative proportions of these activities, however, do not correlate, but this could be accounted for by the marked differences in the assay systems used in the two Laboratories.

Observations reported in the present paper are applicable to several mammalian species (Table 5), and there are marked species differences in the relative activities of the large-granule and super-

nant enzymes. The proportion of the total β -glycerophosphatase activity that is due to the alloxan-inhibitable supernatant enzyme varies widely, being least in rat and greatest in mouse. Further evidence for the existence of a small amount of a non-lysosomal acid monoester phosphohydrolase in the supernatant fraction from rat liver is provided by Shibko & Tappel (1963). Assays of free β -glycerophosphatase activity (Gianetto & de Duve, 1955) will consequently overestimate free lysosomal-enzyme activity. This overestimate will also be least for rat and greatest for mouse among the species listed in Table 5, and would provide an explanation for the observation of Van Lancker & Holtzer (1959) that the percentage free activity for acid β -glycerophosphatase was consistently greater than that for β -glucuronidase in mouse livers undergoing autolysis.

In view of the diversity of substrates used to assay 'acid-phosphatase' activity, and of enzymes that attack these substrates with widely differing affinities, it is strongly recommended that the systematic nomenclature of the *Report of the Commission on Enzymes* (1961) (particularly Rules 8, 14 and 22) should be used wherever possible.

SUMMARY

1. The preparation of pure anhydrous *p*-nitrophenyl phosphoric acid from crude commercial disodium *p*-nitrophenyl phosphate is described.

2. An enzyme, labile at pH 5.0 and 37°, having marked activity on *p*-nitrophenyl phosphate but little on β -glycerophosphate, has been demonstrated in the microsomal fraction from guinea-pig-liver homogenates. This enzyme is very similar to, if not identical with, D-glucose 6-phosphate phosphohydrolase (EC 3.1.3.9).

3. An enzyme, stable at pH 5.0 and 37°, having marked *p*-nitrophenyl phosphate-phosphohydrolase but only slight β -glycerophosphate-phosphohydrolase activity, has been demonstrated in the supernatant fractions from cat-, rat-, rabbit-, mouse- and guinea-pig-liver homogenates.

4. The supernatant enzyme is inhibited considerably by alloxan and formaldehyde (group II compounds) but only slightly by fluoride, tartrate and molybdate (group I compounds). The lysosomal enzyme is extensively inhibited by group I compounds but little inhibited by group II compounds.

5. pH-activity determinations have demonstrated broad regions of maximal activity for lysosomal β -glycerophosphate phosphohydrolase (pH 4.6–5.4), lysosomal *p*-nitrophenyl phosphate phosphohydrolase (pH 4.5–5.5) and supernatant *p*-nitrophenyl phosphate phosphohydrolase (pH 4.9–5.9).

6. These findings are discussed with reference to current methods of determining 'acid-phosphatase'

activity, and to the nomenclature of the enzymes responsible.

The authors acknowledge with gratitude the encouragement of Professor F. L. Warren and the receipt of a grant from the Royal Society for the purchase of apparatus.

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