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EFFECT OF SODIUM DEOXYCHOLATE ON 5′-NUCLEOTIDASE

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SUMMARY

The 5′-nucleotidase localized in rat liver plasma membranes was purified to a single protein, which contained phospholipid. The molecular weight and the sedimentation constant were about 150 000 and 7 S in the presence of sodium deoxycholate, while the enzyme protein was aggregated when the preparation was dialyzed thoroughly. The purified 5′-nucleotidase exhibited the same properties as the 5′-nucleotidase in plasma membranes. The 5′-nucleotidase activity was increased by the addition of various bile salts or by the solubilization of membranes with trypsin, papain or phospholipase C. The solubilized and aggregated forms of the enzyme showed different substrate specificity for nucleotides, pH optimum, heat stability and K_m. The purified enzyme catalyzed an exchange reaction between AMP and adenosine, which was diminished by the addition of sodium deoxycholate.

INTRODUCTION

Membrane-bound 5′-nucleotidase has been purified by Widnell et al. [1, 2] and Evans et al. [3, 4]. However, there are several inconsistent points between the two authors, especially in the functional role of lipid component. The importance of lipid has been emphasized for many enzymes localized in the membranes [5]. Morphological and enzymological examinations have revealed remarkable changes in the structure and activity of membrane-bound enzymes by the addition of bile salts [6–8]. In this report the properties of the further purified enzyme were studied and the role of phospholipid in the activity of 5′-nucleotidase was presented by comparing the catalytic properties of the solubilized and aggregated enzymes.

MATERIALS AND METHODS

Chemicals

5′-[8-^{14}C]AMP and [8-^{14}C]adenosine were purchased from the Radiochemical Centre and purified by a Dowex-1 column chromatography. Bile salts, trypsin, papain, phospholipase C and standards for molecular weight determination were obtained from Sigma Chemical Company.

Determinations

Protein and inorganic phosphate were determined by the method of Lowry et al. [9] and Lowry and Ropez [10], respectively. The determination of phospholipid was carried out according to the method described by Gurd et al. [11]. Packard Tricarb liquid scintillation spectrometer was employed to determine the radioactivity in a Bray's solution as scintillator [12]. Molecular weight was determined using 5 % polyacrylamide gel electrophoresis in 0.2 % sodium deoxycholate according to the method by Dewald et al. [13], using RNA polymerase, collagenase, catalase, serum albumin and lysozyme as standards. Sephadex G-200 column chromatography was carried out in 0.2 % sodium deoxycholate to determine molecular weight, using catalase, alcohol dehydrogenase, serum albumin and cytochrome *c* as standards. Sedimentation coefficient was determined by the method of Martin and Ames [14] using the same standards.

Enzyme assay

The activity of 5'-nucleotidase was determined by two assay methods. Methods 1 and 2 measure the liberation of P_i from 5'-AMP and [^{14}C]adenosine from 5'-[^{14}C]AMP, respectively. For method 1, reaction mixtures contained 2 μmol of 5'-AMP, 50 μmol of Tris/acetate (pH 8.5), 2 μmol of $MgCl_2$ and the enzyme in a final volume of 0.5 ml. The reaction was run for 15 min at 37 °C and terminated by adding 0.1 ml of 25 % trichloroacetic acid. The supernatant solution obtained by centrifuging the mixture was used for the determination of inorganic phosphate. The unit of activity is defined as 1 μmol Pi released/min at 37 °C.

For method 2, reaction mixtures contained 5 nmol of 5'-[8-^{14}C]AMP (30 000 cpm/nmol), 50 μmol of Tris/acetate (pH 8.5), 5 nmol of $MgCl_2$ and the enzyme in a final volume of 0.5 ml. The reaction was carried out and stopped as described above. The supernatant solution was neutralized with NaOH and put on a small column (1 ml bed volume) of Dowex-1 (formate form, $\times$2, 200–400 mesh). Radioactive adenosine produced was eluted from the column with 10 ml of 0.05 M formic acid, while 5'-AMP remained on the column. The radioactivity in an aliquot (1 ml) of the eluate was determined as described above.

The increase in P_i formation (method 1) coincided with an increase in the production of [^{14}C]adenosine (method 2). The reaction was linear with time up to 40 min and with the enzyme volume up to 0.05 unit, either in the presence or in the absence of 0.4 % sodium deoxycholate.

RESULTS

Purification of 5'-nucleotidase

Rat liver plasma membranes were prepared according to the method described by Song et al. [15] with a modification. Minced liver (20 g) of male Wistar rats was homogenized gently in 40 ml of 1 mM $NaHCO_3$ with a Teflon-glass homogenizer (radial clearance 0.7 mm). The homogenate was diluted with 500 ml of 1 mM $NaHCO_3$ and filtered through gauze. The filtrate was centrifuged for 10 min at 1500 $\times g$ and precipitate was suspended in 20 ml of 1 mM $NaHCO_3$. The suspension was added to 110 ml of 70.74 % sucrose ($d = 1.22$) and the homogenized suspension (32 ml each) was placed in three swinging buckets. Sucrose solutions of densities 1.18(14 ml)

and 1.16 (10 ml) were layered over the suspensions. After centrifugation for 60 min at 66 000 $\times g$, three bands were observed and fractions were separated using a tube slicer. The highest 5'-nucleotidase activity was obtained in the fraction near the top (6 ml), which was diluted with 24 ml of 1 mM $NaHCO_3$ and centrifuged for 10 min at 1500 $\times g$. The resulting precipitate was washed again with 1 mM $NaHCO_3$ and the final precipitate was suspended in 5 ml of 1 mM $NaHCO_3$. The membrane fraction thus obtained yielded 15 % of total 5'-nucleotidase activity in the initial homogenate with 30-fold increase in specific activity. The preparation thus obtained showed no glucose-6-phosphatase activity [16] but a small activity of nonspecific alkaline phosphatase [17].

The first three steps of purification were carried out essentially according to the method described by Widnell et al. [1, 2]. The suspension of plasma membranes (20 ml) was added to 80 ml of a solution containing 0.1 M Tris/acetate (pH 7.5), 12.5 % saturated ammonium sulfate solution, 1 mM $MgCl_2$, 5 mM 5'-AMP, 1 % deoxycholate and 2 % Triton X-100. Solid ammonium sulfate was added to a final concentration of 45 % saturated and the mixture was centrifuged for 10 min at 10 000 $\times g$. The resulting precipitate and a plug on the surface were combined and suspended in a solution containing 0.1 M Tris/acetate (pH 7.5), 1 mM $MgCl_2$ and 5 mM 5'-AMP. The mixture was heated for 5 min at 50 °C and centrifuged for 20 min at 10 000 $\times g$ at 0 °C. Saturated ammonium sulfate solution was added slowly to the supernatant solution to a final concentration of 28 % saturated. The mixture was centrifuged for 20 min at 10 000 $\times g$ and saturated ammonium sulfate solution was further added to the supernatant solution to a concentration of 40 % saturated. The surface plug which was obtained by centrifugation was dissolved in 2 ml of a solution containing 0.1 M Tris/acetate (pH 7.5), 0.1 mM $MgCl_2$ and 1 mM 5'-AMP. The solution was put on a column (35 $\times$ 1.7 cm) of Sepharose 4B equilibrated with a solution containing 0.2 % deoxycholate and 0.05 M KCl. The elution was carried out with the same solution at a flow rate of 8 ml/h. The enzyme activity was observed in the fraction from 62 to 68 ml, which was diluted with 24 ml of 5 mM Tris/acetate (pH 7.5) and layered over a column (20 $\times$ 1.0 cm) of DEAE-cellulose equilibrated with 5 mM Tris/acetate (pH 7.5). The column was washed with 40 ml of 5 mM Tris/acetate (pH 7.5) and eluted with 30 ml each of 0.02, 0.05 and 0.1 M NaCl in 5 mM Tris/acetate (pH 7.5) successively. The 5'-nucleotidase activity was observed in 0.05 M NaCl eluate. The enzyme solution was dialyzed against 4 l of 5 mM Tris/acetate (pH 7.5) for 4 h and lyophilized. The lyophilized preparation (0.5 ml) was placed on a column (12 $\times$ 0.7 cm) of 5'-AMP-Sepharose 4B which had been suspended in 5 mM Tris/acetate (pH 7.5), 0.05 M NaCl, 0.2 % sodium deoxycholate. The enzyme was eluted by applying the linear concentration gradient of 5'-AMP. The mixing chamber and the reservoir contained 50 ml each of 5 mM Tris/acetate (pH 7.5), 0.05 M NaCl, 0.2 % deoxycholate and 0.15 Mm AMP in the same solution, respectively. The 5'-nucleotidase activity was observed in the fraction from 38 ml to 44 ml. The purification was 250-fold over the plasma membrane fraction with a yield of 25 % (Table I).

The purified preparation proved to be a single protein preserving 5'-nucleotidase activity on polyacrylamide gel electrophoresis and centrifugation on a sucrose density gradient in sodium deoxycholate when 20-130 μg of protein were applied. The molecular weight was 145 000–158 000 when determined by polyacrylamide gel electrophoresis and 150 000–160 000 by Sephadex column chromatography. The purified prepa-

TABLE I

PURIFICATION OF 5′-NUCLEOTIDASE

Step	Total (units)	Specific activity (units/mg)	Yield (%)
Homogenate	418	0.026	100
Plasma membranes	63	0.78	15
First ammonium sulfate treatment	54	2.5	13
Heat treatment	50	4.9	12
Second ammonium sulfate treatment	38	17.2	9
Sepharose 4B	33	48.5	8
DEAE-cellulose	25	71	6
5′-AMP-Sepharose 4B	17	193	4

ration contained about 2 μmol phospholipid/mg protein and it showed a glycoprotein band on the same electrophoretogram stained according to the method described by Neville and Glossmann [18].

Centrifugation on sucrose density gradient

When the dialyzed preparation of the pure enzyme or plasma membranes was centrifuged, 5′-nucleotidase activity was detected exclusively at the bottom of the tube. Centrifuged in 0.4 % sodium deoxycholate, 5′-nucleotidase activity appeared as a symmetrical peak (7 S) which was not altered by adding 5′-ATP to the sucrose medium. The membrane preparation treated with phospholipase C, papain or sodium dodecyl sulfate exhibited various sedimentation patterns (Fig. 1).

Maximum velocity and K_m

The addition of deoxycholate to the purified enzyme caused an increase in the 5′-nucleotidase activity ($K_a = 0.09\,\%$). Plasma membrane preparations also showed the increment of the activity when solubilized with various bile salts, with proteolytic enzymes (Fig. 2) or with phospholipase C. No activation was observed after deoxycholate solubilized enzyme was treated with trypsin or phospholipase C. The apparent K_m values were $4.7 \cdot 10^{-5}$ and $1.4 \cdot 10^{-5}$ M in the absence and in the presence of 0.4 % sodium deoxycholate, respectively, when calculated from the double reciprocal plot [19].

Substrate specificity

The purified 5′-nucleotidase did not hydrolyze phosphorylated compounds other than 5′-mononucleotides, or the phosphodiester bond of 3′,5′-cyclic AMP. Although the dialyzed enzyme showed the highest activity toward 5′-UMP, the activity toward 5′-AMP was greater than that toward 5′-UMP on addition of deoxycholate or on incubating with trypsin (Table II).

Effect of pH and heat stability

Maximum 5′-nucleotidase activity occurred near pH 7.6 in the absence, and pH 8.5 in the presence, of 0.4 % sodium deoxycholate. The activity of purified

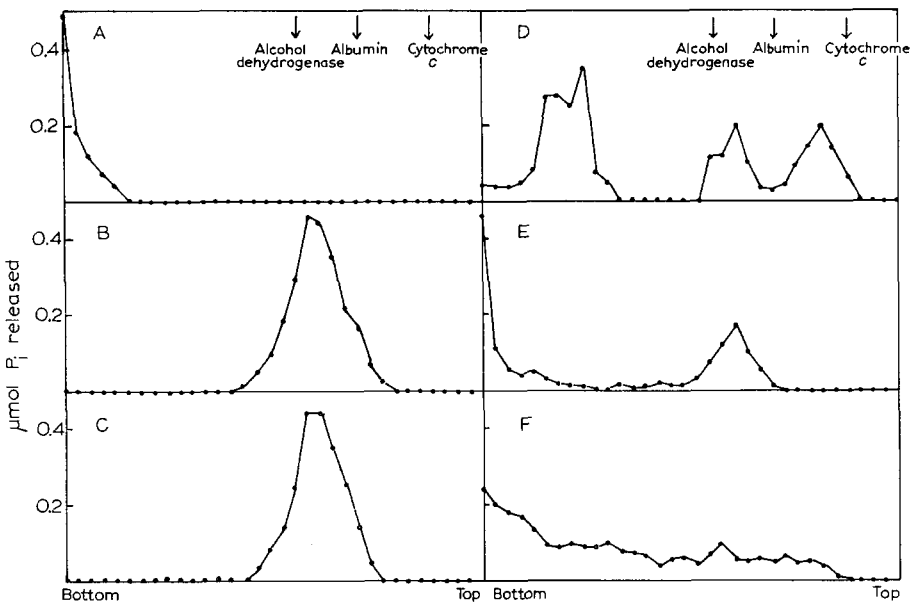

Fig. 1. Sucrose density gradient centrifugation. Linear sucrose density gradient (4.5 ml) from 5 to 20 % was prepared in 20 mM Tris/acetate (pH 7.5). Tube B and C contained 0.4 % sodium deoxycholate and Tube D 0.1 % sodium dodecyl sulfate in the sucrose medium. The dialyzed preparation of the purified enzyme was used in Tubes A and B and plasma membranes in Tubes C, D, E and F as samples. Plasma membranes (1 mg) were incubated with 20 μg of heated phospholipase C for 3 h E or with 125 μg of papain for 1 h F. Internal standards were mixed with samples and 0.1 ml of mixtures was layered over the gradient. Centrifugation was carried out at 40 000 rev./min (114 500 × *g*) for 14 h at 4 °C. Fractions (0.13 ml) were collected from the bottom of the tube and enzymes and proteins were determined. Peaks of internal standards are indicated by arrows.

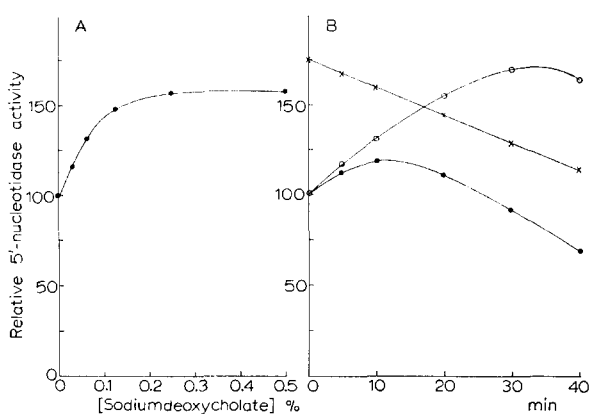

Fig. 2. Activation of 5′-nucleotidase. (A) Various concentrations of sodium deoxycholate were added to the reaction mixture containing dialyzed enzyme. (B) Plasma membranes (1 mg) were preincubated with 125 μg of papain (○), 1mg of trypsin (●) or 1 mg of trypsin and 0.4 % deoxycholate (×). To preincubated enzymes (●) and (×), were added 3 mg of trypsin inhibitor. The activity was determined with an aliquot of preincubated enzyme by method 1. Deoxycholate (0.4 %) was added to the reaction mixture (×).

TABLE II

SUBSTRATE SPECIFICITY

The enzyme activity was measured using method 1, except 5'-AMP. Dephosphorylation of 5'-AMP was assigned a value of 100. Sodium deoxycholate was added to a final concentration of 0.4 %. Plasma membranes (1 mg) were digested with 1 mg of trypsin for 30 min at 37 °C and the digestion was stopped by the addition of 3 mg trypsin inhibitor.

System	5'-AMP	5'-GMP	5'-UMP	5'-CMP	5'-IMP	2'-AMP
Plasma membranes	100	31	111	89	41	1
Purified enzyme	100	32	110	92	40	0
Plasma membranes +sodium deoxycholate	100	42	83	80	54	1
Plasma membranes digested with trypsin	100	38	96	83	49	1
Purified enzyme +sodium deoxycholate	100	44	84	82	50	0

5'-nucleotidase was reduced by 38 % and 94 % on heating for 5 min at 60 °C in the absence and in the presence of deoxycholate, respectively (Fig. 3). An identical observation was obtained using plasma membranes solubilized with trypsin.

Inhibition by nucleotides and exchange reaction

The formation of [^{14}C]adenosine from 5'-[^{14}C]AMP was inhibited competitively by 5'-ADP [20] ($K_i = 0.5 \cdot 10^{-4}$ M), 5'-ATP [21, 22] ($K_i = 0.18$ mM), 3',5'-cyclic AMP [2] ($K_i = 0.54$ mM), adenosine [23] ($K_i = 0.8$ mM) and 3'-AMP ($K_i = 1.15$ mM), but not by adenine or by β-glycerophosphate. 5-'ADP or 5'-ATP

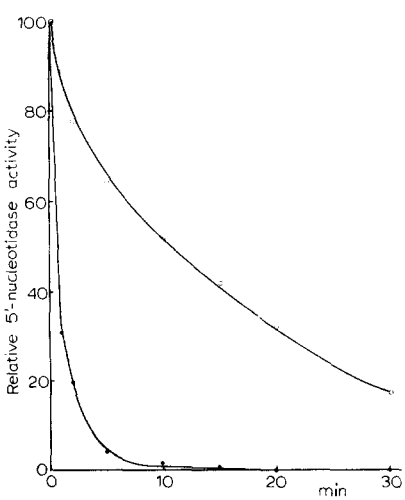

Fig. 3. Effect of preincubation at 60 °C on the 5'-nucleotidase activity. The purified enzyme was incubated for the indicated periods at 60 °C without (○) or with (●) 0.4 % sodium deoxycholate and cooled in iced water. The 5'-nucleotidase activity of preincubated preparations was measured by method 2.

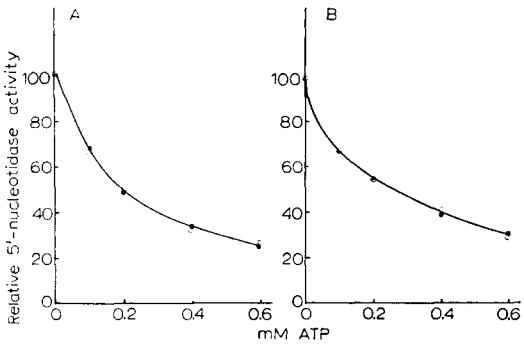

Fig. 4. Effect of sodium deoxycholate and phospholipase C on the inhibition of 5'-nucleotidase by ATP. (A) Various concentrations of ATP were added in the absence (○) or in the presence (●) of 0.4 % sodium deoxycholate. (B) Plasma membranes (1 mg) were incubated without (○) or with (●) phospholipase C (20 μg) for 90 min. The enzyme activity was measured by method 2.

TABLE III

EXCHANGE REACTION BETWEEN ADENOSINE AND 5'-AMP

The reaction was carried out using method 1, except for the addition of [8-^{14}C]adenosine (2.5 nmol, $4.8 \cdot 10^5$ cpm).

System	5'-AMP (cpm)
Complete	738
−5'-AMP	53
−5'-AMP+β-glycerophosphate	45
−5'-AMP+5'-ATP	52
+0.4 % sodium deoxycholate	305

amplified the substrate inhibition by 5'AMP at higher concentrations. The inhibitory effect of ATP was not changed by addition of deoxycholate or solubilization with phospholipase C (Fig. 4).

The exchange reaction between adenosine and 5'-AMP was observed using a purified preparation of 5'-nucleotidase, although the rate was much smaller than that catalyzed by purified nonspecific alkaline phosphatase. The purified 5'-nucleotidase preparation did not exhibit the exchange between 5'-AMP and P_i. The addition of deoxycholate reduced the exchange rate (Table III).

DISCUSSION

Methods for the isolation of plasma membranes from rat liver have been reported from many laboratories [24]. The present procedure based on the method by Song et al. [15] seems to be more reproducible with higher 5'-nucleotidase activity and takes less time. Morphological studies revealed that the preparation retained the intact structure of bile canaliculi, such as desmosomes and terminal bars. The dialyzed preparation of purified 5'-nucleotidase showed vesicular structures similar to purified cytochrome oxidase [25]. Structures became amorphous on the addition of 0.4 % sodium deoxycholate [6, 25].

Various solubilization procedures convert membrane-bound enzymes to solubilized ones with different catalytic and physicochemical properties [5]. In this study the enzyme solubilized with phospholipase C exhibited almost the same properties as the purified 5'-nucleotidase prepared in deoxycholate. However, treatment with sodium dodecyl sulfate or papain produced a protein with a smaller S value than 7 S, which exhibited 5'-nucleotidase activity (Fig. 1). The 5'-nucleotidase purified from brain [22] or pituitary gland [23] showed a sedimentation coefficient of 7 S. But several investigators [2, 4, 22, 23, 26] have reported different molecular weights, varing from 52 000 to 237 000. Evans et al. [3] reported two entities of 5'-nucleotidase with different molecular weights, detected by a polyacrylamide gel electrophoresis in 0.1 % dodecyl sulfate, while by electrophoresis in deoxycholate we observed only one 5'-nucleotidase with the same molecular weight as that calculated from Sephadex G-200 column chromatography. It is conceivable that solubilization using dodecyl sulfate or protease resulted in a disintegrated 5'-nucleotidase with a smaller molecular weight.

The purified preparation contained phospholipid which might make 5'-nucleotidase easy to aggregate into a large molecule by dialysis, as suggested by Green and Tzagoloff [6]. The properties of 5'-nucleotidase in plasma membranes were identical with those of the dialyzed preparation of purified enzyme. The rate of activity toward different substrates remained almost constant throughout purification steps from plasma membranes, suggesting that only one 5'-nucleotidase exists in the plasma membrane preparation used in this study. The treatment of plasma membranes with phospholipase or protease caused similar catalytic alterations to those brought about by the addition of deoxycholate. Tryptic digestion of the solubilized 5'-nucleotidase with deoxycholate did not cause an increase in activity, suggesting that treatment with trypsin may activate the enzyme through the same mechanism as deoxycholate. The effect of deoxycholate was also displayed by other bile salts to the same extent. Therefore it is more likely that the change of catalytic properties of 5'-nucleotidase occurs during the solubilization process than that deoxycholate attaches to the catalytic site, exerting stimulatory effects.

The dialyzed enzyme or the enzyme in plasma membranes may seem to catalyze the reaction as a part of a gigantic molecule. When sodium deoxycholate is added, the enzyme appears to be dispersed in the mixture and behave as a free single enzyme [5]. This hypothesis is consistent with the experimental results, suggesting the high susceptibility of the soluble form, such as elevated heat lability or the decrease of K_m. The same line of study has been reported with membrane bound phosphodiesterase [8].

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