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PREPARATION AND PROPERTIES OF 5'-NUCLEOTIDASES FROM BOVINE MILK FAT GLOBULE MEMBRANES*

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SUMMARY

The 5'-nucleotidase (5'-ribonucleoside phosphohydrolase, EC 3.1.3.5) from bovine milk fat globule membranes was partially purified. Two separate peaks of activity were obtained from a Sepharose column and the two fractions, designated V and VI in order of elution, were collected and characterized separately. Both V and VI exhibited pH optima between 7.0 and 7.5 for AMP, GMP and CMP in the absence of metal ions. In the presence of Mg^{2+}, a second pH optimum at 10.0 was observed with both fractions. Low concentrations of $MnCl_2$ activated Fraction V but not Fraction VI. $HgCl_2$ was a potent inhibitor of both fractions. The relative rates of hydrolysis of various 5'-mononucleotides differed comparing the two fractions. Optimum temperature for P_i release was 69 °C for both fractions. Activation energies were 10400 cal/mole and 9600 cal/mole for Fractions V and VI, respectively. For V, calculated K_m values for AMP, GMP and CMP were 0.94, 2.5 and 1.16 mM, respectively. Calculated K_m values for Fraction VI for AMP, GMP and CMP were 5.0, 3.95 and 1.73 mM, respectively. ATP was a competitive inhibitor of AMP hydrolysis by Fraction V and a noncompetitive inhibitor of AMP hydrolysis by Fraction VI. Both fractions contained chloroform–methanol-extractable phospholipid. The phospholipid distribution pattern of Fraction VI was similar to that of milk fat globule membranes. Fraction V contained only sphingomyelin and phosphatidylcholine. It is proposed that milk fat globule membranes contain two separate 5'-nucleotidases.

INTRODUCTION

The enzyme 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) has been shown to be localized in the plasma membrane of rat liver and is widely used as a marker enzyme for plasma membranes[1,2]. Although 5'-nucleotidases derived from many plant, bacterial and animal sources have been studied in detail (reviewed in ref. 3), Widnell and Unkeless[4] appear to be the only authors to have purified 5'-nucleotidase from isolated plasma membranes. They found the 5'-nucleotidase from rat liver plasma membranes to be a sphingomyelin-containing enzyme. No functional role in activity of the enzyme was ascribed to the lipid component.

Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid.

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It is technically difficult to isolate the quantities of liver plasma membrane needed for recovery of sufficient purified enzyme to permit determination of the functional role of endogenous lipid. In an attempt to circumvent this difficulty, we have studied the properties of 5′-nucleotidase partially purified from the milk fat globule membrane. This membrane is known to contain several enzymes characteristic of plasma membranes[5] and the available morphological and biochemical evidence (reviewed in ref. 6) demonstrates direct derivation of the fat globule membrane from the plasma membrane, which envelops the fat globule during its secretion from the mammary acinar cell. The present paper is concerned with determining the properties of the 5′-nucleotidase isolated from the milk fat globule membrane.

MATERIALS AND METHODS

Materials

Water deionized by passage through a double bed ion exchange resin was used throughout the experiment. ATP and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) buffer were from Calbiochem. Other substrates, Triton X-100, sodium deoxycholate and Tris were from Sigma. Crystalline bovine serum albumin was from Nutritional Biochemicals. Other chemicals were of the highest grade obtainable. Sepharose 4-B was a product of Pharmacia. Solvents used in recovery and separation of lipids were redistilled in glass before use.

Chemical analyses

Phosphorus was determined by the method of Fiske and SubbaRow[7]. Protein was measured with the Folin phenol reagent with bovine serum albumin as the standard[8]. Lipids were extracted from membranes and from purified fractions with chloroform–methanol, separated and analyzed as described previously[9,10].

Electrophoresis

Polyacrylamide gels were prepared and subjected to electrophoresis essentially according to the method of Davis[11]. Electrophoresis was carried out in a cold room at 4 °C. Gels were stained with 0.5 % aniline black and destained in 7 % acetic acid to localize proteins. Duplicate gels were soaked in a reaction mixture consisting of 10 mM AMP, 50 mM Tris–HCl buffer (pH 7.5) and 10 mM $CaCl_2$. Phosphoesterase activity was detected as a white band of calcium phosphate.

Enzyme assay

Unless stated otherwise, 5′-nucleotidase was assayed in a reaction mixture consisting of 50 mM Tris–HCl (pH 7.5), 3.5 mM $MgCl_2$ and 7.5 mM AMP in a final volume of 2.0 ml. Except in those experiments where the effect of temperature on reaction velocities was studied, incubations were conducted at 37 °C for 15 min in a Dubnoff metabolic incubator with agitation at a rate of 80 strokes per min with air as the gas phase. Reactions were stopped by addition of equal amounts of a 10 % trichloroacetic acid solution.

Purification of enzyme

Four preparations of 5′-nucleotidase were made from milk samples obtained within a few minutes of recovery from the animals. Fat globules were harvested

either by centrifugation at 20000 × *g* for 25 min or by passing the milk through a small mechanical separator. Fat globules were washed twice with 4 vol. of 0.9 % NaCl at 37 °C to remove entrained milk serum. Fat globule membranes were obtained by subjecting the washed fat globules to one cycle of freezing and thawing as described previously[6]. All further steps in purification were carried out at 0–4 °C unless otherwise specified. The fat globule membranes were collected by centrifugation at 30000 × *g* for 50 min and were washed once with 0.1 M Tris, pH 7.5. The membrane fraction was suspended in a solution containing 0.1 M Tris–HCl buffer (pH 7.5), 1 mM $MgCl_2$, 5 mM AMP, 1 % sodium deoxycholate, 2 % Triton X-100 and $(NH_4)_2SO_4$ was gradually added to a final concentration of 10 % saturation. After stirring for 30 min, solid $(NH_4)_2SO_4$ was gradually added with stirring to a final concentration of 35 % saturation. The suspension was allowed to stand for at least 1 h and the insoluble material, which contained the enzyme activity, was recovered by centrifugation at 17000 × *g* for 30 min and was suspended in the buffer described above. After stirring for 10 min, the suspension was sonicated for 3 consecutive 20-s periods with a Branson Model B-110 sonicator operated at maximum output. The suspension was cooled for 2 min in ice water between sonication cycles. The suspension was then heated for 5 min in a water bath at 50 °C, cooled to 0 °C and the sonication cycle was repeated. Particulate matter was removed by centrifugation at 145000 × *g* for 15 min and was discarded. Solid $(NH_4)_2SO_4$ was slowly added to 30 % saturation with stirring. After removal of the precipitate, the concentration of $(NH_4)_2SO_4$ was raised to 60 % saturation and the enzyme activity was recovered in the surface plug formed on centrifugation at 20000 × *g* for 30 min. This fraction was dissolved in a minimum volume of a solution containing 0.1 M Tris buffer (pH 7.5), 0.1 mM $MgCl_2$ and 1 mM AMP. This solution was applied to a Sepharose 4-B column (2.5 cm × 36 cm) previously equilibrated with the eluant buffer which contained 0.05 M Tris (pH 7.5), 0.05 M KCl, 0.1 mM $MgCl_2$, 1 mM AMP and 1 % Triton X-100. Fractions (5 ml) containing enzyme activity were pooled and dialyzed against a minimum of 3 changes of 0.05 M Tris buffer (pH 7.5), during 24 h. To collect fractions free of Triton for lipid analysis, protein was precipitated by bringing the respective fractions eluted from the Sepharose column to 80 % saturation with $(NH_4)_2SO_4$. All experiments were performed with this partially purified enzyme. The above procedure is a modification of that described by Widnell and Unkeless[4].

RESULTS

Purification of 5'-nucleotidase

Table I gives a summary of the yield and purification of 5'-nucleotidase from milk fat globule membranes. Chromatography of the active fraction obtained on $(NH_4)_2SO_4$ fractionation, heat treatment, sonication and centrifugation on Sepharose 4-B resulted in the elution of two clearly separated peaks of activity (Fig. 1). The specific activity of the peak tube of Fraction VI was always approx. 1.8 times the specific activity of the peak tube of Fraction V in three separate preparations. The two fractions were characterized separately and will be referred to as Fractions V and VI hereafter (Fig. 1).

Considering the additive of Fractions V and VI together, a purification of 14 times with respect to the original membrane was obtained with a yield of approx.

18 % of the activity in the original membrane preparation. On electrophoretic separation, 4–5 distinct protein bands were observed in both Fraction V and VI from different membrane preparations. However, by soaking gels in an incubation mixture containing AMP and $CaCl_2$, it was found that only one protein band in each fraction displayed 5′-nucleotidase activity. Attempts to purify the active fractions on DEAE-

TABLE I

PURIFICATION OF 5′-NUCLEOTIDASES FROM MILK FAT GLOBULE MEMBRANE

Preparations containing approx. 1 g of milk fat globule membrane protein were fractionated as described in Materials and Methods. 5′-Nucleotidase activity was assayed in a reaction mixture containing 50 mM Tris–HCl (pH 7.5), 3.5 mM $MgCl_2$ and 7.5 mM AMP in a final volume of 2.0 ml. A unit of activity corresponds to the liberation of 1 μmole of P_i per min.

Fraction	*Total volume (ml)*	*Total activity (units)*	*Specific activity (units/mg protein)*
I. Fat globule membrane	100	438.7	0.67
II. First $(NH_4)_2SO_4$	100	343.3	1.22
III. Heat treatment	90	314.7	1.83
IV. Second $(NH_4)_2SO_4$	16	195.3	3.63
V. Sepharose*, 11–14	15	24.7	4.09
VI. Sepharose*, 25–29	25	52.7	5.53

* Two separate peaks were obtained, V and VI. Tubes 11–14 and 25–29 were combined for Fractions V and VI, respectively (Fig. 1).

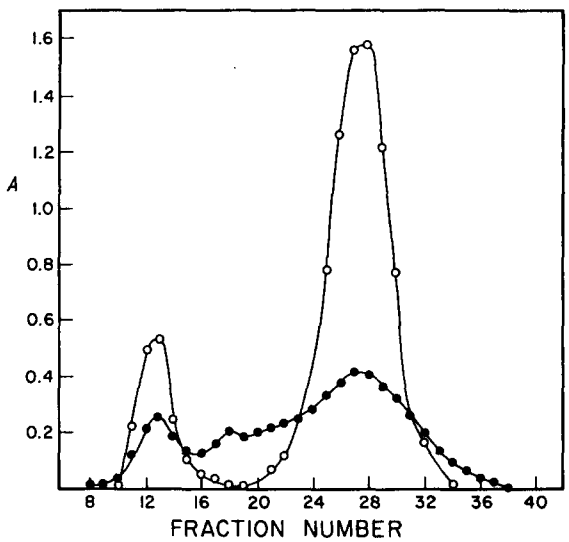


Fig. 1. Elution profile of bovine milk fat globule membrane 5′-nucleotidases from a 2.5 cm × 36 cm Sepharose 4-B column. The column was charged with 54 mg of protein and was eluted with a buffer containing 0.1 M Tris–HCl (pH 7.5), 0.1 mM $MgCl_2$, 0.05 M KCl, 1 mM AMP and 1% Triton X-100. Flow rate was maintained at 12 ml/h and 5-ml fractions were collected. Protein was determined with the Folin phenol reagent (absorbance at 750 nm, ●—●) and enzyme activity was determined by measuring the amount of P_i released from AMP in 15 min at 37 °C in a reaction mixture containing 7.5 mM AMP, 50 mM Tris–HCl (pH 7.5), and 3.5 mM $MgCl_2$ (absorbance at 660 nm, ○—○). Fractions 11–14 and 25–29 were combined, designated Fractions V and VI, respectively, and were used for all further studies.

cellulose and DEAE-Sephadex columns were unsuccessful. Although this method resulted in only a low level of purification, it accomplished the major purpose of yielding enzyme fractions free of non-specific phosphatase activity.

Substrate specificity of 5'-nucleotidase fractions

At constant amounts of enzyme and substrate, the highest relative activity was observed with CMP and GMP with Fractions V and VI, respectively (Table II). Both fractions were active with several other 5'-nucleotides, but the relative activities differed with different substrates comparing the two fractions. Little or no activity was observed when either fraction was incubated with ADP, ATP, IDP, GDP, guanosine 2'+3' mixed monophosphates, and cytidine 2'+3' mixed monophosphates. Neither fraction hydrolyzed β-glycerophosphate, glucose 6-phosphate or fructose 6-phosphate to any significant extent (Table II).

pH optima of the fractions

The pH optima for Fractions V and VI are shown in Fig. 2. With Tris–HCl buffer and AMP as substrate, the pH optima were between 7.0 and 7.5 for both fractions. With HEPES buffer and AMP as substrate, both fractions exhibited a pH optimum of 7.0. When GMP was used as the substrate in HEPES buffer, both fractions displayed a pH optimum of 7.5. With CMP as substrate in HEPES buffer, the pH optima were 7.5 and 7.8 for Fractions V and VI, respectively. Both fractions displayed double pH optima in the presence of Mg^{2+} with AMP as the substrate. Under these conditions, activity maxima were observed at pH 7.0 and 10.0 with both enzyme fractions. The appearance of a second optimum at pH 10.0 in the presence of 4.5 mM Mg^{2+} was observed in both 0.05 M Tris–HCl and 0.05 M glycine–NaOH buffers.

TABLE II

SUBSTRATE SPECIFICITY OF 5'-NUCLEOTIDASES ISOLATED FROM MILK FAT GLOBULE MEMBRANES

The reaction mixtures contained 5 mM of the appropriate substrate, 5 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5) and 40 μg of enzyme protein in a final volume of 2.0 ml.

Substrate	*Relative activity* (%)	
	Fraction V	*Fraction VI*
GMP	100.0	100.0
IMP	110.6	88.5
CMP	121.3	90.8
AMP	69.8	84.5
IMP	7.5	10.5
XMP	29.4	39.2
dAMP	16.4	34.0
dCMP	26.5	32.9
ADP	0.4	0.3
ATP	0.2	0.1
IDP	0.0	1.3
GDP	2.8	1.4
Guanosine 2'+3'-monophosphate	1.1	0.1
Cytidine 2'+3'-monophosphate	1.1	0.3
β-Glycerophosphate	1.1	0.1
Glucose 6-phosphate	0.3	0.4
Fructose 6-phosphate	0.6	2.1

Effect of enzyme protein concentration and incubation time

The activity of both 5'-nucleotidase fractions was linear with respect to enzyme protein up to at least 60 μg of protein. The rate of P_i liberation from 5'-AMP was linear up to at least 60 min incubation at 37 °C with both enzyme fractions.

Effect of temperature on 5'-nucleotidase

Maximum rates of P_i liberation were achieved on incubation at 69 °C for 15 min with both Fractions V and VI; the rate of hydrolysis decreased at higher incubation temperatures (Fig. 3). Rate of P_i liberation increased as the incubation temperature was increased between 17 and 69 °C with both fractions. Activation energies, calculated by the method described by Bodansky[12] from reaction velocities obtained over a temperature range of from 23–48 °C, were 10400 cal/mole for Fraction V and 9600 cal/mole for Fraction VI (averages of 6 determinations). The presence of $MgCl_2$ did not appreciably effect the activation energies of either fraction.

Heat stability of 5'-nucleotidase

To determine the heat stability of the 5'-nucleotidase fractions, they were incubated at 60 °C for periods up to 60 min, immediately cooled in ice water and assayed for ability to liberate P_i from AMP and CMP. Fig. 4 shows that Fraction V

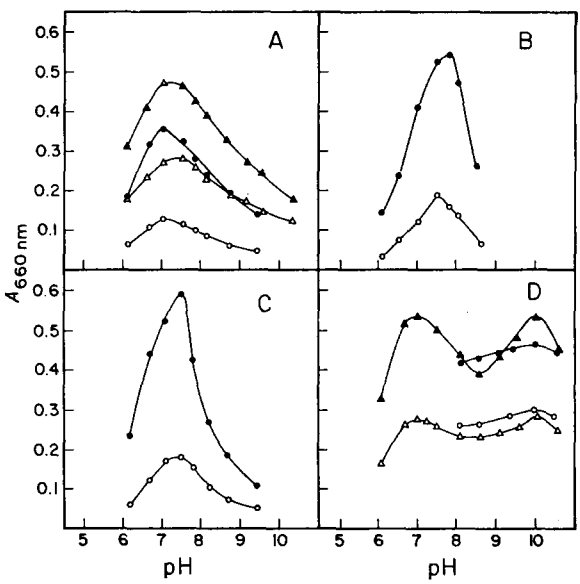
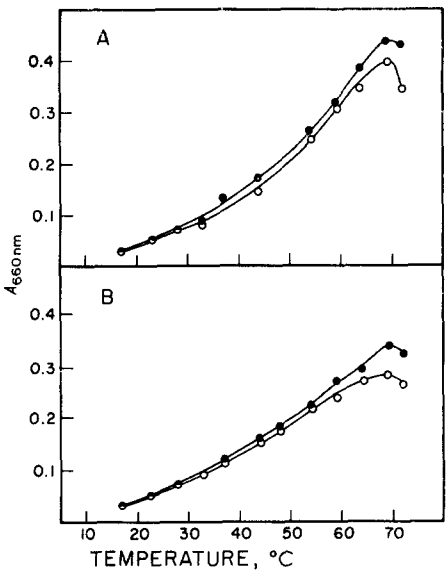


Fig. 2. Effect of pH on the activity of 5'-nucleotidases isolated from milk fat globule membranes. (A) AMP as substrate in a final concentration of 7.5 mM and in the absence of metal ions. ▲—▲, Fraction VI in 50 mM Tris–HCl; ●—●, Fraction VI in 90 mM HEPES buffer; △—△, Fraction V in 50 mM Tris–HCl; ○—○, Fraction V in 90 mM HEPES buffer. (B) CMP as substrate in a final concentration of 9.0 mM and in the absence of metal ions in 90 mM HEPES buffer. ●—●, Fraction VI; ○—○, Fraction V. (C) GMP as substrate in a final concentration of 7.5 mM and in the absence of metal ions in 90 mM HEPES buffer. ●—●, Fraction VI; ○—○, Fraction V. (D) Reaction mixtures contained 4.5 mM $MgCl_2$ and 7.5 mM AMP. ▲—▲, Fraction VI, 50 mM Tris–HCl; ●—●, Fraction VI, 50 mM glycine–NaOH buffer; △—△, Fraction V, 50 mM Tris–HCl; ○—○, Fraction V, 50 mM glycine–NaOH buffer. P_i release was measured after 15 min at 37 °C.



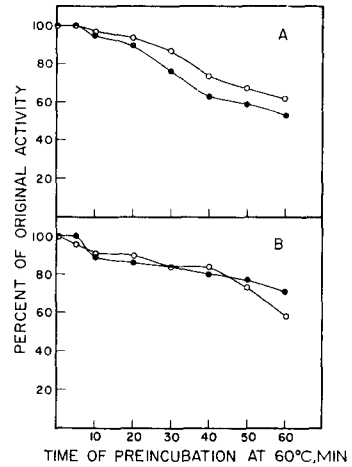


Fig. 3. Effect of incubation temperature on P_i release from AMP by 5′-nucleotidases isolated from milk fat globule membranes. Final concentrations of reactants were 7.5 mM AMP, 50 mM Tris–HCl (pH 7.5) and in a final volume of 2.0 ml. Velocities were determined after 15 min in the presence (●—●) and in the absence (○—○) of 3.5 mM $MgCl_2$. A, Fraction VI; B, Fraction V.

Fig. 4. Effect of preincubation at 60 °C on the rate of P_i release from AMP (○—○) and CMP (●—●) by 5′-nucleotidases isolated from milk fat globule membranes. The partially purified fractions were incubated for the indicated times at 60 °C, cooled in ice water, and added to reaction mixtures containing 7.5 mM AMP or CMP, 50 mM Tris–HCl (pH 7.5) and 3.5 mM $MgCl_2$. Incubations were for 15 min at 37 °C. A, Fraction V; B, Fraction VI.

lost 38 % and 48 % of the original activity with AMP and CMP, respectively, after preincubation for 60 min. In contrast, Fraction VI lost a greater percentage of the original activity with AMP (41 %) than with CMP (31 %). There was progressive loss of activity in both fractions with both substrates for treatment times ranging from 10 to 60 min.

Effect of divalent metal ions on 5′-nucleotidase activity

$HgCl_2$ was a potent inhibitor of the 5′-nucleotidase activity of Fractions V and VI (Table III). At concentrations of both 1 and 5 mM, the activity of Fraction V was inhibited to a greater extent than was Fraction VI. Fraction V was slightly inhibited by 1 and 5 mM $CaCl_2$; activity in Fraction VI was unchanged at 1 mM and slightly activated at 5 mM $CaCl_2$. At pH 7.5, $MgCl_2$ at 1 and 5 mM activated Fraction V with the greatest effect at 1 mM. The activity of Fraction VI was essentially unchanged by 1 and 5 mM $MgCl_2$ at pH 7.5. At 1 mM, $BaCl_2$ slightly inhibited 5′-nucleotidase activity of both fractions but had little effect at 5 mM. At 1 mM, both $MnCl_2$ and $CoCl_2$ activated the 5′-nucleotidase of Fraction V while at 5 mM, both $MnCl_2$ and $CoCl_2$ inhibited 5′-nucleotidase of Fraction V. In contrast, both levels of $MnCl_2$ and $CoCl_2$ inhibited the 5′-nucleotidase of Fraction VI. Fig. 5 shows the effects of $MnCl_2$ on the 5′-nucleotidase activity of Fraction V. At low levels, the enzyme is

activated with a maximum at 1 mM. Inhibition is noted when the $MnCl_2$ concentration approaches 5 mM.

Effect of substrate concentration on reaction velocity

Results of studies on effects of AMP concentration on reaction velocity for both fractions showed straight line relationships when plotted according to the method of Lineweaver and Burk[13] (Fig. 6). The calculated apparent K_m values for AMP were

TABLE III

EFFECT OF DIVALENT METAL IONS ON THE ACTIVITY OF 5′-NUCLEOTIDASES FROM MILK FAT GLOBULE MEMBRANE

The reaction mixture contained 5 mM GMP, 50 mM Tris–HCl (pH 7.5), the indicated amount of metal ion and 40 μg of enzyme protein. Incubation was at 37 °C for 15 min.

Metal	*Concentration (mM)*	*Relative activity (%)*	
		Fraction V	*Fraction VI*
None		100.0	100.0
$HgCl_2$	1	41.7	69.8
	5	0.3	12.8
$CaCl_2$	1	92.3	100.0
	5	98.2	102.0
$MgCl_2$	1	108.0	100.0
	5	102.8	100.7
$BaCl_2$	1	95.1	96.6
	5	100.9	102.0
$MnCl_2$	1	118.2	98.4
	5	86.4	40.0
$CoCl_2$	1	103.1	89.4
	5	80.3	60.3

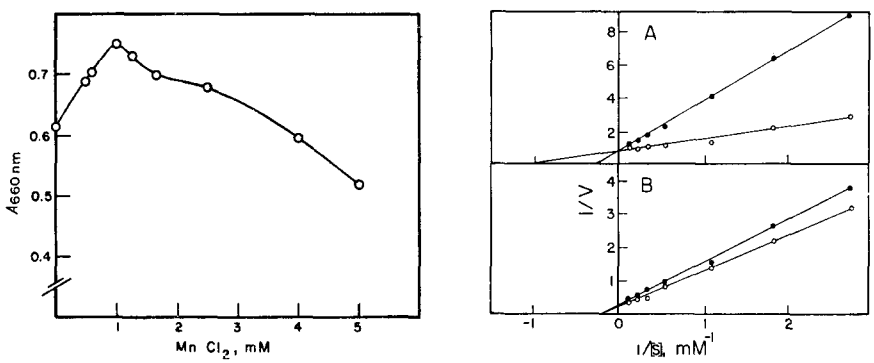


Fig. 5. Activation of the 5′-nucleotidase of Fraction V by $MnCl_2$. The rate of P_i release was determined in a reaction mixture containing 5 mM GMP, 50 mM Tris–HCl (pH 7.5) and the indicated amount of $MnCl_2$ in a final volume of 1.0 ml. Incubations were at 37 °C for 15 min.

Fig. 6. Lineweaver–Burk plots of the rate of P_i release from AMP by 5′-nucleotidases isolated from fat globule membranes. Final concentrations of reactants were 0.4–10 mM AMP, 50 mM Tris–HCl (pH 7.5) and 3.5 mM $MgCl_2$ in a final volume of 2.0 ml. Velocities were determined after 15 min at 37 °C in the absence (○—○) or presence (●—●) of $1.5 \cdot 10^{-5}$ M ATP. A, Fraction V; B, Fraction VI.

0.94 and 5.0 mM for Fractions V and VI, respectively. Straight line relationships were also obtained when CMP and GMP were used as substrates. With CMP the apparent K_m values were 2.5 and 3.95 mM for Fractions V and VI, respectively. For GMP, calculated values for K_m were 1.16 and 1.73 mM for Fractions V and VI, respectively.

Effect of ATP on hydrolysis of AMP

Table IV shows that ATP was a potent inhibitor of AMP hydrolysis by both fractions. Nearly 40% of the original activity of both fractions was lost at ATP concentrations as low as 17 μM. AMP hydrolysis by both fractions was totally inhibited at ATP concentrations of 8.3 mM. Plots of $1/v$ *vs* $1/[AMP]$ revealed ATP to be a competitive inhibitor of AMP hydrolysis by Fraction V (Fig. 6). For this fraction, the K_i for ATP was computed as $5 \cdot 10^{-6}$ M. In contrast, ATP was a noncompetitive inhibitor of AMP hydrolysis by Fraction VI (Fig. 6). The computed K_i for Fraction VI was $4.76 \cdot 10^{-5}$ M.

Lipid composition of the enzyme fractions

Although neither fraction was electrophoretically homogeneous, it was of

TABLE IV

EFFECT OF ATP ON HYDROLYSIS OF AMP BY 5′-NUCLEOTIDASES ON MILK FAT GLOBULE MEMBRANE

The reaction mixture contained 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM AMP, the indicated amount of ATP and 13.5 μg of Fraction V protein or 16 μg of Fraction VI protein in a final volume of 2.0 ml. Incubations were at 37 °C for 15 min.

ATP concentration (M)	*Relative activity (%)*	
	Fraction V	*Fraction VI*
None	100.0	100.0
$1.7 \cdot 10^{-5}$	59.2	63.3
$1.7 \cdot 10^{-4}$	34.4	30.5
$8.3 \cdot 10^{-4}$	12.0	11.2
$1.7 \cdot 10^{-3}$	7.2	5.9
$8.3 \cdot 10^{-3}$	0.0	0.0

TABLE V

PHOSPHOLIPID DISTRIBUTION IN 5′-NUCLEOTIDASE FRACTIONS ISOLATED FROM MILK FAT GLOBULE MEMBRANES

Lipids were extracted with chloroform–methanol–ammonia[10] and phospholipid distribution was determined by phosphorus analysis of components separated by two-dimensional thin-layer chromatography[9].

Phospholipid	*Percent of total lipid phosphorus*		
	Fraction V	*Fraction VI*	*Globule membrane*
Sphingomyelin	78.0	21.1	22.8
Phosphatidylcholine	22.0	34.7	37.1
Phosphatidylserine	—	8.8	4.4
Phosphatidylinositol	—	7.5	6.5
Phosphatidylethanolamine	—	27.9	29.2

interest to determine if the 5′-nucleotidase activity was recovered in a fraction containing lipoprotein. Both fractions contained chloroform–methanol-extractable lipid. Fraction VI contained nearly 4 times more phospholipid (30.5 μg lipid P per mg protein) than did Fraction V (8.2 μg lipid P per mg protein). Sufficient lipid material was recovered to permit thin-layer chromatographic separation and analysis of phospholipid distribution. Results of these analyses are recorded in Table V. All five of the phospholipids present in the milk fat globule membrane were present in Fraction VI. Except for the fact that the enzyme fraction contained slightly higher levels of phosphatidyl serine (8.8 % of the total lipid phosphorus) than does the milk fat globule membrane (2–6 % of the total lipid phosphorus), the phospholipid distribution in this fraction is similar to that repeatedly observed for milk fat globule membranes[14–17]. In contrast, only two phospholipids, sphingomyelin and phosphatidylcholine, were recovered from Fraction V. Sphingomyelin accounted for 78 % of the total lipid phosphorus in this fraction. The low amount of lipid recovered coupled with the presence of residual Triton prevented assessment of the neutral lipid content of these fractions.

DISCUSSION

To our knowledge, there have been no previous reports of attempts to purify and characterize 5′-nucleotidase from either milk or mammary tissue. The observation that the fat globule membrane contains high specific activity in 5′-nucleotidase coupled with the known subcellular localization of this enzyme in the plasma membrane of several cell types[1,2,18–20] provides additional evidence for the concept of direct derivation of the milk fat globule membrane from plasma membrane of mammary secretory cells[6,21–24]. In preliminary experiments, we have found that 5′-nucleotidase activity is enriched in plasma membrane-rich fractions from bovine mammary gland (C. M. Huang and T. W. Keenan, unpublished observation).

When our preparation was chromatographed on Sepharose 4-B, two well separated peaks containing 5′-nucleotidase activity were obtained. One of these, Fraction V, was eluted shortly after the void volume and the second, major peak, Fraction VI, was retained on the column. Widnell and Unkeless[4] observed a similar elution profile for rat liver plasma membrane 5′-nucleotidase on a 6 % agarose column. However, these authors obtained the major portion of the activity in the peak eluting just after the void volume. They ascribed the second peak to a degradation product of the enzyme, since the proportion of the activity in this peak increased when the preparative procedure was spread over longer time periods. We obtained nearly constant ratios of activities comparing the two peaks in three separate preparations, even though preparation times varied widely. It would thus appear that Fractions V and VI represent different enzymes. Additional support for this interpretation comes from the observations that Fractions V and VI differed in K_m value, in effects of $MnCl_2$ on enzyme activity and in different K_i values with ATP. Further, ATP inhibited Fraction V competitively and Fraction VI noncompetitively. The relative rates of hydrolysis 5′-mononucleotides also differed. The relative order of hydrolysis at 5 mM concentration, of Fraction V was CMP > IMP > GMP > AMP > XMP > dCMP > dAMP > TMP, while for Fraction VI the order was GMP > CMP > IMP > AMP > XMP > dAMP > dCMP > IMP. All of these criteria appear to suggest that Fractions V and VI contain different 5′-nucleotidases.

With regard to relative rates of hydrolysis of various substrates, both Fractions V and VI differ from the 5′-nucleotidases of rat liver plasma membranes[4], human liver[25] and rat cerebellum[26]. However, both fractions share many features in common with 5′-nucleotidases of other tissues. The double pH optima observed with both fractions in the presence of Mg^{2+} has previously been demonstrated by Bodansky with 5′-nucleotidases from bull seminal plasma[27], rat liver plasma membrane[1] and human liver[25]. Activation at low Mn^{2+} concentrations, exhibited by Fraction V but not by Fraction VI, has been observed with 5′-nucleotidases from bull seminal plasma[27], human liver[25] and human aorta[2]. Another similarity between the 5′-nucleotidases of the fat globule membrane and those from other sources is inhibition of hydrolysis of 5′-mononucleotides by ATP, which has been observed with several 5′-nucleotidases[26,29–32].

The K_m values for Fractions V and VI were higher, by more than an order of magnitude, than those reported for several other 5′-nucleotidases. With AMP as substrate under reaction conditions closely resembling those employed herein, reported K_m values for bull seminal plasma, rat liver plasma membrane, rat cerebellum, and human liver 5′-nucleotidases range from 12 to 80 μM[1,4,25–27]. This compares to K_m values of 940 and 5000 μM for Fractions V and VI, respectively. Values in this range were also calculated when CMP and GMP served as substrates. The reasons why the enzymes from the milk fat globule membrane differ so greatly in substrate binding ability from other 5′-nucleotidases, especially that of rat liver plasma membranes, are not obvious. This is especially true in view of the general similarity of 5′-nucleotidases comparing other properties as discussed previously.

Finally, our results suggest that the 5′-nucleotidase of Fraction V is similar to that from rat liver plasma membranes in lipid composition. Widnell and Unkeless[4] observed this 5′-nucleotidase to be a lipotrotein containing essentially only one phospholipid, sphingomyelin. Our results show a large enrichment of sphingomyelin in this fraction. While our preparation was not homogeneous, it would be expected that if the 5′-nucleotidase contained a characteristic liproprotein, it would be enriched in the active fraction. In contrast, there was little or no enrichment of any of the five fat globule membrane phospholipids in Fraction VI.

Since completion of this work, Patton and Trams[33] have reported detection of 5′-nucleotidase as well as nucleotide pyrophosphatase and Mg^{2+}-activated ATPase in milk fat globule membranes. These authors compared specific activities of intact fat globules and isolated milk fat globule membranes in an attempt to explore "sidedness" in the plasma membrane. They tentatively concluded that the 5′-nucleotidase is located on the outer surface of the membrane (the surface of the fat globule membrane which is exposed). The milk fat globule appears to be an attractive system for studying differences in the inner and outer surfaces of the mammary cell plasma membrane. In this regard it would compare favorably with erythrocyte ghosts and cultured cells. Additionally, the fat globule represents a rich source of plasma membrane uncontaminated by other subcellular membranes.

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