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Properties of Choline and Ethanolamine Kinases Paritally Purified from Rat Liver Supernatant

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Introduction

In the pioneering study of choline kinase (EC 2.7.1.32) by Wittenberg and Kornberg[1], it has been demonstrated that purified enzyme preparation from yeast and acetone powders of animal tissues catalyze the phosphorylation of choline and its structural analogues in the presence of ATP. Schneider *et al.*[2] have also described a method for the preparation of labeled phosphorylcholine and phosphorylethanolamine by incubating labeled choline and ethanolamine with rat liver supernatant fraction and ATP.

Some investigators suggesed the occurrence of two enzymes which catalyze the phosphorylation of choline and ethanolamine but no one succeeded in separation of these enzymes.

In the present paper, partial purification and some properties of choline and ethanolamine kinases were investigated.

Materials and Methods

Chemicals

Methyl-^{14}C-choline chloride and ethan-1-ol-2-amine-2-^{14}C hydrochloride were purchased from The Radiochemical Centre Amersham, Bucks., U.K. ATP disodium salt tris (hydroxy methyl) aminomethane, phosphorylcholine and phosphorylethanolamine were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. 3-Phosphoglycerate was a product of C.F. Boehringer und Soehne G.m.b.H., Mannheim Germany. Choline, ethanolamine, monomethylethanolamine and dimethylethanolamine were obtained from Tokyo Kasei Co., Tokyo, Japan. Sepharose 6B and Sephadex G-200 were purchased from Pharmacia Ltd., Uppsala, Sweden.

Preparation and partial purification of enzyme

Overnight fasted Wistar rats weighing rats 180–250 g were decapitated and livers were perfused with cold 0.9% NaCl solution and homogenized with 9 volumes of 0.25 M sucrose-1 mM EDTA. The homogenate was centrifuged at 700×g for 15 min to remove cell debris and nuclei. Mitochondrial fraction was precipitated at 7,500×g for 10 min, washed once and suspended in 0.25 M sucrose. The supernatant was recentrifuged at 105,000×g for 60 min. The precipitate was defined as microsomal fraction and the supernatant fluid thus obtained was defined as 'supernatant'.

Ammonium sulfate fractionation of the 'supernatant'

The fractionation was carried out at 4°C by adding saturated ammonium sulfate

solution to the 'supernatant'. The precipitates were dissolved in 0.02 M Tris-HCl buffer pH 7.4 and dialyzed overnight against the same buffer. The acid treatment was performed in the manner in which the ammonium sulfate fraction of 25–50% saturation was dialyzed against 0.02 M Tris-HCl pH 3 for about 30 min. Then dialysis was continued overnight against 0.02 M Tris-HCl pH 7.4. The precipitated protein was discarded by centrifugation and clear supernatant was used as enzyme preparation.

Sepharose 6B column chromatography

A 2 mℓ portion of the acid treated supernatant was applied to a Sepharose 6B column (2.5×42 cm) and eluted by ascending chromatography with 0.02 M Tris-HCl buffer pH 8.0. Fractions of 3 mℓ were collected with the aid of a fraction collector.

Assay of choline and ethanolamine kinases

Incubations were carried out at 37°C for 30 min in a shaking water bath according to the method of Schneider *et al.*[2] The reaction mixture contained, unless otherwise stated, 1.2 mM of methyl-^{14}C-choline or 0.6 mM of 2-^{14}C-ethanolamine (1 μCi/μmole), 2.3 mM of ATP, 12 mM of magnesium chloride, 16 mM of 3-phosphoglycerate, and 20 mM of Tris-succinate buffer pH 8.0. 0.2–1 mg of enzyme protein was used per 0.25 mℓ of incubation medium.

The reactions were terminated by heating for 5 min at 100°C. After cooling and centrifuging, aliquots of the supernatant fluids were applied to Toyo No. 51A paper and chromatographed with following two solvent systems. One was butanol-acetic acid-water (4 : 4 : 1, by vol.). The Rf values of 0.85, 0.75, 0.63 and 0.44 were obtained for choline, ethanolamine, phosphorylcholine and phosphorylethanolamine, respectively. The other solvent was ethanol-conc. ammonia-water (61 : 29 : 10, by vol.). The Rf values of choline, ethanolamine, phosphorylcholine and phosphorylethanolamine were 0.76, 0.71, 0.30 and 0.19, respectively.

Enzyme activity was calculated from the percentage of the radioactivity of the product, phosphorylcholine or phosphorylethanolamine, estimated by Packard Model 7201 Radiochromatogram Scanner and expressed as m unit (n moles of choline or ethanolamine phosphorylated per mg protein per min). Protein was estimated by biuret method[3] or ultra violet method[4].

Results and Discussion

Intracellular localization of choline and ethanolamine kinases were shown in Table 1. Both kinase activities were mainly concentrated in 105,000×g supernatant.

Table 1 *Intracellular localization of choline and ethanolamine kinases in rat liver*

Subcellular fraction	Specific activity of	
	Choline kinase (m unit)	Ethanolamine kinase (m unit)
Homogenate	0.23	0.08
Mitochondria	trace	trace
Microsomes	trace	trace
'Supernatant'	1.68	0.60

Table 2 *Ammonium sulfate fractionation of choline and ethanolamine kinases*

Ammonium sulfate fraction	Choline kinase			Ethanolamine kinase		
	Specific activity (m unit)	Relative specific activity	Recovery of activity (%)	Specific activity (m unit)	Relative specific activity	Recovery of activity (%)
'Supernatant'	1.49	1	100	0.66	1	100
0–25% saturation	0.13	0.09	4.3	—	—	—
25–40% saturation	3.01	2.02	22.6	0.62	0.80	12.1
40–50% saturation	2.38	1.60	23.4	0.35	0.53	11.6
50% supernatant	0.24	0.16	7.0	0.03	0.04	3.6
25–50% saturation, acid treated	3.97	2.66	38.2	1.21	1.83	19.1

The specific activities increased up to 7.5 times of that of homogenate by ultracentrifugation. The 'supernatant' was further fractionated with ammonium sulfate. As shown in Table 2, most of the activity was precipitated between 25–50% saturation, though the recovery of the activity was incomplete. The acid treatment of the fraction precipitated by 25–50% saturation raised the specific activity by 2 to 3 fold. Choline kinase was purified approx. 20 fold and ethanolamine kinase was approx. 14 fold by these procedures described above.

Supernatant fraction of rat liver has been known to catalyze the syntheses of phosphorylcholine, phosphorylethanolamine, CDP-choline and CDP-ethanolamine. As regards cytidyltransferases (EC 2.7.7.15 and EC 2.7.7.14), two enzymes catalyzing phosphorylcholine and phosphorylethanolamine transfer were separated by Sephadex G–200 column chromatography[5] but choline and ethanolamine kinases were not yet separated. In the present experiments, both Sephadex G–200 and Sepharose 6B column chromatography failed to separate two enzymes as shown in Fig. 1. The increament of the specific activities was only 1.5 times. The overall purification resulted in 30 fold increase in the specific activity of choline kinase and 20 fold increase in ethanolamine kinase activity. Similar results have been obtained by other investigators[1,6] using different procedures.

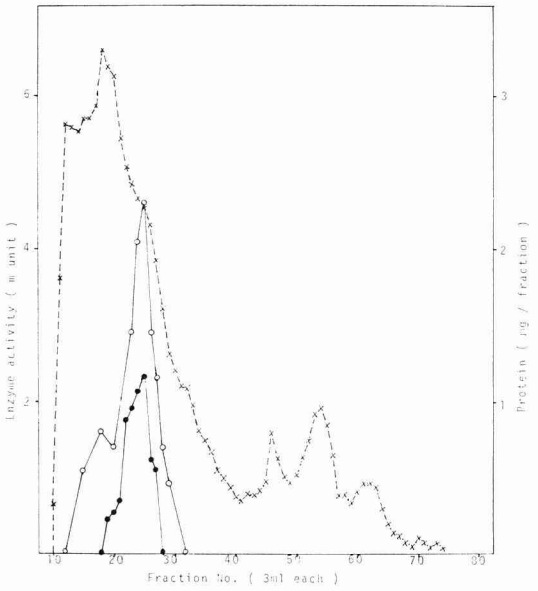

Fig. 1 Fractionation of choline and ethanolamine kinases by Sepharose 6B.
Details in the text
(×) amount of protein (mg/fraction)
(○) specific activity of choline kinase (m unit)
(●) specific activity of ethanolamine kinase (m unit)

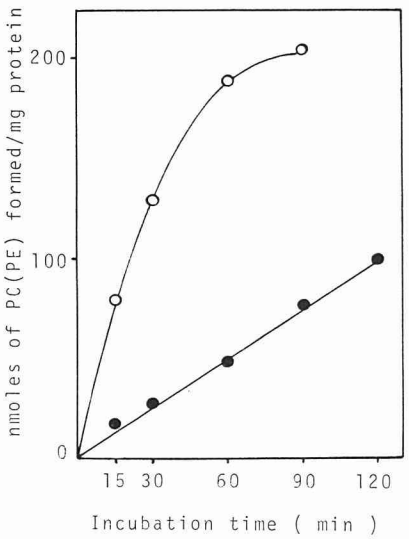

Fig. 2 Time courses of choline and ethanolamine kinase activities.
The incubation mixture contained 20 mM each of Tris and succinate pH 8.0, 12 mM of $MgCl_2$, 2.4 mM of ATP, 16 mM of 3-phos phoglycerate, 1.2 mM of Me-^{14}C-choline or 0.6 mM of 2-^{14}C-ethanolamine and 0.72 mg of enzyme protein in a final volume of 0.25 mℓ.
(○) choline kinase
(●) ethanolamine kinase
PC: phosphorylcholine
PE: phosphorylethanolamine

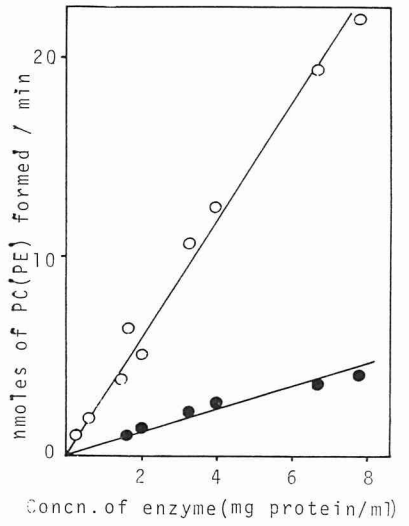

Fig. 3 Enzyme concentration-activity curves of choline and ethanolamine kinases.
The composition of the incubation mixture is present in Fig. 2 Incubation period; 30 min.

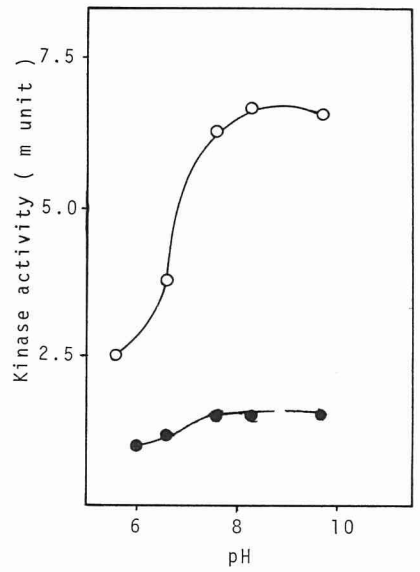

Fig. 4 PH-activity curves of choline and ethanolamine kinases.
The compositon of the incubation mixture is present in Fig. 2 with the exception of varing pH of the buffer. Incubation period; 30 min.

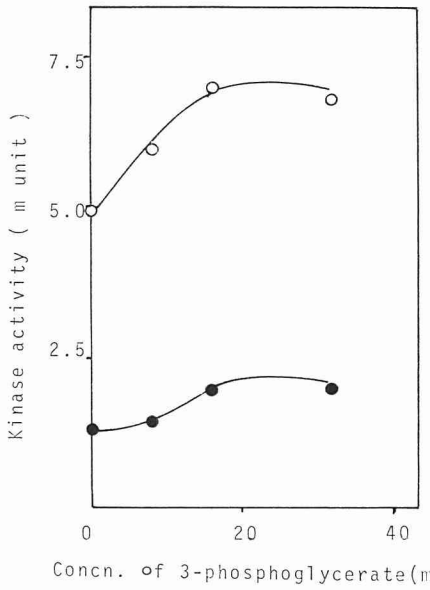

Fig. 5 Effec tof 3-phosphoglycerate on choline and ethaonlamine kinase activities.
The incubation conditions were the same as in Fig. 2 except that the concentrations of 3-phosphoglycerate were varied. Incubation period; 30 min.

Time courses and the influence of protein concentration on choline and ethanolamine kinase activities were shown in Figs. 2 and 3. Phosphorylation of ethanolamine was slow but linearly increased during 120 min of incubation period.

Both choline and ethanolamine were preferably phosphorylated at alkaline pH ranging 8–10 (Fig. 4). Similar alkaline pH optima were reported by other workers[1,6,7] for choline kinase activity. As shown in Fig. 5, stimulant effect of added 3-phosphoglycerate on choline and ethanolamine phosphorylation was observed. Maximal stimulation up to 145% was obtained by the addition of 16 mM of 3-phosphoglycerate.

The effect of ATP concentrations on the rate of phosphorylation was shown in Figs. 6 and 7. Choline kinase activity was markedly inhibited at higher concentrations

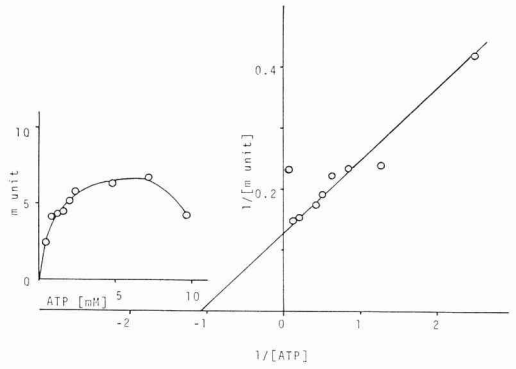

Fig. 6 ATP saturation curve for choline kinase.
The reaction mixture contained: 20 mM-Tris-succinate buffer, pH 8, 12 mM-$MgCl_2$, 1 mM-Me-^{14}C-choline, 1.5 mg-enzyme protein/mℓ, and ATP as stated. Incubation period; 15 min.

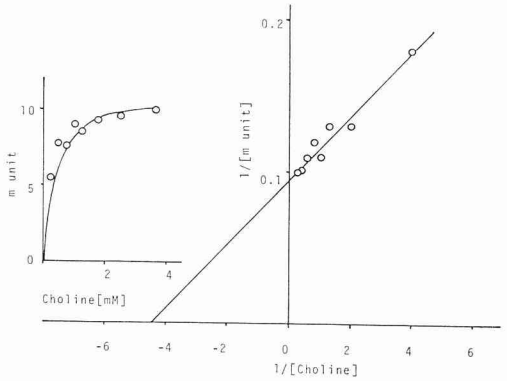

Fig. 8 Choline saturation curve for choline kinase.
The reaction mixture contained: 20 mM-Tris-succinate, pH 8, 12 mM-$MgCl_2$, 16 mM-3-phosphoglycerate, 2.4 mM-ATP, 1.5 mg-enzyme protein/mℓ and choline chloride as stated. Incubation period; 15 min.

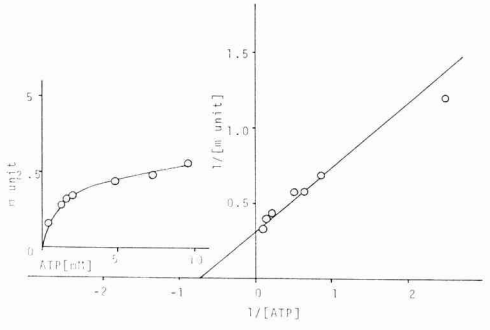

Fig. 7 ATP saturation curve for ethanolamine kinase.
The reaction mixture contained: 20 mM-Tris-succinate, pH 8, 12 mM-$MgCl_2$, 1.1 mM-2-^{14}C-ethanolamine, 1.5 mg-enzyme protein/mℓ, and ATP as stated. Incubation period; 15 min.

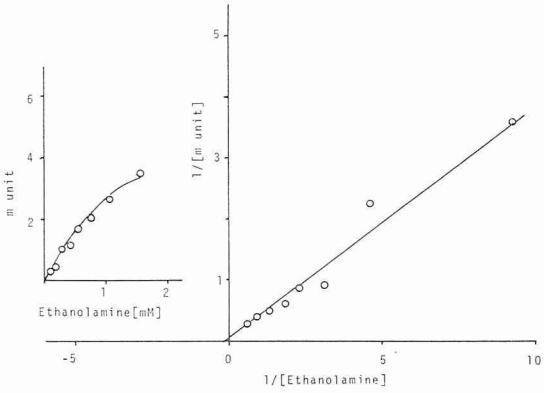

Fig. 9 Ethanolamine saturation curve for ethanolamine kinase.
The incubation conditions were the same as in Fig. 8 except that ethanolamine was used instead of choline.

of ATP but ethanolamine kinase was not inhibited. Apparent Michaelis constants (Km) for ATP estimated from the double reciprocal plots were 0.9 mM for choline kinase and 1.4 mM for ethanolamine kinase.

The influences of concentration of choline and ethanolamine were shown in Figs. 8 and 9. Some kinetic parameters were summerized in Table 3. According to the data obtained by Wittenberg and Kornberg[1], Km values for choline and ethanolamine were 0.02 mM and 10 mM, respectively. However, the maximal rates with two substrates differed only by a factor of 4. In the present data, Km value for choline was 0.23 mM and for ethanolamine was 5.0 mM but maximal velocities (V_{max}) were not so significantly different between choline and ethanolamine kinase. The lower value of V_{max} obtained in the case of ethanolamine kinase, listed in Table 3, seems to be owing to the lower concentration of ethanolamine added in the incubation medium.

Table 3 *Kinetic parameters of choline and ethanolamine kinases*

Substrate	Choline Kinase		Ethanolamine Kinase	
	Km (mM)	V_{max} (m unit)	Km (mM)	V_{max} (m unit)
ATP	0.9	8.0	1.4	3.3
Choline or ethanolamine	0.23 (0.22)	10.5 (3.3)	5.0 (2.2)	10.0 (2.6)

Figures in parentheses represent values obtained from the enzyme preparation of rat brain. Experimental conditions were present in Figs. 6–9.

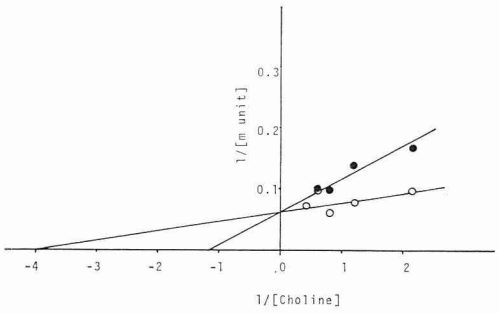

Fig. 10 a) Double-reciprocal plots of choline and ethanolamine kinases against various choline and ethanolamine concentrations.
Effect of added ethanolamine (80 mM) on choline kinase activity.
The reaction mixture contained : 20 mM-Tris-succinate, pH 8.0, 12 mM-$MgCl_2$, 4 mM-ATP, 16 mM-3-phosphoglycerate, 2 mg-enzyme protein/mℓ, with or without 80 mM-ethanolamine and Me-^{14}C-choline as stated. Incubation period ; 15 min. (○) without inhibitor (●) with ethanolamine.

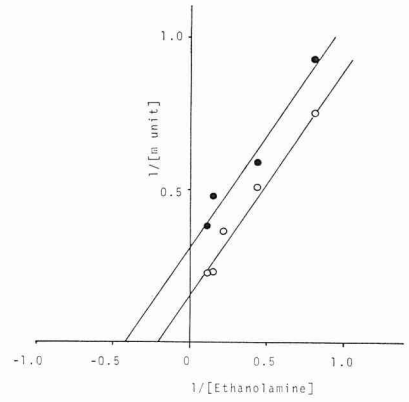

Fig. 10 b) Double-reciprocal plots of choline and ethanolamine kinases against various choline and ethanolamine concetrantions.
Effect of added choline (0.4 mM) on ethanolamine kinase activity.
The reaction mixture contained : 20 mM-Tris-succinate, pH 8.0, 24 mM-$MgCl_2$, 8 mM-ATP, 16 mM-3-phosphoglycerate, 3.9 mg-enzyme protein/mℓ, with or without 0.4 mM-choline and 2-^{14}C-ethanolamine as stated. Incubation period ; 60 imn. ○ without inhibitor (●) with cholide.

The addition of the structural analogues of choline and ethanolamine somewhat affected the phosphorylation rates (Figs. 10, 11). Choline kinase was scarcely inhibited by the addition of ethanolamine even in higher concentrations. 200 mM of ethanolamine at most reduced the choline phosphorylation by 60%. In contrast, the addition of choline significantly inhibited the ethanolamine kinase at lower concentrations. Double reciprocal plots, represented in Fig. 10. revealed that ethanolamine (80 mM) competitively inhibited choline kinase activity (apparent Ki=40 mM), while ethanolamine kinase activty was affected by choline (0.4 mM) in a manner of uncompetitive inhibition (apparent Ki=0.33 mM). Among the structural analogues, dimethylethanolamine exhibited the greatest inhibition both on choline and ethanolamine kinases (Fig. 11). As for the product inhibition, phosphorylcholine and phosphorylethanolamine inhibited the choline kinase activity but ethanolamine kinase activiy was, by contraries, stimulated by phosphorylethanolamine up to 125%. The mechanism of the stimulation remains to be unknown. The stimulating effect was disappeared in the presence of CTP (4 mM). In the latter case, a certain amount of phosphorylethanolamine would be converted into CDP-ethanolamine. However, the convertion of phosphorylethanolamine into CDP-ethanolamine could not explaine the disappearance of the stimulation of ethanolamine kinase activity because the incubation medium contained too much phosphorylethanolamine to be completely converted.

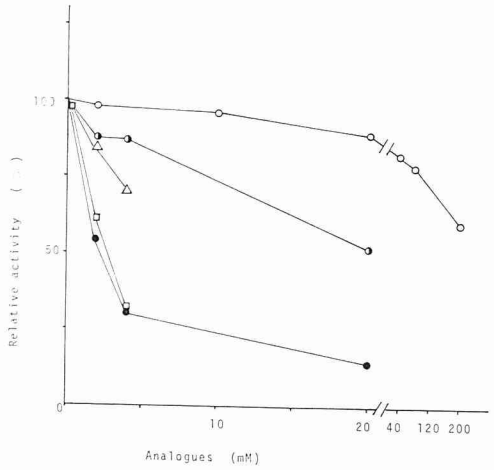

Fig. 11 a) Effect of structural analogues on choline and ethanolamine kinase activities. Inhibition of choline kinase activity. The reaction mixture contained : 20 mM-Tris-succinate, pH 8, 12 mM-$MgCl_2$, 4 mM-ATP, 16 mM-3-phosphoglycerate, 1.2 mM-Me-^{14}C-choline, 2 mg-enzyme protein/mℓ and a structural analogue as stated. Incubation period ; 30 min. Control value without inhibitor was 3.64 m unit. (○) ethanolamine, (◑) monmethylethanolamine, (●) dimethylethanolamine, (△) phosphorylethamolamine, (□) phosphorylcholine.

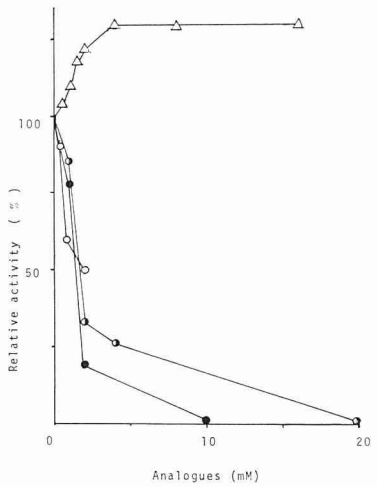

Fig. 11 b) Effect of structural analogues on choline and ethanolamine kinase activites. Inhibition of ethanolamine kinase activity. The reaction mixture contained : 20 mM-Tris-succinate, pH 8, 24 mM-$MgCl_2$, 8 mM-ATP, 16 mM-3-phosphoglycerate, 4.2 mM-2-^{14}C-ethanolamine, 3.9 mg-enzyme protein/mℓ, and a structural analogue as stated. Incubation period ; 90 min. Control value without inhibitor was 2.49 m unit. (○) choline, (◐) monomethylethanolamine, (●) dimethylethanolamine, (△) phosphorylethanolamin.

Ramasarma and Wetter[8] reported that choline kinase from Polish rapeseed exhibited maximal activity only when the Mg^{++}: ATP ratio was 1 : 1. In the enzymes partially purified from rat liver, the effect of Mg^{++} was not significant. Choline kinase exhibited somewhat greater activity when the Mg^{++}: ATP ratio was higher than 2, but ethanolamine kinase activity was not affected with the Mg^{++}: ATP ratio.

Although a separation of two enzymes was not achieved, the above data suggested the possibility that different enzymes or active centers are involved in the phosphorylation of choline and ethanolamine.

Summary

Choline and ethanolamine kinases were partially purified from rat liver homogenate by 30 fold and 20 fold, respectively. Though the separation of two enzymes were not achieved, some properties were observed the suggesting possibility that different enzymes or active centers are involved in the phosphorylation of choline and ethanolamin.

An apparent Michaelis constant (Km) for choline was 0.23 mM and for ethanolamine was 5 mM. Both enzymes showed nearly the same values of maximal velocities or Km for ATP. However, some structural analogues of choline or ethanolamine affected choline and ethanolamine kinase activities in somewhat different manners.

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