Partial Purification and Properties of an Acid Nucleotidase from the Postmicrosomal Supernatant of Rat Spleen

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1. The dephosphorylation of 3'-AMP, 3'-dAMP, 3'-CMP and 3'-dCMP was studied in the postmicrosomal supernatant of rat spleen and liver. In both organs 3'-AMP and 3'-dAMP were dephosphorylated at an appreciable rate, in both the presence and the absence of Mg^{2+} . The pH optimum for this dephosphorylation was in the range 4.5-5.0. 3'-CMP and 3'-dCMP were very slowly degraded, though the activity towards 3'-dCMP increased somewhat in the presence of Mg^{2+} . The optimum pH for this Mg^{2+} -dependent dephosphorylation was 5.5-6.0. 2. The rate of dephosphorylation of 3'-AMP and 3'-dAMP per mg of protein was about 5 times as high in spleen as in liver. 3. The dephosphorylation of 3'-AMP could be ascribed to a single enzyme with pH optimum about 4.5. The activity towards 3'-dAMP could be resolved into one component coinciding with the 3'-dAMP-degrading enzyme, and one Mg^{2+} -requiring component probably identical with the soluble deoxyinosine-activated nucleotidase. The dephosphorylation of 3'-dCMP seemed to be performed only by the latter enzyme. 4. The enzyme dephosphorylating 3'-AMP was purified 200-fold from the postmicrosomal supernatant and its physical and catalytic properties were compared with those of acid nucleotidase (EC 3.1.3.31) purified from rat liver lysosomes. The two enzymes were identical in all properties tested (substrate specificity, K_m , molecular weight, response to phosphatase inhibitors), but some of the data differed from earlier reports on the acid nucleotidase. 5. The subcellular localization of the acid nucleotidase, its relationship to the acid phosphatase(s) and its role in the breakdown of nucleic acid constituents are discussed.

In a number of mammalian tissues it has been shown that the breakdown of nucleic acids, at least partially, gives rise to nucleoside 3'-phosphates by the combined action of endo- and exo-nucleases (Koerner, 1970; Bernardi & Bernardi, 1971; Aronson & Yannarell, 1975). Independently of whether these nucleoside 3'-phosphates are to be excreted from the cells or to be reutilized, they have to be dephosphorylated.

The dephosphorylation of 3'-nucleotides has been ascribed largely to the action of lysosomal enzymes. Arsenis & Touster (1967, 1968) have described an enzyme in rat liver lysosomes that dephosphorylates both 3'- and 5'-nucleotides of both the ribo- and the deoxyribo- series. Though the digestive action of lysosomes under special circumstances such as phagocytosis (Simson & Spicer, 1973) and cell death (Lockshin & Beaulaton, 1975) is well documented, no direct information exists as to the role of lysosomes in the breakdown of endogenous nucleic acids in vital cells. Since a number of enzymes believed to take part in the breakdown and reutilization of purines and pyrimidines are known to be located

in the cytosol (Murray, 1971), it seems likely that the cytosol is also the site for the dephosphorylation of nucleoside 3'-phosphates. Thus a nucleotidase with activity towards nucleoside 3'-phosphates (both ribo- and deoxyribo-nucleotides) as well as deoxyribonucleoside 5'-phosphates has been described in soluble fractions of a number of rat tissues (Fritzson & Smith, 1971; Fritzson, 1972, 1973; Tjernshaugen & Fritzson, 1976). This enzyme, however, seems to lack activity towards the adenine nucleotides and shows a rather low activity towards the cytosine nucleotides. Several reports of the breakdown of 3'-AMP by soluble enzyme(s) have appeared (Lewis & Spencer, 1962; Denner *et al.*, 1973), but because of the vigorous homogenization procedures used the true non-particulate nature of those preparations may be questioned. It was therefore decided to find out whether the cytosol also contains 3'-nucleotidases with activity towards the adenine and cytosine nucleotides.

The present paper reports the finding of one such nucleotidase in the postmicrosomal supernatant of rat spleen and liver, and it is shown with a 200-fold-

purified spleen enzyme that it is probably identical with the lysosomal enzyme previously mentioned (Arsenis & Touster, 1968).

Materials and Methods

Materials

DEAE-cellulose DE 52 was purchased from Whatman (Maidstone, Kent, U.K.). Sephadex G-25, G-100 and G-150 were from Pharmacia (Uppsala, Sweden). Marker proteins for gel filtration were obtained from Serva Entwicklungslabor (Heidelberg, West Germany). Calcium phosphate gel, 3% (w/v) suspension in water, was purchased from BDH Chemicals (Poole, Dorset, U.K.). Folin-Ciocalteu Phenol Reagent was a commercial preparation from E. Merck (Darmstadt, West Germany). Dithiothreitol and deoxythymidine 3',5'-bisphosphate were from Calbiochem (San Diego, CA, U.S.A.), deoxyribonucleoside 3'-phosphates and adenosine 3',5'-bisphosphate from P-L Biochemicals (Milwaukee, WI, U.S.A.), and deoxythymidyl-3'→5'-deoxythymidine was from Boehringer Mannheim G.m.b.H. (Mannheim, West Germany). Other nucleotides, nucleosides and their derivatives, as well as bovine serum albumin, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Preparation of particle-free supernatant

Male Wistar rats, about 60 days old and weighing about 200g, were used. Liver was homogenized as described earlier (Tjernshaugen & Fritzson, 1976). Spleen was homogenized in ice-cold 0.2M-KCl/5mM-Tris adjusted to pH 7.4 with HCl. Homogenization was carried out in a glass homogenizer surrounded by ice, with a tight-fitting Teflon pestle with a clearance of 0.2mm. The postmicrosomal supernatant was prepared by centrifugation for 1h at 3°C and 10⁵g.

Preparation of lysosomal acid nucleotidase

A lysosomal extract was prepared from the lysosomal pellet (de Duve *et al.*, 1955) by using 0.1% (w/v) Triton X-100 in water as described by Arsenis & Touster (1968). The lysosomal extract was applied to a column (0.9cm×12cm) of DEAE-cellulose, and the acid nucleotidase eluted by 10mM-5'-dAMP as described by Arsenis & Touster (1968). *p*-Nitrophenyl phosphate was used for measurements of enzyme activity, and the peak fractions were desalted on a column (1.5cm×25cm) of Sephadex G-25 by elution with 50mM-Tris/HCl, pH 7.5.

Enzyme assays

Unless otherwise stated, acid nucleotidase activity was measured in a total volume of 250μl, containing 2.5mM substrate, 50mM-malonic acid adjusted to pH 5.0 with NaOH, 5mM-dithiothreitol, 0.05mg of

bovine serum albumin, and 25–100μl of the enzyme source, adjusted to a concentration of enzyme that did not hydrolyse more than 20% of the substrate. To avoid significant pH changes, care was taken to adjust all the solutions added to pH 5.0±0.5, if they contained acid or alkaline substances in amounts that gave concentrations of 5mM or more in the incubation mixture. The incubation was generally carried out for 15min at 37°C after 5min of pre-incubation without substrate. The reaction was stopped by the addition of 200μl of 15% (w/v) trichloroacetic acid, and the tube was placed on ice. P_i was determined by the method of Chen *et al.* (1956) with the modification described earlier (Fritzson & Smith, 1971). When ATP or other labile organic phosphoric compounds were present, the method of Lowry & Lopez (1946) was used for the determination of P_i.

Dephosphorylation of *p*-nitrophenyl phosphate was determined with 100mM substrate as described by Fritzson & Smith (1971), except that the incubation conditions were as stated above.

Glycerol 2-phosphatase activity was assayed by the method of Gianetto & de Duve (1955).

Deoxyinosine-activated nucleotidase was determined as described by Tjernshaugen & Fritzson (1976).

One unit of enzyme is defined as the amount that hydrolyses 1nmol of phosphate ester/min under the specified incubation conditions. Specific activity is expressed in units per mg of protein.

Assay of protein

Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Samples containing Tris, (NH₄)₂SO₄, dithiothreitol or Triton X-100 were desalted on a column (0.9cm×10cm) of Sephadex G-25 before protein determination. Sodium phosphate buffer (5mM), pH 8.0, was used for the elution from the column.

Results

Measurements of 3'-nucleotidase activities in the cytosols of spleen and liver

The ability of the cytosols of spleen and liver to dephosphorylate the 3'-nucleotides of adenine and cytosine was studied at various pH values (Figs. 1 and 2). The cytosols of both spleen and liver were able to dephosphorylate 3'-AMP and 3'-dAMP, the optimum pH being about 5.0. The general pattern of activity was very similar in the two organs, but the dephosphorylating activity per mg of protein was about 5 times as high in the spleen as in the liver. Further experiments were therefore carried out with spleen cytosol. The pH-activity curves of the dephosphorylating activity in the postmicrosomal supernatant of spleen were determined both in the

Fig. 1. Effect of pH on the dephosphorylation of various nucleoside 3'-phosphates in the cytosol of rat liver

Supernatant from liver prepared as described in the Materials and Methods section was used as enzyme source. The incubations were carried out for 15 min at 37°C in the presence of 20 mM-MgCl₂ (except for the dashed curve for 3'-dCMP, where MgCl₂ was omitted). pH was varied as indicated. At low pH values 50 mM-sodium malonate buffer was used (open symbols), and at higher pH values 50 mM-Tris/maleate buffer (closed symbols). pH was measured at 20°C in the complete incubation mixture. The dephosphorylation rates are given relative to that obtained with 3'-dAMP in Tris/maleate buffer, pH 4.9. Substrates used were: ○, ●, 3'-AMP; □, ■, 3'-dAMP; △, ▲, 3'-dCMP.

Fig. 2. Effect of pH on the dephosphorylation of various nucleoside 3'-phosphates in the cytosol of rat spleen Supernatant from spleen, prepared as described in the Materials and Methods section, was used as enzyme source. The incubations were carried out as described in the legend to Fig. 1 in the absence of MgCl₂ (a) or in the presence of 20 mM-MgCl₂ (b). For further information see the legend to Fig. 1. The absolute value of the dephosphorylation rate towards 3'-dAMP in Tris/maleate buffer, pH 4.9, was 13.6 units/mg of protein. Substrates used were: ○, ●, 3'-AMP; □, ■, 3'-dAMP; ▽, ▼, 3'-CMP; △, ▲, 3'-dCMP.

absence of bivalent cations (Fig. 2a) and in the presence of 20 mM-Mg²⁺ (Fig. 2b). In the absence of Mg²⁺ the dephosphorylation of 3'-AMP and 3'-dAMP was optimum between pH 4 and 5. The activity towards 3'-CMP and 3'-dCMP was much lower, but the optimum pH was roughly the same. Dephosphorylation of all four substrates occurred at pH values up to 9 or 10, but at a much lower rate than at pH 5 (results not shown).

When the dephosphorylation was studied in the presence of Mg²⁺ (Fig. 2b), the curves for 3'-AMP and 3'-CMP were essentially unchanged, whereas there was a marked increase in the rate of dephosphorylation of 3'-dCMP and a simultaneous shift in the pH optimum towards 5.5. With 3'-dAMP the changes were similar but relatively smaller. These data suggest the presence of two different enzymes, one independent of Mg²⁺, with a pH optimum of 5.0 or less, and one which requires Mg²⁺ and has a higher pH optimum. The latter enzyme is probably identical with the previously described deoxyinosine-activated nucleotidase (Fritzson & Smith, 1971; Fritzson, 1973).

To characterize the 3'-nucleotidase activities further the spleen cytosol was fractionated by gel filtration. On Sephadex G-100 the dephosphorylating activity towards 3'-dAMP at pH 5.0 was eluted in the void

volume as a narrow fraction clearly separated from the deoxyinosine-activated enzyme described above. The application of Sephadex G-150 (Fig. 3) separated the deoxyadenosine 3'-phosphatase activity from the void volume, but it was still eluted as a single peak of activity different from the deoxyinosine-activated enzyme. To test the dependence on Mg²⁺ or other bivalent cations the enzyme fractions from gel filtration were tested in the presence and absence of MgCl₂ in various concentrations. In one experiment EDTA was included as well. Neither of these factors had any influence on the dephosphorylation of 3'-dAMP at pH 5.0.

Dephosphorylating activity towards 3'-dCMP was exerted by gel-filtration fractions corresponding to the deoxyinosine-activated nucleotidase peak and to a much lower extent by the fractions containing the deoxyadenosine 3'-phosphatase activity (see above).

Purification of enzyme from spleen

All operations were carried out at 0–4°C. The steps of a typical purification experiment are summarized in Table 1.

Step 1: preparation of the 10⁵g supernatant. The spleens from ten rats were homogenized by ten strokes of a tight-fitting Teflon/glass homogenizer, as described in the Materials and Methods section. The volume was adjusted to 30ml with homogenization medium, and the particle-free supernatant (fraction E 1) was prepared by centrifugation for 1 h at 10⁵g_{av.}

Step 2: fractionation with (NH₄)₂SO₄. To the 10⁵g

Fig. 3. Gel filtration of rat spleen cytosol

Particle-free supernatant was prepared from one spleen as described in the Materials and Methods section, and was applied to a column (2.5 cm × 36 cm) of Sephadex G-150. The column was equilibrated with 50 mM-Tris/HCl (pH 7.5)/1 mM-MgCl₂/10 mM-(NH₄)₂SO₄/1 mM-dithiothreitol and eluted with the same mixture at a flow rate of 16 ml/h. The dephosphorylation of 3'-AMP (○) and the deoxinosine-activated dephosphorylation of 3'-UMP (▲) were measured under standard conditions, except for the presence of 20 mM-MgCl₂. Dephosphorylation of 3'-dCMP (●) was measured at pH 6.0 with 20 mM-MgCl₂.

supernatant from step 1 was added 0.2 M-Tris/maleate buffer, pH 6.3, to a final concentration of 20 mM. A saturated solution of (NH₄)₂SO₄, containing 1 mM-EDTA and adjusted to pH 6.3 with aq. NH₃, was then added dropwise under stirring to give 60% saturation. The precipitate was removed by centrifugation (30000g for 5 min) and discarded. The supernatant was adjusted to 75% saturation by the addition of (NH₄)₂SO₄ in the same manner. The precipitate was collected by centrifugation (30000g for 5 min) and dissolved in 6 ml of 5 mM-Tris/maleate buffer, pH 6.0, containing 1 mM-dithiothreitol. (NH₄)₂SO₄ was removed by gel filtration on a column (1.5 cm × 25 cm) of Sephadex G-25, by using the same buffer for the elution (fraction E 2). Table 1 shows that most of the enzyme was precipitated between 60 and 75% saturation with (NH₄)₂SO₄.

Step 3: calcium phosphate gel treatment. To fraction E 2 was added calcium phosphate gel (about 250 μl of 3% gel/mg of protein), and after stirring for 15 min the gel was centrifuged (30000g for 5 min) and discarded. The supernatant, which contained most of the enzyme, constituted fraction E 3.

Step 4: gel filtration on Sephadex G-150. The column (2.5 cm × 37 cm) was equilibrated with 50 mM-Tris/HCl buffer (pH 7.5) containing 1 mM-dithiothreitol. Fraction E 3 (approx. 10–12 ml) was applied to the column and eluted with the same buffer. The enzyme was eluted as a sharp peak (fraction E 4). The purification procedure has been repeated a number of times and showed fairly good reproducibility. Total recovery of applied enzyme activity varied from 25 to 40%, the relative purification from 180- to 300-fold.

Homogeneity of the nucleotidase preparation

The data in Table 2 indicate that during purification of the enzyme the dephosphorylation rates for 3'-AMP, 3'-dAMP, 5'-AMP, 5'-CMP and possibly glycerol 2-phosphate were in nearly constant proportions. This finding suggests that all these dephosphorylating activities may be ascribed to the same protein.

Table 1. Purification of acid nucleotidase from rat spleen

Fractions were assayed for activity with 3'-AMP as substrate, as described in the Materials and Methods section. The amount of enzyme used was approx. 150 μg of fraction E1, 30 μg of fraction E2, 4 μg of fraction E3 and 0.5 μg of fraction E4.

Fraction	Approx. volume (ml)	Total protein (mg)	Total enzyme activity (units)	Specific activity (units/mg)
E1 (10 ⁵ g supernatant)	15	163	1884	11.5
E2 [60–75%-satd.-(NH ₄) ₂ SO ₄ precipitate]	8	15.8	1240	79
E3 (Supernatant after calcium phosphate gel adsorption)	10	0.72	799	1120
E4 (Sephadex G-100 eluate)*	25	0.30	761	2560

* The data refer to tubes with peak activity.

Table 2. *Relative specific activities*

The enzyme fractions from a purification experiment were assayed for respective activities as described in the Materials and Methods section. The amounts of enzyme protein used were as in Table 1. 3'-AMP and 3'-dAMP were tested at 2.5mM, 5'-AMP and 5'-CMP at 5.0mM and glycerol 2-phosphate at 100mM, all under standard conditions. 3'-dCMP (2.5mM) was tested in the presence of 20mM-MgCl₂ at pH6.0. 3'-UMP with deoxyinosine was tested as described for deoxyinosine-activated nucleotidase. All activities are expressed relative to that of 3'-AMP in the same fraction.

Fraction	Relative activity						
	3'-AMP	3'-dAMP	5'-AMP	5'-CMP	Glycerol 2-phosphate	3'-dCMP + MgCl ₂	3'-UMP+ deoxyinosine
E1	100	111	78	99	183	56	136
E2	100	125	65	99	214	19	5
E3	100	128	65	115	236	12	1
E4	100	131	63	110	200	11	—

Fig. 4. *Effect of pH on enzyme activity*

Fraction E 4 (approx. 0.5 μ g of enzyme protein) was used as enzyme source, and dephosphorylation of the different substrates was measured under standard conditions, except that pH was varied as indicated. Open symbols, malonate buffer; closed symbols, Tris/maleate buffer. pH was adjusted to the desired value with NaOH and was measured at 20°C in the complete incubation mixture. Δ , $\blacktriangle$, 5'-dAMP (6mM); $\square$, $\blacksquare$, 3'-dAMP (6mM); $\circ$, $\bullet$, 3'-AMP (2.5mM); ∇ , $\blacktriangledown$, 3'-dCMP (2.5mM).

described above that the deoxyinosine-activated enzyme is responsible for most of the dephosphorylation activity towards 3'-dCMP.

Stability of the enzyme during storage and incubation

The purified enzyme could be stored for 1–2 weeks at 0°C without substantial decrease in activity. Though freezing and thawing lead to a slight inactivation (5–10%), the enzyme might be kept for about 2 months at –20°C without further decrease in activity. Serum albumin (0.1 mg/ml) or dithiothreitol (2mM) had no effect on enzyme stability at 0 or –20°C, but exerted some protective effect on the enzyme during incubation at 37°C. When both components were added together, the production of P_i from 3'-AMP was linear with time for at least 60 min.

Effect of pH and buffer substance on enzyme activity

The enzyme exhibited a rather broad activity optimum between pH 4 and 5 when 3'-AMP was used as substrate (Fig. 4). With 5'-dAMP or 3'-dAMP the pH-activity curves exhibited a sharper optimum at pH 5.0 or slightly below. With 3'-dCMP the activity was very low, but although diffuse, the pH optimum apparently occurred about pH 4–5. Mg²⁺ did not change this pattern.

Fig. 4 shows that Tris/maleate buffer leads to higher dephosphorylation rates than malonate buffer at the same pH values, probably owing to a slight inhibitory effect of malonate. The use of citrate buffers leads to a marked shift in pH optimum towards higher pH values for all substrates and gave higher K_m values for 3'-AMP and 5'-dAMP.

Effect of some phosphatase inhibitors and cofactors

The most common activators and inhibitors known to affect nucleotidase and phosphatase activities in general were tested for their effect on the nucleotidase of spleen supernatant. Of the cations tested Zn²⁺ showed a weak, but reproducible, inhibition (approx. 15% decrease at 10mM concen-

In contrast, the activity of the deoxyinosine-activated nucleotidase totally disappeared from the preparation during the first two purification steps. The dephosphorylation of 3'-dCMP, measured under optimum conditions, also decreased markedly during the first two purification steps. This observation supports the conclusion drawn from the experiments

Table 3. *Effect of some phosphatase inhibitors on the acid nucleotidases of spleen cytosol and liver lysosomes*

A portion (50 μ l) of either spleen cytosol enzyme or lysosomal enzyme purified as described in the Materials and Methods section was used as an enzyme source. The incubation was carried out with 6 mM-5'-dAMP as substrate under standard conditions, except for the indicated additions (all adjusted to pH between 4.5 and 5.5). The dephosphorylation rate without additions is taken as 100%. With the spleen enzyme with 3'-AMP as substrate, results identical with those listed were obtained.

Addition (mM)	Activity (%)	
	Spleen cytosol enzyme	Liver lysosome enzyme
None	100	100
Sodium citrate (2)	103	102
Sodium citrate (10)	100	101
Sodium/potassium tartrate (0.5)	35	28
Sodium/potassium tartrate (1.0)	18	18
Sodium oxalate (2)	89	97
Sodium oxalate (10)	73	77
EDTA (1)	104	101
EDTA (5)	106	104
KF (5)	12	10

tration) but the others (including K^+ , Na^+ , NH_4^+ , Ca^{2+} , Cu^{2+} , Fe^{3+}) did not interfere significantly. Further, EDTA, which traps metal ions effectively by chelation, had no effect on the enzyme activity (Table 3). Of the inorganic anions tested Cl^- , SO_4^{2-} and CN^- were without effect, whereas F^- (Table 3) was an effective inhibitor. Tartrate, another well-known phosphatase inhibitor, also exhibited a strong effect at low concentrations. Somewhat less effective were the other dicarboxylic acids, oxalic acid (Table 3) and malonic acid (see Fig. 4). In contrast, acetic acid, citric acid and EDTA were without effect. Ethylene glycol (containing vicinal hydroxy groups, like tartrate) also showed a marked inhibitory effect (results not shown), whereas nucleosides had no significant effect.

Determination of molecular weight

Molecular-weight determinations were performed on the spleen nucleotidase as well as on the liver lysosome enzyme. For the spleen enzyme determinations were carried out both with the 10^5g supernatant (fraction E 1, see Fig. 3) and with the purified enzyme preparation (fraction E4). Portions (2 ml) of each sample were applied in separate runs on a

Table 4. *Relative enzyme activity, K_m and V_{max} with various substrates*

Fraction E4 (approx. 0.05 μ g of enzyme protein) was used as enzyme source. The relative activity toward the substrates were tested at 6 mM concentration (except CoA) under standard conditions specified in the Materials and Methods section. The dephosphorylation rates and V_{max} values are given relative to those of 3'-AMP (100). The absolute value of V_{max} for 3'-AMP was 4900 units/mg of protein. The K_m values were estimated from Lineweaver-Burk plots on the basis of activity measurements described in the legend to Fig. 5. The standard deviation for the K_m determinations was generally about 10%.

Substrate	Relative activity	K_m (mM)	V_{max}	Substrate	Relative activity
3'-AMP	100	0.7	100	3'-UMP	14
5'-AMP	49	0.9	78*	3'-CMP	6
3'-dAMP	131	1.7	160	3'-dCMP	19
5'-dAMP	128	2.6	156	3'-IMP	37
				5'-IMP	28
3'-GMP	49	1.7	63	5'-dIMP	46
5'-GMP	31	4.7	55	2'-AMP	68
3'-dGMP	142	1.7	155	NADP ⁺	4
5'-dGMP	37	12	108	CoA (1 mM)	37
3'-dTMP	77	2.6	81	Adenosine 3',5'-bisphosphate	76
5'-dTMP	60	3.2	93	Deoxythymidine 3',5'-bisphosphate	131
5'-UMP	22			ATP	40
5'-dUMP	28	8	64	Deoxythymidyl-3'→5'-deoxythymidine	0
5'-CMP	97	3.7	156	Cyclic AMP	5
5'-dCMP	101	6.2	150	Ribose 5-phosphate	5
Glycerol 2-phosphate	19	25	155	Glucose 6-phosphate	6
p-Nitrophenyl phosphate	140	4	160	Pyridoxal phosphate	14

* Theoretical value of V_{max} . As this substrate is inhibitory in higher concentration, the maximum value actually reached is lower (approx. 49% of V_{max} for 3'-AMP).

column (2.5cm×37cm) of Sephadex G-150, which was calibrated with the following marker proteins (molecular weights given in parentheses): horse myoglobin (17800), chymotrypsinogen A (25000), egg albumin (45000), bovine serum albumin (67000), γ -globulin from human serum (160000). The proteins showed close adherence to the linear relationship between partition coefficient and logarithm(molecular weight) (Andrews, 1964).

Three experiments with the spleen nucleotidase (both in the crude supernatant and in the purified sample) gave an average molecular weight of 125000 (range 120000–135000), on the basis of the partition coefficient. The value for the lysosomal enzyme (117000) was within the 95% confidence interval of the values for the spleen enzyme, and in a run with a combined sample containing both enzymes they were eluted as one peak.

Effect of substrate structure and concentration

The relative activity of the nucleotidase towards a number of nucleotides and other phosphate compounds is shown in Table 4, first column. The enzyme exerted a high activity towards 3'-dAMP and 5'-dAMP as well as the adenine ribonucleotides independently of whether the phosphate was attached in the 2', 3', or 5'-position. Table 4 suggests that the K_m values for the adenine nucleotides are lower than those for the corresponding nucleotides containing the other bases. In contrast, the V_{max} values were more or less independent of the nature of the base.

Fig. 5 shows the relationship between substrate concentration and enzyme activity in double-reciprocal plots (Lineweaver & Burk, 1934) for the nucleotides mentioned (except for 2'-AMP). Whereas three of the nucleotides showed linear plots and thus exhibited Michaelis-Menten kinetics, substrate inhibition occurred with 5'-AMP at higher concentrations.

The dephosphorylation rate of nucleotides containing bases other than adenine was lower and more dependent on the nature of the sugar phosphate moiety. Thus guanine compounds had rather low activity as substrates, except 3'-dGMP, which showed the highest activity of all the nucleotides tested. Of the cytosine derivatives the 5'-phosphates were effectively dephosphorylated, whereas the 3'-phosphates were virtually unaffected. It is noteworthy that the uracil nucleotides were rather poor substrates, whereas those of thymine were fairly good. The most striking correlation between substrate structure and value of V_{max} is that the deoxyribonucleotides generally have higher V_{max} values than the ribonucleotides. However, the ribonucleotides, owing to their lower K_m values, are more readily dephosphorylated at low concentrations. Further, the 3'-isomers have lower K_m values than the 5'-isomers.

Fig. 5. Dependence of enzyme activity on substrate concentration

The data are plotted in double-reciprocal plots as described by Lineweaver & Burk (1934). The rate of dephosphorylation of the adenine nucleotides was measured under the assay conditions specified in the Materials and Methods section, except that the substrate concentration (S) was varied as indicated. At each substrate concentration incubations were carried out for 10 and 20min in duplicate. The amount of enzyme (approx. 250ng of enzyme protein from fraction E 4) was adjusted so that less than 15% of the substrate was dephosphorylated. In all experiments the amount of phosphate released was proportional with time over the assay period. The substrate concentration (S) is the mean concentration during the incubation period. ○, 3'-AMP; ●, 3'-dAMP; △, 5'-AMP; ▲, 5'-dAMP.

In addition to ordinary nucleoside monophosphates several other phosphate compounds were tested. Deoxythymidine 3',5'-bisphosphate was a particularly good substrate, whereas adenosine 3',5'-bisphosphate was less active. The difference between these two substances may indicate that adenosine 3',5'-bisphosphate shows substrate inhibition at higher concentrations, just as does 5'-AMP. A high dephosphorylation rate similar to that of thymidine 3',5'-bisphosphate could not be obtained by incubating with mixtures of 3'-dTTP and 5'-dTTP.

Even if the base specificity of the enzyme is very broad, the minimum activity towards ribose 5-phosphate indicates that the presence of a heterocyclic component in the glycosidic linkage greatly stimulates the dephosphorylating activity, and of the non-nucleotide substances tested, only *p*-nitrophenyl phosphate is dephosphorylated at a rate comparable with that of the adenine nucleotides. This is not surprising, as most phosphohydrolases known show high activity towards substituted phenyl phosphates. The enzyme also hydrolysed phosphoric anhydrides such as ATP to a certain degree, but lacked

phosphodiesterase activity, as shown by its inability to liberate P_i from thymidyl-3'→5'-thymidine and cyclic AMP.

Comparison of nucleotidase from cytosol and lysosomes

The effect of some organic and inorganic inhibitors on the spleen enzyme and the lysosomal enzyme (Table 3) has been compared with the data reported for the lysosomal enzyme (Arsenis & Touster, 1968). The strong inhibitory effect of tartrate and fluoride found by the latter workers agrees with our findings, but we have been unable to verify their report of citrate and EDTA as potent inhibitors. Oxalate, though slightly inhibitory to both enzymes, showed a definitely weaker effect than that suggested by Arsenis & Touster (1968).

K_m values for the lysosomal acid nucleotidase were 2.9 mM for 5'-dAMP and 0.9 mM for 3'-AMP. These values agree well with those for the supernatant enzyme. The K_m values reported by Arsenis & Touster (1968) for the lysosomal acid nucleotidase were much lower.

Further evidence for the identity of the enzymes from the two sources are given by the similarity in relative activity towards different substrates (Table 4), by the pH optimum (Fig. 4) compared with the data for the lysosomal acid nucleotidase (Arsenis & Touster, 1968), and by the determinations of molecular weight (see the text). No acceptable explanation of the dissimilarity in the data of the present paper and those of Arsenis & Touster (1968) can be offered so far.

Discussion

A number of nucleotidases with activity towards 3'-AMP have been described in micro-organisms and plants (Drummond & Yamamoto, 1971; Polya, 1974; Voltattorni & Ipata, 1975), but the knowledge of such enzymes in animals is more limited. The only specific 3'-nucleotidase that has been purified from mammalian tissues and further characterized is one (EC 3.1.3.30) that seems to be specific for derivatives of 3'-AMP with a charged group (phosphate or sulphatophosphate) in the 5'-position (Farooqui & Balasubramanian, 1970; Denner *et al.*, 1973).

The breakdown of 3'-AMP in mammalian tissues may thus be ascribed largely to less specific enzymes, either to the non-specific phosphatases (alkaline phosphatase, EC 3.1.3.1, and acid phosphatase, EC 3.1.3.2) or non-specific nucleotidase (EC 3.1.3.31). The latter has been purified from rat liver lysosomes (Arsenis & Touster, 1968) and was particularly active towards the adenine nucleotides. A similar enzyme of uncertain subcellular localization has been found in pig spleen (Chersi *et al.*, 1966). As this enzyme has been classified as an acid phosphatase,

the question arises about the delineation of the terms 'nucleotidase' and 'non-specific phosphatase'. The investigations by Arsenis & Touster (1967, 1968) show that much of what has been considered measurements of non-specific acid phosphatase has indeed been of their nucleotidase instead, as this enzyme also possesses activity towards both glycerol 2'-phosphate and *p*-nitrophenyl phosphate, which are commonly used as substrates to measure acid phosphatase activity. The present paper also shows that the nucleotidase described shares two important features with some acid phosphatases: (1) the lack of metal ion requirement; (2) a response to phosphatase inhibitors which is almost identical with that of the human prostate acid phosphatase (Kilsheimer & Axelrod, 1957). This similarity is so striking that it might suggest identity or a very close genetic relationship between the two enzymes.

Since the enzyme purified from the postmicrosomal supernatant of spleen was identical with the enzyme purified from liver lysosomes in all properties tested, it is a puzzling question whether the enzyme really stems from the cytosol or if it only leaked out of the lysosomes during homogenization and centrifugation. Measurements of enzyme latency (results not shown) indicated that the lysosomal membranes were left fairly intact by the homogenization procedure applied, but it is not established whether this means that the lysosomes are really unbroken. Substantial amounts of lysosomal enzymes, ranging from 12–15% of the total activity in liver (de Duve *et al.*, 1955) to about 50% of that in spleen (Bowers *et al.*, 1967; Bowers & de Duve, 1967), are always found in the postmicrosomal fraction after homogenization. Whether this represents real extralysosomal enzyme activity *in vivo* or loss from the lysosomes owing to manipulation is an unanswered question.

Regardless of its origin the acid nucleotidase described in the present paper seems to be the only enzyme with significant activity towards 3'-AMP in the postmicrosomal supernatant of rat spleen, as the pH-activity curve of crude cytosol showed only one peak of activity, and the experiments with gel filtration of the cytosol gave only one peak of dephosphorylation activity toward 3'-AMP. Thus the deoxyinosine-activated nucleotidase (Fritzson & Smith, 1971) and the present acid nucleotidase seem to be the only enzymes in the cytosol of liver and spleen catalysing the breakdown of nucleoside 3'-phosphates. It may therefore be assumed that the cytosol of the cells in these organs shows great differences in its ability to catabolize the various 3'-phosphates. The physiological significance of this finding remains to be elucidated. In this connection it is pertinent to consider the possible deamination of adenine and cytosine 3'-nucleotides. So far no systematic study of this problem has been made, though Lewis & Spencer (1962) claim to have observed the

appearance of 3'-IMP as an intermediate in the breakdown of 3'-AMP by both mitochondrial and microsomal fractions as well as the postmicrosomal supernatant. This may be due to an analytical error, as the nucleotide and nucleoside deaminases known have been shown to be inactive towards nucleoside 3'-phosphates (Scarano *et al.*, 1962; Cory & Suhadolnik, 1965; Askari & Franklin, 1965; Zielke & Suelter, 1969; Wisdom & Orsi, 1969).

In addition to the difference in substrate specificity there are striking differences between the present enzyme and the previously described deoxyinosine-activated nucleotidase, including subcellular localization (Tjernshaugen & Fritzson, 1976), pH optimum, requirement for Mg^{2+} , order of magnitude of K_m (Fritzson & Smith, 1971), and allosteric regulation (Fritzson, 1972, 1973). These differences seem to indicate different physiological roles for the two enzymes. The possible physiological significance of the deoxyinosine-activated nucleotidase has been discussed in previous studies (Fritzson & Smith, 1971; Tjernshaugen & Fritzson, 1976; Tjernshaugen & Gautvik, 1976). The significance of the present acid nucleotidase at the cellular and whole-organism levels may possibly be sought in the manifold roles similar to those of the lysosomal enzymes (de Duve & Wattiaux, 1966; Dean & Barrett, 1976). The role of the lysosomal acid nucleotidase in nucleic acid catabolism has been discussed by Arsenis *et al.* (1970).

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