The presence and activity in normal and regenerating rat liver postmicrosomal supernatant fraction of an enzyme with properties similar to those of membrane-bound 5'-nucleotidase

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An alkaline 5'-nucleotidase with properties similar to those of membrane-bound 5'-nucleotidase was recovered in soluble form in the postmicrosomal supernatant fraction (cytosol) of rat liver. The enzyme seems to constitute a quantitatively distinct fraction, since the activity in postmicrosomal supernatants was increased by a further 10% by additional homogenization of livers. Lysosomal acid phosphatase activity increased similarly, whereas other membrane-bound marker enzymes alkaline phosphatase, phosphodiesterase I and glucose-6-phosphatase showed no increase when homogenization of liver tissue was continued. Gel-permeation chromatography and pH-dependence studies indicated that enzyme activity in the supernatant fraction with 0.3 mM-UMP or -AMP as substrate at pH 8.1 was about 85 or 100% specific respectively. In regenerating liver the enzyme recovered in soluble form showed decreased specific activity, in contrast with alkaline phosphatase measured for comparison. The nucleotidase activity per mg of cytosolic protein was 2.1 nmol/min with AMP as substrate. The total activity measured in the postmicrosomal supernatant was 1.5% of the homogenate activity measured in the presence of detergent.

INTRODUCTION

During studies of the activity of cytosolic 5'-nucleotidase (EC 3.1.3.5) in regenerating rat liver with IMP as substrate at the pH optimum for this enzyme of 6.3 (Tjernshaugen & Fritzson, 1984) we observed that the postmicrosomal supernatant fraction also contained another nucleotidase with alkaline pH optimum and with activity that differed radically from that of the cytosolic 5'-nucleotidase. A similar alkaline nucleotidase activity was also observed in earlier studies of nucleotide dephosphorylations in this subcellular fraction (Fritzson, 1967). The only known nucleotidase of this type in rat liver is the membrane-bound 5'-nucleotidase, which is considered to be an integral part of the plasma membrane (Emmelot *et al.*, 1964; Widnell *et al.*, 1982) and which has also been detected in the membranes of cytoplasmic elements such as the endoplasmic reticulum, lysosomes, intracellular vesicles and the Golgi apparatus (Widnell, 1972; Farquhar *et al.*, 1974; Little & Widnell, 1975; Stanley *et al.*, 1980; Maguire & Luzio, 1985). The occurrence of the alkaline nucleotidase activity in the postmicrosomal supernatant fraction could point to the presence of a soluble pool of the membrane-bound enzyme either in the cytoplasm or within intracellular vesicles, or to the existence of an enzyme fraction with a loose membrane association. The present studies of nucleotide metabolism in the postmicrosomal supernatant fraction provide new information about the soluble alkaline nucleotidase. Here we describe a relationship between tissue homogenization conditions and enzyme activity, properties of the enzyme and its activity in regenerating liver.

MATERIALS AND METHODS

Chemicals

Nucleotides, nucleosides, *p*-nitrophenyl phosphate,

concanavalin A, serum albumin, α -methyl D-mannoside, α -methyl D-glucoside and Lubrol PX were obtained from Sigma Chemical Co., and dithiothreitol was from Calbiochem-Behring Corp. Other chemicals were of analytical grade. [U-¹⁴C]UMP (515 mCi/mmol), [2-¹⁴C]uridine (56 mCi/mmol), [8-¹⁴C]AMP (50 mCi/mmol), [8-¹⁴C]adenosine (58 mCi/mmol), [8-¹⁴C]IMP (61 mCi/mmol) and [U-¹⁴C]inosine (575 mCi/mmol) were obtained from The Radiochemical Centre.

Animals

Male Wistar rats weighing about 240 g were used. Partial hepatectomy was performed by the procedure of Higgins & Anderson (1931) after the animals had been anaesthetized with diethyl ether or with pentobarbital (50 mg/ml; 50 mg/kg) injected intraperitoneally.

Tissue preparations

The livers were homogenized and the 123000 *g* supernatant fraction was prepared as described previously (Tjernshaugen & Fritzson, 1984). Portions of the supernatant were stored at -20 °C for enzyme assays. No significant change in enzymic activities could be detected after 1 week in the frozen state.

Liver plasma-membrane fragments were purified from the nuclear and debris fraction of liver homogenate as described by Aronson & Touster (1974).

Enzyme assays

Alkaline 5'-nucleotidase activity was assayed colorimetrically with 0.3 mM-nucleotide as substrate (UMP unless otherwise stated) under the conditions described previously (Tjernshaugen & Fritzson, 1984) for determination of IMP phosphohydrolase activity at pH 8.1. In some experiments ¹⁴C-labelled nucleotide (0.75 μ Ci) was used as substrate in total incubation volume 250 μ l. Labelled nucleoside formed was determined essentially

as described by Van den Berghe *et al.* (1977), small columns of neutral alumina being used to remove unchanged nucleotide. The recovery of ^{14}C -labelled uridine, adenosine and inosine in 10 ml of eluate was 83, 98 and 93% respectively. The enzyme values obtained were identical with those determined colorimetrically.

Alkaline phosphatase (EC 3.1.3.1) activity was determined with 10 mM-*p*-nitrophenyl phosphate as substrate in a reaction mixture (500 μl) containing 100 mM-glycine/NaOH buffer, pH 10.5, 0.2 mg of serum albumin, 5 mM-MgCl₂, 1 mM-dithiothreitol and enzyme source. After incubation for 10 min at 37 °C, the reaction was stopped with 400 μl of 15% (w/v) trichloroacetic acid, and the *p*-nitrophenol released was determined as described for the assay of phosphodiesterase I (Aronson & Touster, 1974).

Lactate dehydrogenase (EC 1.1.1.27) activity was determined by the method of Wróblewski & LaDue (1955), deoxyribonucleoside-activated nucleotidase activity by the specific method described by Tjernshaugen & Fritzson (1976), glucose-6-phosphatase (EC 3.1.3.9) activity by the method of Nordlie & Arion (1966) except that 50 mM-sodium maleate buffer was used, phosphodiesterase I (EC 3.1.4.1) activity as described by Aronson & Touster (1974) and acid phosphatase (EC 3.1.3.2) activity by the method of Gianetto & de Duve (1955).

One unit of enzyme is defined as the amount that converts 1 nmol of substrate/min under the specific incubation conditions. Specific activity is expressed in units/mg of protein.

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as the standard.

RESULTS

Effect of homogenization on enzyme activities in post-microsomal supernatant fraction

A Teflon pestle with radial clearance 0.2 mm was found most suitable for disruption of the liver cells with a minimum release of enzyme activity from particulate structures. Virtually all liver cells were broken after eight

pestle strokes, as indicated by the recovery in the postmicrosomal supernatant fraction of a maximum amount (122000 units/ml) of lactate dehydrogenase, a cytosol marker. After three pestle strokes as much as 93% of the enzyme activity had been liberated. Table 1 shows the activities of various phosphohydrolases after eight pestle strokes as well as the activity increase that may be measured after an additional eight strokes. It can be seen that the alkaline nucleotidase (AMP or UMP as substrate) and the acid phosphatase showed somewhat increased activity after prolonged homogenization. The plasma-membrane marker enzymes phosphodiesterase I and alkaline phosphatase, as well as the endoplasmic-reticulum marker enzyme glucose-6-phosphatase, showed no increase. This was also the case with the cytosolic deoxyribonucleoside-activated nucleotidase and soluble protein. Similar results were obtained when pestles with radial clearance 0.3 mm or 0.5 mm were used, except that ten or 20 pestle strokes respectively were required for complete cell breakage. With the manufacturer's standard radial clearance 0.1 mm the release per pestle stroke of alkaline nucleotidase and acid phosphatase was very rapid even after complete cell disruption (Fig. 1), which seemed to occur during the first stroke with this pestle.

Properties of the rapidly released nucleotidase

The studies were carried out with supernatant fraction from liver homogenates prepared by eight strokes of the loose-fitting pestle. Gel filtration through Sephadex G-25 was performed immediately before use to remove endogenous ions and nucleotides. Fig. 2 shows that the alkaline nucleotidase has optimum activity at pH 7.8 with Tris/HCl or glycine/NaOH buffer, and at pH 8.4 with Tris/maleate buffer. Possible contribution of alkaline phosphatase and acid phosphatase activities to the pH-dependence profile of the nucleotidase was investigated by measuring *p*-nitrophenyl phosphate dephosphorylation at various pH values. Neither the acid phosphatase activity, which was optimal at pH 5.8, nor the very small alkaline phosphatase activity at

Table 1. Enzyme activities in postmicrosomal supernatant fraction after homogenization with eight and 16 pestle strokes

Homogenization, enzyme assays and protein measurements were performed as described in the Materials and methods section. Portions of the homogenate were removed during homogenization for preparation of supernatant fraction and analysis. All cells were disrupted after eight pestle strokes, as indicated by a maximal amount of lactate dehydrogenase activity (results not shown). The enzyme values are the means for three experiments and were calculated as described in the legend to Fig. 1. The alkaline nucleotidase activity is measured with both AMP and UMP as substrate.

Enzyme activity measured	Enzyme activity (units/ml of supernatant)	
	8 pestle strokes	Increase after additional 8 pestle strokes
Alkaline nucleotidase (AMP)	36.7	4.5
Alkaline nucleotidase (UMP)	63.9	5.6
Acid phosphatase	78.2	4.8
Phosphodiesterase I	14.6	0.0
Alkaline phosphatase	34.3	0.0
Glucose-6-phosphatase	31.3	0.0
Deoxyribonucleoside-activated nucleotidase	85.3	0.0
Protein (mg/ml of supernatant)	20.3	0.0

Fig. 1. Effect of homogenization of liver with tight-fitting pestle on enzyme activities and protein content of the postmicrosomal supernatant fraction

Homogenization was carried out with the pestle with manufacturer's standard radial clearance 0.1 mm after a single stroke with the loose-fitting pestle. The temperature of the homogenate did not exceed 4 °C during homogenization. Alkaline 5'-nucleotidase (substrate UMP) (●), acid phosphatase (○), deoxyribonucleoside-activated nucleotidase (■), alkaline phosphatase (□) and glucose-6-phosphatase (▲) activities are expressed in nmol/min per ml of supernatant fluid, and protein content (△) in mg/ml. The activities and protein content are mean values for three different experiments normalized as follows. If $e_1, e_2, \dots, e_5$ are the values obtained for a particular enzyme in the supernatant fractions after homogenization of a liver with 1, 3, 20, 40 and 80 pestle strokes respectively, $E = \sum_{i=1}^5 e_i$ is used as a measure of the supernatant enzyme activity of that liver. E_{mean} for the three livers was calculated, and each set of enzyme values $e_1, e_2, e_3, \dots$ were normalized by multiplying by the factor $f = E_{\text{mean}}/E$. S.E.M. was smaller than the point symbol.

pH 10.1 seems to influence the nucleotidase activity. The pH optimum for membrane-bound alkaline phosphatase was 10.1 (Fig. 2) and was determined in a separate experiment with purified plasma-membrane fragments as enzyme source.

The nucleotidase was active in the absence of added Mg^{2+} ions but was stimulated 4-fold when 1 mM- Mg^{2+} was added. At this concentration the activity was 80% of the optimum value, which occurred at 10 mM- Mg^{2+} . Almost the same Mg^{2+} -dependence was observed when we used purified plasma-membrane fragments as enzyme source.

Double-reciprocal plots of initial reaction velocity

Fig. 2. Effect of pH on alkaline nucleotidase and *p*-nitrophenyl-phosphatase of postmicrosomal supernatant fraction

Postmicrosomal supernatant fraction gel-filtered through Sephadex G-25 was used as enzyme source for measurement of alkaline nucleotidase activity (●, ▲ and ■). *p*-Nitrophenyl-phosphatase activity was assayed both with the supernatant fraction (-----, ○ and □) and with purified plasma-membrane fragments (—, ○ and □) as enzyme source. Incubation conditions were as described in the Materials and methods section except that pH, measured at 20 °C in the complete reaction mixture, was varied as indicated. ● and ○, 0.1 M-Tris/maleate buffer; ▲, 0.05 M-Tris/HCl buffer; ■ and □, 0.1 M-glycine/NaOH buffer. A relative enzyme activity of 100 corresponds to 815 and 55 units/ml of supernatant fluid for acid *p*-nitrophenyl-phosphatase and alkaline nucleotidase respectively, and to 131 units/mg of plasma-membrane protein for alkaline *p*-nitrophenyl-phosphatase.

versus nucleotide concentration in the range of 5–60 μM were linear and gave the following apparent K_m values: for AMP, 20 μM ; for UMP, 25 μM ; for IMP, 29 μM . The relative enzyme activities towards the nucleotides (0.3 mM) were: AMP, 100; UMP, 168; IMP, 39.

The enzyme was inhibited 50% by 8 μg of concanavalin A/ml (0.073 μM) with UMP as substrate. Inhibition at 500 μg of the lectin/ml was 90% with UMP and 96% with AMP as substrate. Most of the inhibition was released by the addition of the competitive sugars (So & Goldstein, 1968) α -methyl D-mannopyranoside or α -methyl D-glucopyranoside at a concentration of 50 mM. ATP (0.1 mM) inhibited the enzyme by 50%. Maximum inhibition was 92% and occurred at 2 mM-ATP. [α -Methylene]ADP (0.5 mM) inhibited the nucleotidase activity by 79% with either AMP or UMP as substrate.

Gel-permeation chromatography of nucleotidase activity

Fig. 3 shows the results of chromatography on Sephacryl S-300 of supernatant fractions from rat livers homogenized by (a) two strokes and (b) 80 strokes of the pestle with radial clearance 0.2 mm. Nucleotidase activity measured with UMP as substrate at pH 8.1 was eluted in three distinct peaks, which are denoted I, II and III from left to right. Comparison of the elution pattern with that of M_r standards, the positions of which are indicated by arrows, showed that peak I is eluted at the

Fig. 3. Gel-permeation chromatography of postmicrosomal supernatant fractions from rat liver homogenates

The livers were homogenized by (a) two pestle strokes and (b) 80 pestle strokes as described in the Materials and methods section. A 5 ml portion of the supernatant fraction (approx. 100 mg of protein) was applied to a 2.5 cm × 80 cm column of Sephacryl S-300 equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 1 mM-dithiothreitol. Elution was carried out at 4 °C with the same buffer at a rate of 20 ml/h. Fractions of volume 7 ml were collected. Alkaline 5'-nucleotidase activity was measured with UMP as substrate, as described in the Materials and methods section. Calibration of the column was carried out with the markers Blue Dextran (M_r 2×10^6), ferritin (M_r 440 000), fructose biphosphate aldolase (M_r 158 000), egg albumin (M_r 45 000) and cytochrome *c* (M_r 12 400). Their positions in the diagram are indicated by arrows in order from left to right.

void volume and may represent enzyme with M_r of the order of 10^6 or higher. Peak II corresponds to an M_r of 280 000. Its marked increase in Fig. 3(b) clearly demonstrates that this peak contains the enzyme responsible for the increase in alkaline nucleotidase activity during homogenization. The size of peak III, taking into account the amounts of protein eluted in Figs. 3(a) and 3(b), seems to be independent of the

number of pestle strokes and may represent cytosolic enzymes with M_r about 45 000.

The activities of the enzyme peaks towards various nucleotides are shown in Table 2. Peaks I and II dephosphorylated 5'-UMP and 5'-AMP efficiently, whereas the activity towards 3'-dTTP and 2'-AMP was low. The 5'-UMP/5'-AMP activity ratio was 1.10–1.12, which is the same as that obtained with purified

Table 2. Dephosphorylation of nucleotides by cytosol fractions eluted from Sephacryl S-300 columns

The eluted peaks are shown in Figs. 3(a) and 3(b) and are denoted I, II and III from left to right in each diagram. Three to five fractions from each peak were mixed and concentrated to approx. 3 ml by ultrafiltration. Dephosphorylation of the nucleotides was measured under the conditions for assay of alkaline 5'-nucleotidase activity as described in the Materials and methods section. Percentage distribution of the nucleotidase activity among the different peaks was estimated from the elution diagrams. The enzyme activity of peak I (two-strokes homogenate) was too low to give a meaningful activity ratio.

Measurement	Substrate	2-strokes homogenate			80-strokes homogenate		
		Peak I	Peak II	Peak III	Peak I	Peak II	Peak III
Specific enzyme activity (nmol/min per mg of protein)	5'-UMP	6	7.4	2.0	12.2	12.3	1.6
	5'-AMP	7	6.7	0.1	11.1	10.9	0.2
	3'-dTTP	0	0.5	7.1	0.3	0.5	7.8
	2'-AMP	0.4	1.3	7.7	0.4	0.9	6.2
Percentage distribution of enzyme activity	5'-UMP	19	62	19	13	77	10
Activity ratio	5'-UMP/5'-AMP		1.12		1.10	1.12	

plasma-membrane 5'-nucleotidase (Nakamura, 1976). Peak III showed high relative activity towards the 3'- and 2'-nucleotides and low activity toward the 5'-nucleotides, demonstrating that this peak contained nucleotide phosphohydrolase(s) different from the 5'-nucleotidase. Some 10–19% of the total supernatant-fraction activity towards UMP was present in this peak.

Enzyme activities in regenerating liver

The nucleotidase activity per mg of cytosolic protein was determined at 1-day intervals during a 7-day period after partial hepatectomy, with UMP, AMP and IMP as substrates. The dephosphorylating activities expressed as percentages of the control values were lower than the control value throughout the experimental period, and showed strikingly parallel oscillatory variations similar to those observed previously for IMP dephosphorylation at pH 8.1 (Tjernshaugen & Fritzson, 1984). In contrast, alkaline phosphatase, which was included for comparison, showed a temporary increase in activity, reaching 140% of the control value after 3 days.

DISCUSSION

The alkaline nucleotidase released by eight strokes of the loose-fitting pestle seems to be a distinct enzyme fraction, since after a further eight strokes the enzyme activity increased by only 9–12%, depending on the substrate used. This rapidly liberated enzyme amounted to about 2% of the activity of the corresponding amount of liver homogenate assayed in the presence of 2% (w/v) of the detergent Lubrol PX. The initial rapid release of enzyme coincided with the quantitative release of the cytosol marker lactate dehydrogenase, a finding that could be taken to indicate a cytosolic location of the nucleotidase. However, other findings attach uncertainty to this interpretation. First, the inhibition by concanavalin A and the reversing effect of appropriate sugars indicate that the enzyme is a glycoprotein. It is generally accepted that proteins located in the cytosol are almost always devoid of carbohydrate. Secondly, the enzyme is similar to or identical with the membrane-bound 5'-nucleotidase, and could be released by the shearing force of the homogenizer.

As to the latter point, there was no increase in plasma-membrane and endoplasmic-reticulum marker enzymes during prolonged homogenization, and the main part of the 5'-nucleotidase released had M_r 280000. Consequently, the enzyme seems to be released in a free form and not as part of membrane fragments. Since the liberation of the nucleotidase during prolonged homogenization paralleled closely that of acid phosphatase, an enzyme located mainly in lysosomes (de Duve *et al.*, 1955), the possibility cannot be excluded that at least the slowly released enzyme is of lysosomal origin (Maguire & Luzio, 1985) or present in other intracellular vesicles (Stanley *et al.*, 1980; Widnell *et al.*, 1982) with a fragility similar to that of lysosomes. Whether acid phosphatase is also located in the cytosol is an open question (Tjernshaugen, 1978). However, although the intracellular location of the enzyme cannot yet be stated, knowledge of its properties and activity is important for studies of nucleotide metabolism in the postmicrosomal supernatant fraction.

Comparison of the properties of the supernatant-fraction alkaline 5'-nucleotidase with those of the

membrane-bound 5'-nucleotidase (Widnell & Unkeless, 1968; Riordan & Slavik, 1974; Widnell, 1974; Burger & Lowenstein, 1975; Nakamura, 1976; Ikehara *et al.*, 1977) showed that the enzymes have the same relative substrate activities, K_m values, pH optimum and Mg^{2+} -dependence, and both enzymes are inhibited by ATP, concanavalin A and [$\alpha\beta$ -methylene]ADP. Judged by these criteria, the enzymes seem to be identical. Gel-permeation chromatography indicated that the major proportion of the supernatant-fraction enzyme had M_r 280000 and that a smaller proportion with higher M_r was also present. It is possible that the two forms are aggregates of smaller enzyme molecules. Membrane-bound 5'-nucleotidase has been shown to form aggregates of monomers with M_r 70000 (Evans & Gurd, 1973; Slavik *et al.*, 1977; Bailyes *et al.*, 1984). If this is the case also for the present enzyme, the predominating form should be a tetramer.

The specificity of our enzyme assay is indicated by the lack of influence of alkaline phosphatase and acid phosphatase activity on the pH-activity curve for alkaline 5'-nucleotidase (Fig. 2) and the entirely different patterns of variation of the activities of this enzyme and alkaline phosphatase in regenerating liver. Gel-permeation chromatography (Table 2) indicated that about 15% of the crude supernatant-fraction activity towards UMP was due to enzyme(s) different from the alkaline 5'-nucleotidase. Such enzymes could be cytosolic 5'-nucleotidase and the deoxyribonucleoside-activated nucleotidase, with pH optima 6.3 and 6.0 respectively, which may both exert some activity also at pH 8.1 (Fritzson, 1969; Fritzson & Smith, 1971). Contribution of these enzymes to the supernatant-fraction activity could explain a slightly smaller decrease in UMP and IMP dephosphorylation in regenerating liver compared with AMP dephosphorylation, since both enzymes show increased activity in regenerating liver (Fritzson, 1978; Tjernshaugen & Fritzson, 1984) and exhibit very low activity towards AMP at the substrate concentrations used. It may be concluded that our assay for alkaline 5'-nucleotidase in the crude supernatant fraction is about 85 or 100% specific, depending on the use of UMP or AMP as substrate respectively.

The experiments with regenerating liver confirm the previously detected difference in expression of the cytosolic 5'-nucleotidase and the alkaline 5'-nucleotidase (Tjernshaugen & Fritzson, 1984), and clearly show the difference in the nature of the two enzymes. The importance of further studies of the nature and origin of the alkaline 5'-nucleotidase is indicated, particularly in view of its possible role as a factor in formation of adenosine in the cytoplasm.

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