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PURIFICATION AND SOME PROPERTIES OF CYTOSOL 5'-NUCLEOTIDASE FROM RAT LIVER

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Summary

A 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) was highly purified from rat liver. The preparation appeared homogeneous on the criteria of disc-gel electrophoresis.

A pH optimum at about 6.5 was observed for all substrates tested. The activity of this enzyme was absolutely dependent on the presence of various bivalent metal salts. The highest V value was attained with $MgCl_2$ and the concentration at half-enzyme saturation was lowest with $MnCl_2$. The enzyme had markedly higher affinities for IMP, dIMP, GMP and dGMP than the other 5'-mononucleotides, although V values for all the substrates tested were in the same order of magnitude.

The activity of this enzyme was stimulated by various alkali metal salts, some carboxylic acids and adenine nucleotides. When AMP was used as substrate, the substrate-velocity plot was sigmoidal and NaCl, Tris-maleate and ATP stimulated the enzyme by decreasing the sigmoidicity of the plot. When IMP was used as substrate, the substrate-velocity plot was hyperbolic and these three activators stimulated the enzyme by increasing the V and decreasing the K_m value.

Some of these results provided consistent evidence for the identity of this enzyme and the cytosol 5'-nucleotidase, the presence of which had been reported in crude preparations from rat liver.

Introduction

A 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) that preferentially hydrolyses IMP and GMP was originally described in chicken liver [1]. We have shown that this enzyme is localized in the cytoplasm [2]. The

physiological significance of this enzyme in uric acid production, from IMP in uricotelic animals, was suggested by the fact that the activity of this enzyme increased in response to a high protein diet, in chicken liver and kidney [3,4]. Results of a kinetic study on a highly purified preparation of chicken liver enzyme [5] have shown that this enzyme is an allosteric protein regulated by various ligands through the cooperative interaction among subunits [6].

Using crude preparations, we suggested the presence of similar 5′-nucleotidases in various animals, including the rat [7]. The activity of this enzyme in rat liver has also been reported to be cytoplasmic [8].

Some investigators have suggested physiologically important roles for cytoplasmic 5′-nucleotidase in mammalian tissues. From a kinetic study on a partially purified preparation from rat liver, Van den Berghe et al. [9] concluded that the degradation of AMP required deamination to IMP, which is then hydrolysed to inosine by a cytosol 5′-nucleotidase [9]. Newby [10] has reported that cytoplasmic IMP-hydrolysing activity in polymorphonuclear leucocytes is sufficient to explain the rate of hypoxanthine formation observed.

For all these studies crude preparations were used and the reaction conditions adopted by these authors were varied. Because of the presence of various types of nonspecific phosphatases and nucleotidases [11], and the low content of the 5′-nucleotidase, which preferentially hydrolyses IMP, it seemed essential to obtain a homogeneous preparation of the cytosol 5′-nucleotidase to provide consistent evidence for the presence of this enzyme in mammalian tissues and to characterize its properties. A study on the catalytic properties of this enzyme is also necessary to find optimal assay conditions.

We have purified this enzyme to apparent homogeneity on the criteria of analytical disc electrophoresis from rat liver. The method of purification and some catalytic and regulatory properties of this preparation are presented in this paper.

Materials and Methods

Chemicals. dIMP and dGMP (sodium salts) were obtained from Sigma Chemical Co. AMP, GMP and IMP (free acids) were purchased from Yamasa Shoyu Co. and used as Tris salts. The other nucleotides were obtained as sodium salts from Yamasa Shoyu Co. Concentrations of all nucleotide solutions were determined spectrophotometrically. Ribose 5′-phosphate (sodium salt) was from Boehringer, Mannheim. [8-^{14}C]AMP (specific activity, 59 Ci/mol) and [8-^{14}C]IMP (60 Ci/mol) were purchased from The Radiochemical Center, Amersham. [8-^{14}C]GMP (40 Ci/mol) was obtained from Schwarz/Mann. Phosphocellulose was a product of Brown Co.

All other chemicals were of reagent grade or the highest quality available.

Assay of 5′-nucleotidase. Assay 1 : 5′-Nucleotidase activity was assayed in the following incubation mixture: 100 mM imidazole-HCl buffer (pH 6.5)/50 mM $MgCl_2$/500 mM NaCl/0.1% bovine serum albumin and appropripate amounts of nucleoside 5′-monophosphate and enzyme preparation in a total volume of 0.5 ml. After incubation at 37°C, P_i liberated was determined as described previously [6].

Assay 2: Enzyme activity was assayed with [8-^{14}C]nucleoside 5'-monophosphate as substrate in the same reaction mixture as Assay 1 in a total volume of 50 μl. Liberated [8-^{14}C]nucleoside was determined as described previously [6].

Even at the lowest concentrations of the substrates, the time course of the reaction was linear under the assay conditions used in the kinetic experiments.

Protein determination. Protein was determined by the method of Bradford [12] using crystalline bovine serum albumin as a standard.

Gel electrophoresis. Analytical disc-gel electrophoresis was carried out by the method of Davis [13] modified by Hjerten et al. [14] in 5% polyacrylamide gel at pH 8.0 and 9.5. The enzyme activity in the gel was determined by the method described by Naito and Tsushima [5].

Estimation of kinetic parameters. When the substrate-velocity plot of the catalysed reaction was hyperbolic, V and K_m values were estimated by the direct linear plot described by Eisenthal and Cornish-Bowden [15]. When the substrate-velocity plot of the reaction was sigmoidal, V, $s_{0.5}$ and n_H were estimated by the method of Atkinson [16].

Results

Purification of 5'-nucleotidase from rat liver. The purification procedure described below resulted in a final purification of the enzyme of about 3600-fold with recovery of 3.1%. Table I shows a summary of data from this procedure. All steps were performed at 0—4°C in solution that contained 50 mM Tris/HCl (pH 7.4)/1 mM EDTA/10 mM β-mercaptoethanol. This solution is referred to as the 'buffer'. Enzyme activity was determined by assay 1 with 3 mM IMP as substrate, except for step 3, in which assay 2 was used with 3 mM [8-^{14}C]IMP.

Step 1. Extraction. Rat liver (180 g) was cut into pieces and homogenized with 4 vol. buffer in Waring Blendor at maximum speed for 5 min. The homogenate was centrifuged at 16 000 × *g* for 15 min and the precipitate discarded.

Step 2. 1st phosphocellulose column chromatography. Phosphocellulose suspension (10 g in 150 ml), previously equilibrated with buffer, was mixed with the extract from step 1. The mixture was allowed to stand overnight with stirring. The phosphocellulose was collected by centrifugation, resuspended in buffer and packed in a column (2.5 × 32 cm). The column was washed with 1 l buffer/200 mM NaCl and a linear gradient of 200—800 mM NaCl in buffer was

TABLE I

PURIFICATION OF RAT LIVER 5'-NUCLEOTIDASE

5'-Nucleotidase activity was determined with 3 mM IMP as substrate under the standard reaction condition described in the text.

Step	Total volume (ml)	Protein (mg/ml)	Total activity (μmol/min)	Specific activity (μmol/min per mg)
1. Extraction	710	31	137	0.006
2. 1st phosphocellulose	45	0.80	25.1	0.698
3. 2nd phosphocellulose	20	0.094	11.9	6.28
4. $(NH_4)_2SO_4$ fractionation	0.5	1.3	10.5	16.1
5. Low ionic strength precipitation	0.5	0.40	4.3	21.5

applied. The volume of the limiting buffers was 150 ml each. Fractions of about 5 ml were collected. The fractions containing high enzyme activity (45 ml) were pooled.

Step 3. 2nd phosphocellulose column chromatography. The pooled fractions from step 2 were diluted 3-fold with buffer and applied to a phosphocellulose column (1.5 × 5 cm) previously equilibrated with buffer. The column was washed with 100 ml buffer/200 mM NaCl and a linear gradient from 200 mM NaCl to 13 mM ATP and 200 mM NaCl in buffer was applied. The volume of the limiting buffers was 30 ml each. Fractions of about 1 ml were collected. The fractions containing high activity (20 ml) were pooled.

Step 4. $(NH_4)_2SO_4$ fractionation. The pooled fractions were brought to 45% saturation by addition of solid $(NH_4)_2SO_4$. After 30 min the precipitate formed was removed by centrifugation and solid $(NH_4)_2SO_4$ was again added to the supernatant to 60% saturation. The precipitate formed was collected by centrifugation and dissolved in 0.5 ml buffer.

Step 5. Precipitation at low ionic strength. The protein solution from step 4 was dialysed against 200 ml buffer for 14 h. The precipitate formed was dissolved in 0.5 ml buffer/500 mM NaCl. The solution was then centrifuged at 20 000 × *g* for 15 min to remove the insoluble precipitate. The supernatant was stored at 4°C and used for the following experiments.

Purity of the enzyme preparation. The purified 5′-nucleotidase was shown to be homogeneous on the criteria of disc-gel electrophoresis at two different pH values. In each case, the protein band corresponded to 5′-nucleotidase activity detected with IMP as substrate.

Some kinetic characteristics of the 5′-nucleotidase

pH-Activity profile. As shown in Fig. 1, the pH optimum was found to be about 6.5 with IMP as substrate in the presence of 2.5 mM $MgCl_2$ in imidazole-HCl buffer. Activity in the acidic range was stimulated by increasing the concentration of $MgCl_2$. Addition of 500 mM NaCl stimulated the activity in the alkaline range. In the presence of 50 mM $MgCl_2$ and 500 mM NaCl, the following substrates exhibited pH maximum at 6.5: GMP, dGMP, dIMP, AMP and UMP.

Effect of various salts on enzyme activity. The addition of NaCl of Tris-maleate caused an increase in the rate of reaction, when either IMP or AMP was used as substrate, at a concentration of 3 mM. Tris-HCl or imidazole-HCl was less effective than NaCl with IMP. When AMP was used as substrate, imidazole-HCl up to the concentration of 300 mM and Tris-HCl up to the concentration of 100 mM had no significant effect on the reaction rate. As shown in Table II, various alkali metal salts were equally effective with IMP as substrate. When AMP was used as substrate, LiCl and Na_2SO_4 were less effective than the other salts tested. The effect of maleate was not specific. At the same concentration, Tris salt of fumaric acid or succinic acid was as effective as Tris-maleate. An equivalent amount of Tris-acetate also stimulated the enzyme activity. Two amino acids tested had almost no effect. The effects of NaCl and maleate were not additive.

As shown in Fig. 2, a plot of the reaction rate with respect to AMP concentration was sigmoidal ($n_H = 1.8$). Addition of 500 mM NaCl or 150 mM Tris-

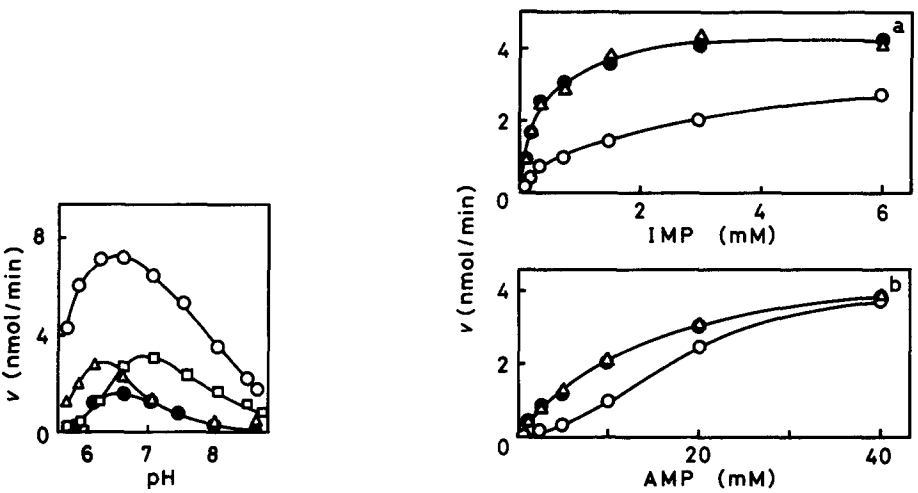

Fig. 1. Effect of pH on the activity of the 5′-nucleotidase with IMP. Reaction mixture contained 100 mM imidazol-HCl buffer/0.1% bovine serum albumin/3 mM IMP/various concentrations of NaCl and $MgCl_2$. ●——●, 2.5 mM $MgCl_2$; △——△, 50 mM $MgCl_2$; □——□, 2.5 mM $MgCl_2$ and 500 mM NaCl; ○——○, 50 mM $MgCl_2$ and 500 mM NaCl.

Fig. 2. Effect of NaCl or Tris-maleate on the 5′-nucleotidase activity as a function of substrate concentration. Reaction mixture contained 100 mM imidazol-HCl buffer (pH 6.5)/50 mM $MgCl_2$/0.1% bovine serum albumin/IMP (a) or AMP (b) and effectors. ○——○, no addition; △——△, 500 mM NaCl; ●——●, 150 mM Tris-maleate (pH 6.5).

TABLE II

EFFECT OF VARIOUS SALTS ON THE 5′-NUCLEOTIDASE ACTIVITY

Reaction mixture contained 100 mM imidazole-HCl (pH 6.5)/50 mM $MgCl_2$/0.1% bovine serum albumin/3 mM IMP or AMP as substrate and effectors, as indicated. The activity in the absence of the effectors was taken as the standard for each substrate. Carboxylic acids were added as solutions of Tris salts at pH 6.5.

Addition	Conc. (M)	Relative activity with 3 mM	
		IMP	AMP
None	—	1.00	1.00
Acetate	0.3	2.12	3.50
Glycine	0.3	1.10	1.08
Maleate	0.15	2.26	4.69
Fumarate	0.15	2.25	5.45
Succinate	0.15	2.22	4.53
Glutamate	0.15	1.29	0.92
LiCl	0.5	2.04	2.23
KCl	0.5	2.38	6.22
KBr	0.5	2.34	7.66
KI	0.5	2.00	6.96
KNO_3	0.5	1.79	7.40
NaCl	0.5	2.27	6.29
Na_2SO_4	0.25	2.22	2.99
Maleate NaCl	0.15 0.5	2.28	4.47

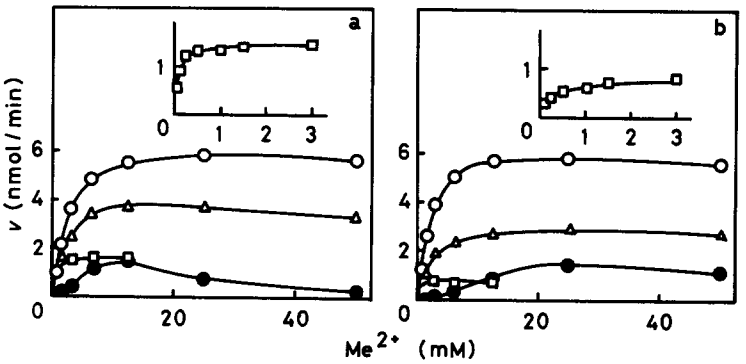

Fig. 3. Effect of bivalent metal salts on the activity of 5'-nucleotidase. Reaction mixture contained 100 mM imidazol-HCl buffer (pH 6.5)/500 mM NaCl/0.1% bovine serum albumin/3 mM IMP (a) or 45 mM AMP (b) and various concentrations of bivalent metal salts as indicated. ○———○, $MgCl_2$; △———△, $CoCl_2$; □———□, $MnCl_2$; ●———●, $NiCl_2$.

maleate yielded curves which approximate rectanglar hyperbola. No change in V values was observed. Substrate concentration of half-enzyme saturation ($s_{0.5}$) decreased from 25 to 14 mM. The substrate-velocity plot with IMP was hyperbolic. In the presence of 500 mM NaCl or 150 mM Tris-maleate, V increased 1.8-fold and the K_m value decreased from 1.0 to 0.2 mM.

Effect of bivalent metal salts. The enzyme is inactive in the absence of bivalent metal ions (Fig. 3). In the presence of 500 mM NaCl, the dependence of the reaction rate on the concentration of $MgCl_2$, $CoCl_2$ or $MnCl_2$ was hyperbolic with 3 mM IMP or 45 mM AMP as substrate. When IMP was used as substrate, the concentrations at half-enzyme saturation of bivalent metal salts were 3.5, 2.7 and 0.1 mM and the relative values of V were 1.00, 0.63 and 0.22 for $MgCl_2$, $CoCl_2$ and $MnCl_2$, respectively. When AMP was used as substrate, the concentrations at half-enzyme saturation were 2.0, 1.1 and 0.15 mM and the relative values of V were 1.00, 0.41 and 0.11 for $MgCl_2$, $CoCl_2$ and $MnCl_2$, respectively. The saturation curves for $NiCl_2$ were sigmoidal for both substrates

TABLE III

SUBSTRATE SPECIFICITY OF THE 5'-NUCLEOTIDASE

Reaction mixture contained 100 mM imidazole-HCl (pH 6.5)/500 mM NaCl/50 mM $MgCl_2$/0.1% bovine serum albumin and various concentrations of nucleoside 5'-monophosphates. V and K_m values were estimated by the direct linear plot [15]. The V with IMP was taken as the standard.

Substrate	V	K_m (mM)
IMP	1.00	0.2
dIMP	1.08	0.7
GMP	0.98	0.7
dGMP	0.91	1.1
XMP	0.88	2.9
AMP	1.15	14
dAMP	1.02	35
UMP	0.98	6.0
CMP	0.79	17
dTMP	0.88	22

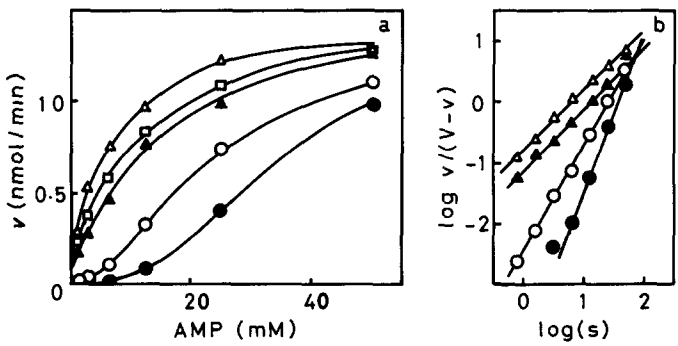

Fig. 4. (a) Effect of ATP, ADP and P_i on the 5'-nucleotidase activity as a function of AMP concentration. Reaction mixtures contained 100 mM imidazol-HCl buffer (pH 6.5)/50 mM $MgCl_2$/0.1% bovine serum albumin/various concentrations of [^{14}C]AMP and effectors as indicated. ○———○, no addition; △———△, 5 mM ATP; □———□, 5 mM ATP and 500 mM NaCl; ▲———▲, 5 mM ADP; ●———●, 1 mM P_i. (b) Hill plots of the data from (a) are presented. △———△, 5 mM ATP (n_H = 1.0); ▲———▲, 5 mM ADP (n_H = 1.0); ○———○, no addition (n_H = 1.8); ●———●, 1 mM P_i (n_H = 2.4).

and a marked inhibitory effect was observed at high concentrations with IMP as substrate.

In the presence of $CaCl_2$, $ZnCl_2$, $CdCl_2$ or $PbCl_2$ at a concentration of 12.5 mM, the enzyme was inactive.

Substrate specificity of the enzyme. In the presence of 500 mM NaCl and 50 mM $MgCl_2$, substrate-velocity plots for all the 5'-mononucleotides tested were hyperbolic. Apparent K_m and V values for each substrate were presented in Table III. K_m values for AMP and pyrimidine nucleotides were much higher than those for IMP, dIMP, GMP and dGMP, although V values for all the substrates tested were in the same order of magnitude. Apparent K_m values for deoxyribonucleotides were higher than those for corresponding ribonucleotides. Reaction rate with 20 mM ribose 5'-phosphate was estimated to be about 1% of the activity with IMP at the same concentration.

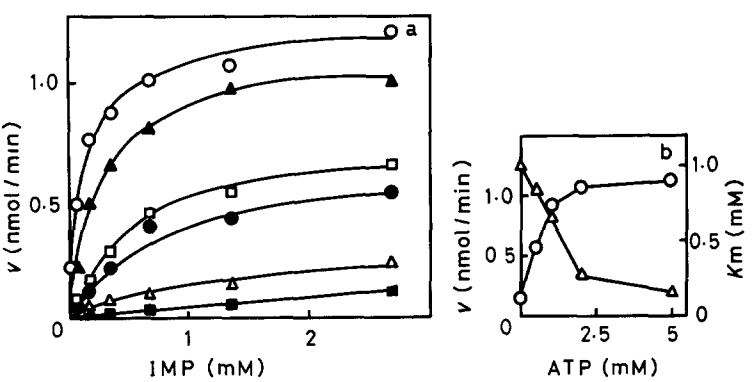

Fig. 5. (a) Effect of ATP and P_i on the 5'-nucleotidase activity as a function of IMP concentration. Reaction mixtures contained 100 mM imidazol-HCl buffer (pH 6.5)/50 mM $MgCl_2$/0.1% bovine serum albumin/various concentrations of [^{14}C]IMP and effectors as indicated. △———△, no addition; ●———●, 0.5 mM ATP; □———□, 1.0 mM ATP; ▲———▲, 2.0 mM ATP; ○———○, 5.0 mM ATP; ■———■, 1.0 mM P_i. (b) Effect of ATP concentration on V (○———○) and K_m values (△———△). The values were estimated from the data of (a) by the direct linear plot [15].

Effect of ATP, ADP and P_i on the enzyme activity. When AMP was used as substrate in the absence of NaCl, the substrate-velocity plot was sigmoidal ($n_H = 1.8$) and $s_{0.5}$ was 25 mM. In the presence of 5 mM ATP or ADP, the curves assumed a hyperbolic behavior ($n_H = 1.0$). ATP and ADP at the concentration of 5 mM decrease the $s_{0.5}$ from 25 to 5.5 and 12 mM, respectively. In the presence of 5 mM ATP with 500 mM NaCl, $s_{0.5}$ was 8.0 mM and n_H was 1.0. P_i at a concentration of 1 mM increased $s_{0.5}$ to 40 mM and n_H to 2.4. Neither ATP, ADP nor P_i altered the V values with respect to AMP (Fig. 4).

When IMP or GMP was used as substrate, saturation curves were hyperbolic even in the absence of 500 mM NaCl. Effect of various concentrations of ATP on the kinetic parameters for IMP are shown in Fig. 5. Increasing concentration of ATP decreased K_m and increased V values. In the presence of 5 mM ATP, the K_m for GMP decreased from 2.1 to 0.3 mM and the V value increased 3.4-fold (data not shown). In the presence of 1 mM P_i, the K_m values for IMP and GMP increased 4.8 and 7.9 mM respectively, but no change in V and n_H was observed.

Interaction between substrates. AMP up to the concentration of 50 mM had a stimulatory effect on the hydrolysis of IMP, whereas IMP was inhibitory on the hydrolysis of AMP up to the concentration of 100 mM.

Discussion

The physiological significance of a cytosol 5′-nucleotidase in purine metabolism in mammals has been discussed by some investigators [9,10]. But it has been impossible to obtain its homogeneous preparation from mammalian sources, mainly because of the low content of this enzyme in tissues. We highly purified IMP-hydrolysing activity from rat liver by a method including elution of the enzyme with ATP from phosphocellulose. The preparation obtained appeared homogeneous on the criteria of disc-gel electrophoresis and the specific activity of this preparation was comparable to that of a homogeneous preparation of the cytosol 5′-nucleotidase from chicken liver [5].

General catalytic properties of this preparation, which has markedly high affinities for IMP, dIMP, GMP and dGMP were studied. Some of these properties were proved to be identical with those of crude preparations of the cytosol 5′-nucleotidase previously described for rat liver [7—9].

The pH-activity profile of this preparation was quite different from those of the other 5′-nucleotidases. Double pH optimum, stimulation of activity in the alkaline range by Mg^{2+} and the difference between pH-activity curves with 5′-ribonucleotides and those with 5′-deoxyribonucleotides have been reported for 5′-nucleotidases from various sources [11]. On the contrary, activity of this preparation was stimulated in the acidic range by increasing concentrations of Mg^{2+}. Only one pH optimum at about 6.5 was observed in the presence of 50 mM $MgCl_2$ and 500 mM NaCl for all the nucleotides tested, including 5′-deoxyribonucleotides.

This preparation is clearly distinguishable from the other 5′-nucleotidases [11] by absolute dependence on bivalent metal salts. With IMP or AMP as substrates, a plot of the reaction rate with respect to the concentration of bivalent metal salts was hyperbolic for $MgCl_2$, $CoCl_2$ or $MnCl_2$. V and K_m values

were highest for $MgCl_2$ and lowest for $MnCl_2$. For the optimum assay conditions, inclusion of $MgCl_2$ at a concentration of higher than 25 mM in the reaction mixture seems necessary.

This enzyme was found to be activated by high concentration of various alkali metal salts and Tris salts of some carboxylic acids. This stimulatory effect of these compounds has not been reported for 5'-nucleotidases. Thus, the ionic strength of the reaction mixture and the buffer system used should be taken into account for the estimation of the activity of this 5'-nucleotidase. In almost all the experiments described in this paper, activity of this enzyme was assayed in the presence of 500 mM NaCl.

Activation of the cytosol 5'-nucleotidase by various adenine nucleotides was first reported by Van den Berghe et al. with a crude preparation from rat liver [9], and confirmed with a highly purified chicken liver enzyme [6]. The stimulatory effect of ATP on this preparation from rat liver is similar to those of NaCl and Tris-maleate. When AMP was used as substrate, the substrate-velocity plot was sigmoidal. These three activators decreased the sigmoidicity of the plot, but did not alter the V value. When IMP was used as substrate, the substrate-velocity plot was hyperbolic and these three compounds activated the enzyme by increasing the V and decreasing the K_m value. AMP, a substrate of this enzyme, showed a stimulatory effect on the activity of IMP-hydrolysis, whereas IMP was inhibitory on the hydrolysis of AMP.

Effect of these activators on the enzyme can be interpreted qualitatively by assauming an equilibrium between two states of the enzyme: an active state, which has high affinity for substrates and high catalytic activity and an inactive state, which has low affinity for substrates and low catalytic activity. NaCl, Tris-maleate and adenine nucleotides, including a substrate AMP, shift the equilibrium between these two states to the active state. To establish a more precise model of this enzyme, detailed studies on catalytic and physical properties will be necessary.

Some of the observations presented in this paper provided consistent evidence for the identity of this preparation and the cytosol 5'-nucleotidases, the presence of which had been reported by various authors in crude preparations from rat liver [2,7—9].

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