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5'-NUCLEOTIDASE OF CHICKEN LIVER

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SUMMARY

1. 5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) was partially purified from chicken liver. This is the first time it has been possible to obtain 5'-nucleotidase from the hepatic tissue of uricotelic animals and it was found to be kinetically distinct from 5'-nucleotidases obtained from other sources.

2. 5'-Mononucleotides having a keto group at position 6 in the purine base are the most active substrates of this enzyme. 5'-IMP is the most active substrate among the 5'-nucleotides tested, and it is about 10 times more active than 5'-AMP.

3. This enzyme has an optimum pH at 6.5 and requires divalent metal ions. In the absence of divalent metal ions, the enzyme is almost inactive.

4. Inosine, guanosine, *p*-chloromercuribenzoate (PCMB) and NaF inhibit this enzyme. Of these inhibitors, PCMB was found to be the most potent.

5. The general properties of the enzyme are described, and its possible metabolic function is discussed.

INTRODUCTION

5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) has been found in a wide variety of tissues[1-7].

In 1960, SEGAL AND BRENNER[8] reported the presence of a highly specific 5'-nucleotidase in the microsomal fraction of rat liver. Recently, reports have appeared on the properties of purified 5'-nucleotidases from calf intestinal mucosa[9], bovine pituitary gland[10] and human liver[11].

In uricotelic animals, uric acid is the end product of nitrogen catabolism and 5'-nucleotidase is assumed to participate in the first step of the degradation of 5'-IMP to uric acid. Very little, however, is known about the properties of 5'-nucleotidase in the hepatic tissue of uricotelic animals.

The partial purification and some properties of the enzyme from chicken liver are described in this paper.

Abbreviation: PCMB, *p*-chloromercuribenzoate.

MATERIALS

5′-IMP was obtained from Kyowa Hakko Kogyo Co. 5′-AMP, 5′-GMP, 5′-dGMP, ADP, 2′(3′)-adenylic acid (mixed isomers, free acid) and D-glucose 6-phosphate were purchased from Sigma Chemical Co. 5′-CMP and ATP were obtained from Nutritional Biochemical Corporation. Phenylphosphate and ribose 5-phosphate (barium salt) were purchased from Daiichi Pure Chemicals Co. β-Glycerophosphate was obtained from Koso Chemical Co.

5′-XMP was prepared from 5′-GMP by deamination with nitrous acid as described by KLEINZELLER[12]. It was precipitated from the reaction mixture as the barium salt, dissolved in dilute HCl, and Ba^{2+} was removed with Na_2SO_4. The crude 5′-XMP was purified chromatographically on Dowex 50 (ref. 13) and finally precipitated as the barium salt. 2′(3′)-IMP (mixed isomers) was also prepared from 2′(3′)-adenylic acid (mixed isomers) by deamination with nitrous acid, and purified by chromatography on Dowex-50.

Where not otherwise specified, chemicals were obtained as the sodium salts. Barium salts were converted to sodium salts with Na_2SO_4.

DEAE-cellulose, a product of Brown Co., was washed thoroughly with 1 M NaOH, 0.01 M HCl and distilled water before use.

All materials not specified were commercial products of reagent grade.

METHODS

Enzyme assay

The 5′-nucleotidase was assayed routinely in the following incubation mixture: 100 μmoles of Tris–maleate–NaOH buffer (pH 6.5), 10 μmoles of $MgCl_2$, 3 μmoles of 5′-IMP and enzyme preparation in a total volume of 1.0 ml.

The reaction mixture for the assay of acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) and alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) contained, in 1 ml, 100 μmoles of acetate buffer (pH 5.5) (for acid phosphatase assay) or 100 μmoles of glycine–NaOH buffer (pH 9.0) (for the alkaline phosphatase assay), 10 μmoles of $MgCl_2$, 3 μmoles of disodium phenylphosphate and enzyme.

The incubation was carried out at 37° for 7–10 min and the reaction was terminated by the addition of trichloroacetic acid to a final concentration of 5%. The precipitated protein was removed by centrifugation, and an aliquot of the supernatant fluid was used for P_i determination by the method of FISKE AND SUBBA-ROW[14]. A unit of activity corresponds to the liberation of 1 μmole of P_i per h and specific activity was defined as units per mg of protein.

Protein was determined according to the method of LOWRY *et al.*[15]. Crystalline bovine serum albumin was used as a standard.

Chromatography of proteins was carried out on DEAE-cellulose, which was packed into a column after alkali regeneration, washed thoroughly with water and equilibrated with an appropriate buffer at 4° before use. After application of the protein solution to the column, stepwise elution was carried out. Fractions were collected automatically in the cold.

Analysis of the products of 5′-nucleotidase action

The deproteinized reaction mixture was subjected to the determination of P_i and chromatographic analysis. An aliquot (0.2 ml) of the solution was streaked on Whatman No. 1 paper and chromatographed by the ascending method in *n*-butanol–acetic acid–water (12:3:5, by vol.)[16] for about 15 h. The regions corresponding to 5′-IMP and inosine were eluted with 0.01 M HCl and 0.001 M HCl, respectively. The eluates were identified as 5′-IMP and inosine chromatographically and spectrophotometrically. The concentrations of 5′-IMP and inosine were calculated by using 11.7 at 249 mμ for 5′-IMP (ref. 17) and 12.2 at 248 mμ for inosine[18] as the molecular extinction coefficients.

RESULTS

Purification of the enzyme

Preparation of chicken-liver acetone powder. Fresh chicken livers were obtained from the local meat market. They were minced coarsely, and then blended with 10 vol. of cold acetone (−20°) for 1 min in a Waring blendor. 30 min later the suspension was filtered through a buchner funnel. The filter cake was washed with cold acetone (−20°) until the filtrate was almost colorless. The washed filter cake was crumbled and spread out to allow the evaporation of all traces of the solvents. The powder was stored in an evacuated desiccator over silica gel at 4°.

Extraction. 100 g of acetone powder were homogenized with 1 l of 0.1 M Tris–HCl buffer (pH 7.5) for 2 min in a Potter homogenizer and stirred for about 30 min. The extract was centrifuged at 17 000 × *g* for 10 min and the supernatant fluid was collected.

n-Butanol treatment and acetone fractionation. 115 ml of *n*-butanol were added gradually to 775 ml of the supernatant fluid, while stirring vigorously. After being stirred for about 90 min the dispersion was centrifuged at 17 000 × *g* for 10 min. The slightly turbid aqueous layer (700 ml) was collected, and the excess butanol and the floating precipitate were discarded. 318 ml of cold acetone (−20°) were added gradually, while stirring, to 700 ml of the aqueous layer. The temperature was kept below 0° during this process. The suspension was allowed to stand for 30 min and centrifuged at 9000 × *g* for 10 min. The precipitate was extracted with 200 ml of 0.1 M Tris–HCl buffer (pH 7.5). The undissolved protein was removed by centrifugation at 77 500 × *g* for 30 min and the supernatant fluid was collected.

$(NH_4)_2SO_4$ fractionation. 59 g of solid $(NH_4)_2SO_4$ were added, while stirring, to 195 ml of the supernatant fluid. 30 min later the precipitate was collected by centrifugation at 9000 × *g* for 10 min. The precipitate was dissolved in 30 ml of 0.05 M Tris–HCl buffer (pH 7.5).

Precipitation at pH 5.6. About 30 ml of $(NH_4)_2SO_4$ fraction were dialyzed against 2 l of 0.05 M Tris–HCl buffer (pH 7.5) overnight. Any precipitate formed during dialysis was removed by centrifugation at 105 000 × *g* for 30 min. The pH of the dialyzed solution (44 ml) was adjusted to 5.6 with 2.0 M acetate buffer (pH 5.0) and allowed to stand for 20 min at 4°. The precipitate formed was collected by centrifugation at 9000 × *g* for 10 min and suspended in 40 ml of 0.1 M Tris–HCl buffer (pH 7.5). After being stirred occasionally for 30 min, the undissolved precipitate was removed by centrifugation at 105 000 × *g* for 30 min and discarded.

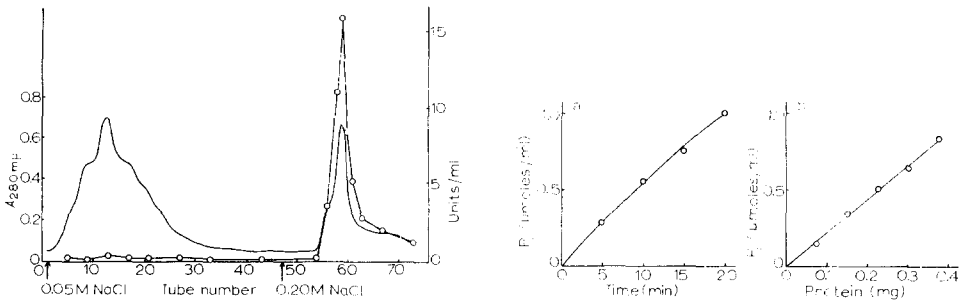

Fig. 1. Purification of chicken-liver 5′-nucleotidase by DEAE-cellulose column chromatography. A sample of 39 ml containing 140 mg of protein was applied to a DEAE-cellulose column (7.1 $cm^2 \times$ 30 cm). Fraction volume, 15 ml; flow rate, 2.5 ml/min. Stepwise elution with Tris–HCl buffer (pH 7.5) containing NaCl of indicated molarity. — —, protein concentration expressed as $A_{220\ m\mu}$; ○ —○, activity of 5′-nucleotidase (units/ml).

Fig. 2. (a) Time course of P_i liberation from 5′-IMP. (b) Effect of enzyme concentration on the reaction rate. (a) The reaction mixture (1 ml) contained 100 μmoles of Tris–maleate–NaOH buffer (pH 6.5), 10 μmoles of $MgCl_2$, 3 μmoles of 5′-IMP and enzyme preparation. (b) The reaction mixture (1 ml) contained 100 μmoles of Tris–maleate–NaOH buffer (pH 6.5), 10 μmoles of $MgCl_2$, 3 μmoles of 5′-IMP and enzyme preparation. Incubation was for 7 min at 0°.

DEAE-cellulose column chromatography. The supernatant fluid was applied to the DEAE-cellulose column (7.1 $cm^2 \times$ 30 cm) which had previously been equilibrated with 0.1 M Tris–HCl buffer (pH 7.5). Elution was carried out at a rate of 2.5 ml/min with the same buffer with stepwise increase in concentration of NaCl from 0.05 to 0.20 M. The elution pattern is shown in Fig. 1. The activity of 5′-nucleotidase was found in the fraction of 0.1 M Tris–HCl buffer containing 0.2 M NaCl.

Concentration. The combined Fractions 56–70 (about 200 ml) were diluted

TABLE I

PURIFICATION OF CHICKEN-LIVER 5′-NUCLEOTIDASE

5′-Nucleotidase activity was determined with 5′-IMP at pH 6.5. A unit of activity corresponds to the liberation of 1 μmole of P_i per h. Activities of acid and alkaline phosphatase were determined with phenylphosphate at pH 5.5 and pH 9.0, respectively. Activities were assayed under conditions described in METHODS.

Fraction	*Total volume (ml)*	*Protein (mg/ml)*	*5′-Nucleotidase activity (units/ml)*	*Specific activity (units/mg protein)*	*Total units*	*Ratio of activity*	
						Acid phosphatase:5′-nucleotidase	*Alkaline phosphatase:5′-nucleotidase*
Crude extract	775	48.4	35	0.7	27 000	1.35	0.52
n-Butanol treatment and acetone fractionation	195	13.6	28	2.0	5400	0.39	0.60
$(NH_4)_2SO_4$ fractionation	44	18.2	102	5.6	4500	0.16	0.50
pH 5.6 precipitation	39	3.6	64	17.8	2500	0.01	0.03
DEAE-cellulose column	19	1.6	44	27.2	830	0.01	0.03

5 times with cold distilled water and applied to the DEAE-cellulose column (3.1 cm^2 × 7 cm) for concentration. Elution was carried out with 0.5 M NaCl in 0.1 M Tris–HCl buffer (pH 7.5) and 19 ml of effluent which had high activity were collected.

All procedures, unless otherwise indicated, were carried out at 0–4°. The purification procedure is summarized in Table I.

In the crude preparation from chicken-liver acetone powder, the presence of 5′-nucleotidase was completely obscured by non-specific phosphatases. The greatest effort was made to separate 5′-nucleotidase from other phosphatases in this purification experiment.

Decrease in the ratio of acid phosphatase activity to 5′-nucleotidase activity and of alkaline phosphatase activity to 5′-nucleotidase activity were taken as a criterion of purification. Non-specific phosphatases (acid and alkaline) were almost completely removed by the chromatography on DEAE-cellulose.

Properties of the enzyme

Analysis of the product of 5′-nucleotidase reaction and stoichiometry. Table II shows that the decrease in 5′-IMP was accompanied by an equivalent increase in inosine and P_i after 60 min and 120 min of reaction. The close agreement among

TABLE II

STOICHIOMETRY

The reaction mixture (1 ml) contained 100 μmoles of Tris–acetate buffer (pH 6.5), 10 μmoles of $MgCl_2$, 5.6 μmoles of 5′-IMP and enzyme preparation. Assays of 5′-IMP, inosine and P_i are described in METHODS.

Incubation time (min)	*5′-IMP (μmoles)*	*Net change of 5′-IMP (μmoles)*	*Inosine (μmoles)*	*P_i (μmoles)*
0	5.6	—	—	—
60	4.1	−1.5	1.4	1.5
120	3.4	−2.2	2.1	2.2

the values in each interval of the reaction suggests that the hydrolytic cleavage of 5′-IMP to inosine and P_i proceeds stoichiometrically with this enzyme preparation.

Time course. Fig. 2a shows that the rate of P_i liberation from 5′-IMP decreases with time. This might be due to an inhibition of the reaction by the reaction product, inosine, as shown in Table V.

Effect of enzyme concentration. Fig. 2b shows that the rate of hydrolysis of 5′-IMP to inosine is directly proportional to the amount of enzyme protein, up to 0.375 mg.

Effect of pH. The effect of pH on enzyme activities in Tris–maleate–NaOH buffer with three different substrates, 5′-IMP, 5′-GMP and 5′-AMP, was tested (Fig. 3). The pH optimum was found to be 6.5, and it was the same for the three substrates. At pH 8.0 there was about 35% as much activity as at pH 6.5. At pH 5.5 almost no activity was observed.

Substrate specificity. The relative activities of the enzyme towards a number of phosphorylated compounds are shown in Table III. The enzyme showed high speci-

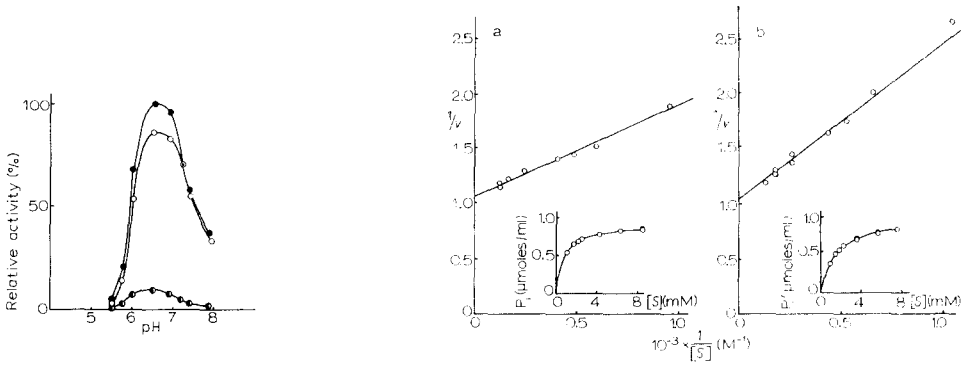

Fig. 3. Effect of pH on the activity of 5′-nucleotidase. The reaction mixture (1 ml) contained 3 μmoles of substrate (5′-IMP, 5′-GMP or 5′-AMP), 10 μmoles of $MgCl_2$, 150 μmoles of Tris–maleate–NaOH buffer (pH 6.5) and enzyme preparation. Incubation was for 10 min at 00°. ●—●, 5′-IMP; ○ ○, 5′-GMP; ◐—◐, 5′-AMP.

Fig. 4. Effect of substrate concentration on reaction velocity (LINEWEAVER–BURK plot). The reaction mixture (1 ml) contained 100 μmoles of Tris–maleate–NaOH buffer (pH 6.5), 10 μmoles of $MgCl_2$, various amounts of 5′-IMP (a) or 5′-GMP (b) as indicated and enzyme preparation. Incubation was for 10 min at 00°.

ficity for 5′-nucleotides. Phosphorylated compounds other than 5′-nucleotide, however, were also hydrolyzed to a slight extent. This appears to be due to the slight contamination by non-specific phosphatases, since non-specific phosphatases could be removed progressively in the process of purification. Among the nucleotides,

TABLE III

SUBSTRATE SPECIFICITY OF CHICKEN-LIVER 5′-NUCLEOTIDASE

The reaction mixture (1 ml) contained 100 μmoles of Tris–maleate–NaOH buffer (pH 6.5), 10 μmoles of $MgCl_2$, 3 μmoles of substrate and enzyme preparation, and was incubated at 37° for 10 min.

Substrate	*Relative activity (%)*
5′-IMP	100
5′-GMP	87
5′-dGMP	64
5′-XMP	31
5′-AMP	9
5′-UMP	11
5′-CMP	6
2′(3′)-IMP*	1
2′(3′)-AMP*	1
ATP	5
ADP	5
Ribose 5-phosphate	2
Glucose 6-phosphate	1
β-Glycerophosphate	1
Phenylphosphate	1

* Mixed isomers.

TABLE IV

EFFECT OF DIVALENT METAL CATIONS ON THE ACTIVITY OF CHICKEN-LIVER 5′-NUCLEOTIDASE

The reaction mixture (1 ml) contained 100 μmoles of Tris–maleate–NaOH buffer (pH 6.5), 3 μmoles of 5′-IMP, enzyme preparation and metal salts (chlorides) as indicated. The reaction mixture was incubated at 37° for 10 min.

Addition	*Final concn. (mM)*	*Relative activity (%)*
Mg^{2+}	10	100
	1	33
Co^{2+}	10	65
	1	53
Mn^{2+}	10	24
	1	21
Ni^{2+}	10	26
	1	16
Ca^{2+}	10	1
	1	1
None	—	1

5′-IMP was by far the best substrate. It was degraded 10 times more rapidly than 5′-AMP. Pyrimidine 5′-nucleotides were less satisfactory substrates.

Effect of substrate concentration. The results of studies on the effect of substrate concentration on reaction velocity were plotted according to LINEWEAVER AND BURK[19], and the apparent K_m values of 0.8 and 1.3 mM were calculated for 5′-IMP and 5′-GMP, respectively (Fig. 4).

Cation requirement. The effect of divalent ions on enzyme activity was tested at 10 and 1 mM. Mg^{2+} was the most effective of several divalent metal ions tested

TABLE V

INHIBITION OF CHICKEN-LIVER 5′-NUCLEOTIDASE ACTIVITY

The reaction mixture (1 ml) contained 100 μmoles of Tris–maleate–NaOH buffer (pH 6.5), 10 μmoles of $MgCl_2$, 3 μmoles of 5′-IMP, enzyme preparation and compounds tested as indicated, and was incubated at 37° for 10 min.

Addition	*Final concn. (mM)*	*Relative activity (%)*
None	—	100
Inosine	3	50
Guanosine	3	72
Adenosine	3	92
Hypoxanthine	3	99
2′(3′)-IMP*	3	112
2′(3′)-AMP*	3	103
NaF	1	46
PCMB	0.01	28
Monoiodoacetic acid	10	98
$HgCl_2$	0.1	12

* Mixed isomers.

(Table IV). 10 mM Co^{2+} sustained a rate 65% of that found with Mg^{2+} at the same concentration. Mn^{2+} and Ni^{2+}, although active, gave a rate only 24 and 26%, respectively, of that obtained with Mg^{2+}. Ca^{2+} had no effect on enzyme activity. The enzyme was almost inactive in the absence of divalent metal ions.

Inhibition of the enzyme activity. Table V summarizes the effect of a number of inhibitors on the 5'-nucleotidase activity. In all experiments, 5'-IMP was used as the substrate and substances tested were added at the concentration indicated in Table V. The reaction was started by addition of the enzyme. Appreciable inhibition was observed when inosine or guanosine was added, as illustrated in Table V, whereas adenosine, hypoxanthine and 2'(3')-nucleotides (mixed isomers) had almost no effect. NaF, commonly used to inhibit phosphatases, was a potent inhibitor.

The marked inhibition by *p*-chloromercuribenzoate (PCMB) could be partially reversed by the addition of cysteine, but when cysteine alone was added the rate was not affected. These observations suggest that the enzyme contains an essential sulfhydryl group. On the other hand, another sulfhydryl reagent, iodoacetate, did not inhibit the reaction.

DISCUSSION

5'-Nucleotidase was partially purified from chicken liver. This is the first time that 5'-nucleotidase has been obtained from the liver of uricotelic animals. Some of the properties of this enzyme were found to be quite different from those of the 5'-nucleotidases obtained from other higher animals. The optimum pH for the 5'-nucleotidase from chicken liver lies close to 6.5 in Tris–maleate–NaOH buffer, when 5'-IMP, 5'-GMP and 5'-AMP are used as substrates. Calf intestinal 5'-nucleotidase has been reported to have an optimum pH between 6.0 and 6.5 (ref. 9). With respect to pH optimum, this enzyme is similar to the 5'-nucleotidase from chicken liver. The optimum pH of the enzyme obtained from rat-liver microsomes[8], bull seminal plasma[1], snake venom[2], the bovine pituitary gland[10] and human liver[11] is different from that of the chicken-liver enzyme.

The substrate specificity of the 5'-nucleotidase from chicken liver is quite different from that of the 5'-nucleotidase from other sources[1–11]. 5'-IMP, 5'-GMP and 5'-XMP are hydrolyzed at a faster rate than other 5'-nucleotides tested, and the rate of hydrolysis of 5'-AMP, 5'-UMP and 5'-CMP is only 5–10% of that attained when 5'-IMP was used as the substrate. This would suggest that the 5'-mononucleotides having a keto group in position 6 of the purine base are the most active substrates for the enzyme. Whether the 5'-nucleotidase from chicken liver is absolutely specific for the purine 5'-mononucleotides mentioned above remains unclear. Further purification of this enzyme preparation will perhaps eliminate its activities with 5'-AMP, 5'-CMP and 5'-UMP.

5'-Nucleotidase from chicken liver shows no activity in the absence of divalent metal ions. This is one of the interesting properties of this enzyme as compared with 5'-nucleotidases obtained from other animal sources. SEGAL AND BRENNER[8] reported that 5'-nucleotidase from rat-liver microsomes required no added Mg^{2+} for activity. Most 5'-nucleotidases from other animal sources have been reported to be active without the addition of divalent metal ions and activated moderately by the addition of such ions[1,2,9,11].

Among the nucleosides tested, inosine is the most potent inhibitor of the 5′-nucleotidase from chicken liver when 5′-IMP is used as the substrate. It inhibits the activity 50% when used at the same concentration as the substrate. Adenosine was reported to be the most potent inhibitor of 5′-nucleotidase from calf intestine[9] and bovine pituitary gland[10]. However, no significant inhibition of the chicken-liver enzyme was observed.

It is of interest to note that the 5′-nucleotidase from the liver of uricotelic animals hydrolyses 5′-IMP and 5′-GMP more rapidly than other nucleotides. This suggests that this enzyme may participate significantly in the reaction of 5′-IMP to inosine in the metabolic pathway of uric acid production in the hepatic tissue of chicken, a uricotelic animal. One cannot exclude the possibility, however, that this enzyme may participate in the dephosphorylation of 5′-mononucleotides in general.

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