Choline Kinase and Ethanolamine Kinase Activity in the Cytosol of Nerve Endings from Rat Forebrain

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Both choline kinase and ethanolamine kinase are present in the cytosol of nerve endings prepared from rat brain as are the products of their action, phosphocholine (84 nmol/g fresh wt. of brain) and phosphoethanolamine (190 nmol/g fresh wt. of brain). In contrast with the enzymes from the cytosol of whole brain, both are as equally active at pH 7.5 as 9.0. Determination of kinase activity in membrane-containing tissue samples at pH 9 gives low values because of the activity of alkaline phosphatase. Choline kinase, but not ethanolamine kinase, requires Mg^{2+} in excess of that required for the formation of the $MgATP$ complex and is inhibited by an excess of free ATP. The K_m for choline is 2.6 mM and for ethanolamine is 2.2 mM. The differing requirements for ATP and Mg^{2+} and the inhibition of choline kinase, but not ethanolamine kinase, by hemicholinium-3 suggest either the presence of two separate enzymes or two different active sites on the same enzyme.

Although choline kinase (EC 2.7.1.32) was discovered in 1953 by Wittenberg & Kornberg (1953) and studied briefly in brain tissue (Berry *et al.*, 1958) the first detailed investigation of the enzyme was by McCaman (1962), who showed that brain was the richest source of the enzyme in mammalian tissues. He also showed that it required Mg^{2+} and had a pH optimum of 10. The fact that ethanolamine could also be phosphorylated by brain tissue *in vitro* was first noted by Ansell & Dawson (1951) before the realization of the significance of phosphoethanolamine and phosphocholine for phospholipid synthesis. Both Wittenberg & Kornberg (1953) and McCaman (1962) suggested that ethanolamine could be phosphorylated by choline kinase, whereas Sung & Johnstone (1967) indicated that the soluble fraction of Ehrlich ascites cells contained separate enzymes for the phosphorylation of each base. However, the first detailed study of ethanolamine kinase (EC 2.7.1.82) was by Weinhold & Rethy (1972), who demonstrated that the pH optimum for the liver enzyme was 8.5 and that the substrate was the $MgATP$ complex. The same authors (Weinhold & Rethy, 1974) separated two enzymes from the soluble fraction of liver: ethanolamine kinase I (mol.wt. 36000), which had no activity towards, and was not inhibited by, choline; ethanolamine kinase II (mol.wt. 160000), which had activity towards, and was inhibited by choline. Kinases I and II had

different K_m values for ethanolamine and ATP. That ethanolamine and choline kinase activities tend to co-purify together has also been demonstrated in liver by Brophy & Vance (1976) and in mammary gland by Infante & Kinsella (1976), who suggest that the two activities are mediated by two distinct active sites on a single protein.

It is clear that in whole-brain tissue the choline kinase is present in the cytosol (McCaman & Cook, 1966; Ansell & Spanner, 1972), though there has been one report to the contrary (Upreti *et al.*, 1976). We have briefly reported the presence of choline and ethanolamine kinases of high specific activity in the cytosol of nerve endings (synaptosomes) isolated from rat forebrain (Spanner & Ansell, 1977). In the present paper the two activities are described in some detail and evidence given that they are distinct.

Experimental

Materials

[Me- ^{14}C]Choline and [γ - ^{32}P]ATP were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The [γ - ^{32}P]ATP was diluted with a neutralized solution of disodium ATP to give a sp. radioactivity of about 300 d.p.s./ μ mol. Disodium ATP, spermine, phosphoethanolamine and phosphocholine were obtained from Sigma (London) Chemical Co., Kingston upon Thames, Surrey KT2 7BH, U.K. The phosphoethanolamine had to be recrystallized from ethanol to decrease the contamination by P_i . All the other chemicals were of A.R. quality where

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

possible. Adult female Wistar rats were used of approx. 250g body wt. Hemicholinium-3 (the bis-hemiacetal form of $\alpha\alpha'$ -dimethylethanolamino-4,4'-bisacetophenone) was supplied by Aldrich Chemical Co. Inc., Milwaukee, WI, U.S.A.

Methods

Preparation of synaptosomal cytosol. Adult female rats were decapitated after light ether anaesthesia. The brain was removed and the forebrain or cerebellum homogenized in 0.25M-sucrose with six passes of a Teflon/glass homogenizer with a clearance of 0.10–0.15mm and at an average speed of 1450 rev./min. The synaptosomal fraction of the forebrain was prepared by a modification of the procedure of Gray & Whittaker (1962) and that of the cerebellum by the method of Rabié & Legrand (1973). The homogenate was diluted with 0.25M-sucrose to 20% (w/v) and centrifuged at either 2000g for 10min (forebrain) or at 900g for 10min (cerebellum). The supernatant was removed, the pellet resuspended in the original volume of 0.25M-sucrose and again centrifuged. From this stage both brain areas were treated in the same way. The two supernatants were pooled and centrifuged at 25000g_{av.} for 20min. The supernatants were removed, the pellets resuspended in the same volume of 0.25M-sucrose and again centrifuged at 25000g_{av.} for 20min. The pellet was suspended in 0.25M-sucrose with light homogenization and made up to a concentration equivalent to 1g of original tissue in 5ml. This was layered on a discontinuous gradient of 5ml of 0.8M-sucrose over 5ml of 1.2M-sucrose and centrifuged at 100000g_{av.} for 90min. The band sedimenting between 0.8M- and 1.2M-sucrose (P_2B) was removed and sufficient 0.25M-sucrose was added with shaking to decrease the sucrose concentration to 0.4M. This was centrifuged at 100000g_{av.} for 40min to give the synaptosomal pellet. The pellet was suspended in water (4ml of water/1g of original tissue) and left for 15min with frequent passage through a very fine bore pipette. The 'water-shocked' material was then homogenized (3 passes at 400 rev./min) and centrifuged at 100000g_{av.} for 40min. The supernatant, which contained the nerve-ending cytosol (P_2B soluble fraction), was removed and kept. In experiments where cortex was used it was fractionated in the same way as forebrain. All centrifuging was carried out in a 6 × 16.5 ml swing-out rotor in an MSE Superspeed 65 ultracentrifuge.

Kinase assays. These were carried out under zero-order conditions. A series of tubes was set up containing 60mM-Hepes/NaOH buffer, pH 7.5, containing 30mM-MgCl₂, 20mM-choline or 20mM-ethanolamine and [γ -³²P]ATP, in a total volume of 1.5ml. The Hepes buffer (0.12M, pH 7.5) was made by adding 12ml of 0.4M-Hepes to 8.5ml of 0.4M-NaOH

and diluting the mixture to 40ml. For the assay of choline kinase the ATP concentration was 10mM and for the ethanolamine kinase assay, 30mM. These tubes were put into a water bath at 37°C for 5min and to each was then added 0.5ml of the P_2B soluble fraction that contained synaptosomal cytosol equivalent to 125mg of original tissue. The contents of the tubes were well mixed and the reaction allowed to continue for 1h before being terminated by the addition of 0.5ml of 50% (w/v) trichloroacetic acid. The tubes were left in ice for 20min, then centrifuged in a bench centrifuge for 15min. A sample (2ml) of the clear supernatant was washed three times with an equal volume of diethyl ether. This removed most of the trichloroacetic acid and brought the pH to between 4 and 5. A drop of phenolphthalein solution [0.1g in 250ml of aq. 70% (v/v) ethyl alcohol] was added and drops of a saturated solution of Ba(OH)₂ added until the contents of the tubes were just pink (pH 8.3). The tubes were left on ice for 15min, then centrifuged for 15min to bring down the insoluble salts of barium ATP and its breakdown products. This removed over 90% of the ATP. The clear supernatant was applied to a 3ml column (0.7cm × 7.0cm) of Dowex 50 (X8; H⁺ form). The remaining ATP, ADP and P_i were washed through with water until the effluent was neutral; then the phosphocholine and phosphoethanolamine were eluted with 15ml of 1M-HCl. Eluates were collected directly into polythene counting vials and their radioactivity was measured by the Cerenkov procedure. Duplicate samples of the [γ -³²P]ATP were taken for counting of radioactivity and for phosphorus determination to establish the specific radioactivity of the [γ -³²P]ATP in each experiment. From this could be calculated the amount of choline or ethanolamine phosphorylated and hence the activity of the kinase. Blank (zero-time) incubations were always put through the whole procedure.

Separation of phosphocholine and phosphoethanolamine on Dowex 50 (H⁺ form). In the early stages of the work when the barium precipitation was omitted it was observed that the eluate containing phosphoethanolamine also contained traces of another ³²P-labelled material that caused blank values to be high and variable. It was concluded that the relatively high concentration of H⁺, generated as the sodium ATP passed over the Dowex 50 (X8; H⁺ form), was sufficient to elute some of the phosphoethanolamine so that the two could not be separated. Precipitation of most of the ATP with Ba²⁺ (the barium salt of phosphoethanolamine is water-soluble) before the separation on the columns eliminated this problem in that 2 bed vol. of water would remove the residual ATP leaving the phosphoethanolamine on the column to be eluted with 1M-HCl. This problem did not arise with phosphocholine, which has a quaternary N atom and is more strongly bound to the Dowex

50 (X8, H⁺ form). It should also be noted that 1M-HCl will partially elute free choline and ethanolamine. If it is desired to separate the bases from the phosphate esters, the latter should first be eluted with 0.1M-HCl, which does not elute the bases (see below).

When the choline kinase assay was being developed, [*Me*-¹⁴C]choline and not [γ -³²P]ATP was used as the radioactively labelled substrate. However, the presence of a small radioactively labelled contaminant that was eluted from the Dowex 50 (X8, H⁺ form) column with the phosphocholine in 0.1M-HCl resulted in high and erratic blank values.

Alkaline phosphatase assay. The incubation system contained 0.025M-glycine/NaOH buffer, pH9, containing 1mM-MgCl₂ and 10mM-phosphoethanolamine (adjusted to pH8 with NaHCO₃) or 10mM-phosphocholine and synaptosomes or synaptosomal membranes equivalent to 125mg to original tissue, all in a total volume of 2ml. Glycine/NaOH buffer (0.05M, pH9) was made by dissolving 375mg of glycine in 25ml of water, adding 8.5 ml of 0.1M-NaOH and diluting to 100ml. Incubations were for 30min at 37°C and the activity stopped by adding 0.5ml of 50% (w/v) trichloroacetic acid. The tubes were left on ice for 20min and then centrifuged for 15min. A sample (1 ml) of the supernatant was taken for the determination of P_i by the method of Swanson *et al.* (1964). Control samples with no substrate added were put through the whole procedure.

Determination of phosphocholine and phosphoethanolamine in synaptosomes. To a synaptosomal homogenate was added trichloroacetic acid to give a final volume of 10% (w/v) trichloroacetic acid. The tube was left in an ice bath for 20min and then centrifuged for 14min. A sample of the supernatant was removed and extracted three times with ether to remove the trichloroacetic acid and to bring the pH to about 5. The pH was then adjusted to pH8 with an ammonia wick (which was prepared by dipping a thin strip of filter paper in 18M-NH₄OH and inserting it into the tube so that it did not touch

the sides of the tube or the solution) and the sample applied to a column of Dowex 50 (X8; H⁺ form; 3 ml). A further 20ml of water was passed through the column and the phosphocholine and phosphoethanolamine were then eluted with 40ml of 0.1M-HCl. This eluate was taken to dryness under reduced pressure and left for at least 1 h in a desiccator under vacuum and over KOH pellets to remove the residual acid. The residue was taken up in a small volume of water and applied to Whatman no. 1 paper for chromatography in ethanol/18M-NH₄OH/water (6:3:1, by vol.). The papers were sprayed for the presence of phosphorus by the method of Hanes & Isherwood (1949) and the phosphocholine [*R_F* 0.58 ± 0.05 (mean ± s.d.; *n* = 40)] and phosphoethanolamine [*R_F* 0.49 ± 0.05 (mean ± s.d.; *n* = 40)] spots cut out, oxidized with HClO₄ and the phosphorus content determined by the method of Ernster *et al.* (1950).

Determination of protein. This was carried out by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Choline kinase

In previous studies of the subcellular distribution of choline kinase, there was an indication of a small amount of activity in the synaptosomal fraction (Ansell & Spanner, 1972). This activity was measured at pH9. By analogy with whole tissue, the enzyme of the synaptosome was assumed to be soluble (McCaman, 1962; McCaman & Cook, 1966; Ansell & Spanner, 1972). This assumption was strengthened in that, when the synaptosomes were hypo-osmotically ruptured and the cytosol isolated free from membranes, mitochondria and vesicles (see under 'Methods'), the phosphocholine was found to be exclusively in the cytosol of the synaptosome (Table 1). According to Marchbanks (1975) the internal fluid volume of synaptosomes prepared

Table 1. *The distribution of phosphocholine, phosphoethanolamine and some enzymes involved in their metabolism between the total soluble and total membranous fractions of the synaptosomal fraction of rat forebrain*
The values given are means ± s.d. with the number of determinations in parentheses.

	Synaptosomes	
	Cytosol	Membranes
Protein (mg/g fresh wt.)	2.02 ± 0.2 (15)	10.97 ± 0.4 (10)
Phosphocholine (nmol/g fresh wt.)	84.36 ± 7.1 (14)	0
Phosphoethanolamine (nmol/g fresh wt.)	189.60 ± 20.5 (12)	0
Alkaline phosphatase (μmol/g fresh wt. per h) activity with phosphocholine or phosphoethanolamine as substrate	0	3.01 ± 0.3 (13)
Choline kinase (μmol/g fresh wt. per h)	1.18 ± 0.11 (9)	0
Ethanolamine kinase (μmol/g fresh wt. per h)	0.48 ± 0.04 (23)	0

from guinea-pig forebrain is $2.9 \mu\text{mol}/\text{mg}$ of synaptosomal protein. If synaptosomes from rat forebrain are similar, then, since 84 nmol of phosphocholine is found in synaptosomes to be equivalent to 13 mg of protein (see Table 1), the concentration of this ester in synaptosomal cytosol is approx. 2.2 mM . The choline kinase activity of 1.18 ± 0.11 (mean $\pm$ s.d.; $n = 9$) $\mu\text{mol}/\text{g}$ of brain per h (Table 1) was very much higher than that found in the intact synaptosome (0.14 – $0.23 \mu\text{mol}/\text{g}$ of brain per h), implying the presence of an inhibitor of the kinase or an enzyme that degraded the newly synthesized phosphocholine. Alkaline phosphatase was found to have an activity of 3.01 ± 0.3 (mean $\pm$ s.d.; $n = 13$) $\mu\text{mol}/\text{g}$ of brain per h at pH 9, the pH value at which the kinase assay was carried out. It was found to be exclusive to the membranes of the synaptosome (Table 1). In the presence of 3 mM -cysteine, an inhibitor of alkaline phosphatase (Cooper & Hawthorne, 1975), the activity of the kinase in the intact synaptosome was the same as that of the isolated cytosol.

Until this time, the conditions for assaying the choline kinase in the synaptosome had been those previously established for the whole-brain soluble fraction, i.e. a choline concentration of 20 mM , an

Fig. 1. The effect of ATP concentration on the activity of choline kinase and ethanolamine kinase in synaptosomal cytosol

Incubations were carried out as described in the text for 1 h in 60 mM -Hepes buffer, pH 7.5, together with 20 mM -choline or ethanolamine, 30 mM - Mg^{2+} , $P_2\text{B}$ soluble fraction equivalent to 125 mg of original forebrain and various amounts of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Symbols: $\blacktriangle$, choline kinase; $\bullet$, ethanolamine kinase. Each point represents the mean for six separate incubations and the variation in activity is approx. 0.5% .

Fig. 2. The effect of Mg^{2+} concentration on the activity of synaptosomal choline kinase

Incubations were carried out as described in the text for 1 h in 60 mM -Hepes buffer, pH 7.5, together with 20 mM -choline, 10 mM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, $P_2\text{B}$ soluble fraction equivalent to 125 mg of original forebrain and various amounts of Mg^{2+} . Each point is the mean for four separate incubations and the variation in activity is approx. 0.5% .

MgATP concentration of 10 mM with the addition of 20 mM - Mg^{2+} and a pH value of 9. These conditions were also found to be optimal for the enzyme of the cytosol of the synaptosome (Fig. 1) with the exception of the pH value. The enzyme activity was found to be the same at pH 7.5 as at pH 9 and at the former pH the alkaline phosphatase activity of the synaptosomal membranes was decreased to almost zero. Increasing the concentration of ATP above 10 mM when the Mg^{2+} concentration was maintained at 30 mM was found to be inhibitory (Fig. 1). Fig. 2 shows clearly that when the ATP concentration is maintained at 10 mM the activity falls as the Mg^{2+} concentration is decreased below 30 mM .

Ethanolamine kinase

Synaptosomes also contain phosphoethanolamine at a much higher concentration than the phosphocholine i.e. 189.6 ± 20.5 (mean $\pm$ s.d.; $n = 12$) nmol/g of brain (Table 1). By using a calculation similar to that described for phosphocholine the concentration of this ester in synaptosomal cytosol is approx. 5 mM . With the conditions found to be optimal for the choline kinase assay, the presence of an ethanolamine kinase in the cytosol of the synaptosomes was

Fig. 3. The effect of MgATP concentration on the activity of synaptosomal ethanolamine kinase

Incubations were carried out as described in the text for 1 h in 60 mM-Hepes buffer, pH 7.5, together with 10 mM-ethanolamine, P_2B soluble fraction equivalent to 125 mg of original forebrain, and various concentrations of MgATP (equimolar amounts of Mg^{2+} and ATP). Each point is the mean for four incubations and the variation in activity is approx. 0.5%.

established. It was, however, found impossible under these conditions to saturate the enzyme with the ethanolamine substrate even at concentrations as high as 100 mM. Various concentrations of Mg^{2+} and ATP were tried and it was found that with equimolar concentrations of Mg^{2+} and ATP of 30 mM the enzyme was optimally active at an ethanolamine concentration of 10 mM (Fig. 3). At this ATP concentration the choline kinase was inhibited by 35% (Fig. 1).

The activity of the ethanolamine kinase in the synaptosome was lower than that of the choline kinase, i.e. 0.48 ± 0.04 (mean $\pm$ S.D.; $n = 23$) μ mol/g of brain per h and the determination of this activity was likewise affected in the intact synaptosome at pH 9 by the presence of alkaline phosphatase. Thus in the intact synaptosome at pH 9 the ethanolamine kinase activity appeared to be as low as 0.06 ± 0.01 (mean $\pm$ S.D.; $n = 6$) μ mol/g per h, whereas the activity in the isolated cytosol was 0.48 ± 0.04 (mean $\pm$ S.D.; $n = 23$) μ mol/g of brain per h. As with the choline kinase, there was no significant difference in activity of the ethanolamine kinase between pH 7.5 and 9.

Fig. 4. The effect of hemicholinium-3 on the activities of synaptosomal choline and ethanolamine kinase (Hanes-Woolf plot)

(a) Incubations were carried out as described in the text for 1 h in 60 mM-Hepes buffer, pH 7.5, together with 20 mM-choline, 30 mM- Mg^{2+} , 10 mM- $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ and P_2B soluble fraction equivalent to 125 mg of original forebrain. Symbols: Δ , with 0.04 mM-hemicholinium-3; $\blacktriangle$, without hemicholinium-3. (b) As for (a) but with 10 mM-ethanolamine instead of 20 mM-choline, 30 mM- Mg^{2+} and 30 mM- $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$. Symbols: $\circ$, with 0.04 mM-hemicholinium-3; $\bullet$, without hemicholinium-3. In (a), $[S]$ is [choline](mM) and v is μ mol of phosphocholine/mg of protein per h; in (b), $[S]$ is [ethanolamine](mM) and v is in μ mol of phosphoethanolamine/mg of protein per h.

Effect of hemicholinium-3 and spermine

Hemicholinium-3 had been shown to be a potent inhibitor of the choline kinase in the total cell cytoplasm of brain *in vitro* (Ansell & Spanner, 1974) and an inhibitor of phosphocholine formation *in vivo* (Ansell & Spanner, 1975a,b). For the synaptosomal cytosol it was found that, whereas there was an

Table 2. *Choline kinase activity in the total soluble fraction and the synaptosomal cytosol of rat cortex and cerebellum*
Values are ranges with the numbers of observations in parentheses.

Fraction ...	Cerebellum		Cortex	
	S_3^*	P_2B soluble†	S_3^*	P_2B soluble†
Protein (mg/g fresh wt.)	38.5–44.0 (3)	0.54–0.56 (3)	32.0–34.0 (3)	0.90–0.93 (3)
Choline kinase ($\mu\text{mol/g}$ fresh wt. per h)	4.4–4.7 (3)	0.42–0.44 (2)	3.4–3.7 (3)	0.24–0.28 (2)
($\mu\text{mol/mg}$ of protein per h)	0.113 (3)	0.78 (2)	0.11 (3)	0.28 (3)

* Total soluble.

† Synaptosomal cytosol.

inhibition of the choline kinase of 28% in the presence of 40 μM -hemicholinium-3, which is apparently non-competitive (Fig. 4a), there was no effect on the ethanolamine kinase (Fig. 4b). The inhibition of the choline kinase of the cytosol from whole-brain tissue by hemicholinium-3 seemed to be uncompetitive (Ansell & Spanner, 1974).

Fukuyama & Yamashita (1976) showed that with partially purified liver choline kinase, there was a stimulation by polyamines and in particular by spermine. In brain we were able to demonstrate a 43% stimulation of the choline kinase activity of the synaptosomes, but a 10 mM concentration of spermine was required. At a concentration of 1 mM there was no significant effect.

Stability of enzymes on storage

The choline kinase activity in the synaptosomal cytosol was stable for 4 days when stored at 4°C. The ethanolamine kinase activity, however, rapidly fell and had decreased to 50% of its initial activity on storage for 16 h at 4°C.

Cerebellum and cortex

Choline kinase activity was measured in two regions of the rat brain, the cortex and the cerebellum. The activity of the enzyme was very similar in the total cytosol of the two regions, i.e. 0.11 $\mu\text{mol/mg}$ of protein per h, but kinase activity of the synaptosomal cytosol of the two regions showed a considerable difference. Choline kinase activity in the synaptosomal cytosol of the cerebellum was 0.79 $\mu\text{mol/mg}$ of protein per h, whereas that of the cortex was 0.29 $\mu\text{mol/mg}$ of protein per h. This difference was not so marked when the results were expressed per g of tissue, but the value for the synaptosomal cytosol of cortex was still only 60% of that of the cerebellum (Table 2).

Discussion

Previous investigations on choline kinase in brain tissue have indicated that it is an enzyme of the

cytosol (McCaman & Cook, 1966; Ansell & Spanner, 1972). Upreti *et al.* (1976) reported that in rat and mouse brain the enzyme is entirely in the microsomal fraction, whereas the ethanolamine kinase is in the cytosol. We have found no evidence for this and in no other tissue has choline kinase been found in the microsomal fraction (Sung & Johnstone, 1967; Weinhold & Rethy, 1974; Infante & Kinsella, 1976). We can only assume that under the conditions of subcellular fractionation used by Upreti *et al.* (1976) the choline kinase became bound to the microsomal fraction. The cytosol derives from both neurons and glial cells and one advantage of studying the cytosol of nerve endings (synaptosomes) therefore is that it is neuronal in origin. Although choline kinase can be assayed in whole-brain tissue (e.g. McCaman, 1962; Burt, 1977) or preparations derived from whole tissue (Haubrich, 1973), there are two clear practical advantages of using the cytosol. First, there is no production of ADP, which inhibits choline kinase (Burt & Brody, 1975), by the membrane-bound ATPases and adenylate kinase with which brain is richly endowed; secondly, when the assay is performed at a pH value more alkaline than 7.5, the product of the reaction, phosphocholine, is not hydrolysed because membrane-bound alkaline phosphatase is absent. When choline kinase was assayed in whole nerve endings the activity at pH 9.0 was much less than that of the cytosol obtained from an equivalent amount of nerve endings, though the values were the same when cysteine, an inhibitor of alkaline phosphatase, was added to the incubation medium. At pH 7.5 there was no hydrolysis of phosphocholine or phosphoethanolamine. This presumably accounts for the fact that there is little post-mortem loss of these esters, and they can be determined in nerve endings with a reasonable degree of accuracy (Dowdall *et al.*, 1972; Table 1 of the present paper).

Assays were routinely carried out on the cytosol at pH 7.5 because, although the optimum pH for the kinase of the cytosol as a whole is 9.0, for the cytosol of synaptosomes the activity is the same at pH 7.5 as at 9.0 (assays at lower pH values were not made). Haubrich (1973), with a partially purified preparation

from an acetone-dried powder of whole-rabbit-brain tissue, noted a choline kinase activity at pH 7.5 with a K_m for choline of $32\mu\text{M}$, whereas the K_m at pH 9.5 was 0.31mM . It is very difficult to compare the results of Haubrich (1973) with those of others because in the preparation of an acetone-dried powder a lot of the cytosol will be lost. Conceivably the powder might be enriched in synaptosomal kinases, which would account for an activity with an optimum pH of 7.5, but not for its K_m . The K_m for choline of the synaptosomal choline kinase was 2.6mM (Fig. 4a) which is the same as that of the cytosol of whole-brain tissue (Ansell & Spanner, 1974).

When the optimum conditions for the assay of choline kinase in the synaptosomal cytosol were used to determine ethanolamine kinase, it rapidly became clear that they were unsuitable. It is known from the work of Weinhold & Rethy (1974) on the liver kinases that the Mg^{2+} and ATP requirements of the two enzymes could well be different, and experiments showed that equimolar ATP and Mg^{2+} were required for optimal activity at an ethanolamine concentration of 10mM (K_m for ethanolamine 2.2mM ; Fig. 4b). Choline kinase differs from the ethanolamine kinase in requiring Mg^{2+} in excess of that required for the formation of the MgATP complex and the possible function of the excess Mg^{2+} in decreasing the K_m of MgATP for liver choline kinase has been discussed in some detail by Infante & Kinsella (1976). The synaptosomal choline kinase is inhibited by an excess of ATP (Fig. 1), as has been observed for the choline kinase from whole brain by McCaman (1962), Burt & Brody (1975) and Haubrich (1973). The ethanolamine kinase does not, however, appear to be inhibited by an excess of free ATP (Fig. 1), which suggests that a different kinase may be involved in the phosphorylation of ethanolamine. Weinhold & Rethy (1972) noted that the ethanolamine kinase activity of liver was inhibited by an excess of free ATP and also by an excess of MgATP complex. From a further study by the same authors (Weinhold & Rethy, 1974) it is apparent that ethanolamine kinase activity in liver is complex. There appear to be two distinct enzymes (I and II), with different K_m values for ethanolamine and choline; both enzymes require Mg^{2+} in excess of that required for the formation of the MgATP complex. Furthermore, ethanolamine kinase II, but not I, was strongly inhibited by choline and also had choline kinase activity.

Fig. 4 shows that the ethanolamine kinase activity of the synaptosomal cytosol was not inhibited by hemicholinium-3, whereas the choline kinase activity was inhibited in a non-competitive manner [cf. whole-brain cytosol (Ansell & Spanner, 1974)]. In a recent study, Burt (1977) has shown that the choline kinase of rat spinal cord is in two forms, one sensitive to hemicholinium-3 and the other not. This was

based on the observation that increasing the concentration of hemicholinium-3 from 0.02 to $0.1\mu\text{M}$ did not increase the inhibition, which remained at 34%. In the experiments illustrated in Fig. 4 a concentration of $0.04\mu\text{M}$ was used which is within the range used by Burt (1977), and we cannot therefore state whether the 28% inhibition observed represents only a hemicholinium-3-sensitive fraction of the choline kinase activity of the nerve ending.

From these observations it is possible to conclude that ethanolamine and choline kinases are either two distinct enzymes in the synaptosomal cytosol or perhaps two different active sites on the same protein.

Choline kinase activity is much higher on a protein basis in the synaptosomal cytosol from cortex than the cytosol of brain cortex as a whole (Table 2). As mentioned above the whole cytosol will derive from a variety of neuronal cells and a variety of glial cells. This enzyme has not been determined in the cytosol of different cell types, but since the amount of choline kinase in white matter is low (McCaman, 1962), it is possible that oligodendroglial cells are low in activity of this enzyme. It certainly cannot be stated as yet that the cytosol of neuronal perikarya is poorer in the enzyme than the cytosol of nerve endings, with which it is continuous.

The presence of high activities of the two kinases in the nerve endings certainly increases the likelihood that phospholipid synthesis can occur there, independent of that which may be transported down axons. This has been discussed elsewhere (Spanner & Ansell, 1978). Another role for choline kinase could be in the regulation of free choline concentrations for acetylcholine synthesis in the cytosol of cholinergic terminals. There is good evidence that acetylcholine synthesis in such terminals is controlled by Na^+ -dependent high-affinity uptake system for choline (Barker & Mittag, 1975), but this is probably only true when acetyl-CoA concentrations are not limiting (Jope *et al.*, 1978). It has been generally accepted that in the presence of low concentrations of external choline the choline that is taken up by synaptosomal preparations is primarily converted into acetylcholine, not phosphocholine (Yamamura & Snyder, 1973). However, the results of Yavin (1976) indicate that the reverse is true for differentiating cells from rat cerebral hemispheres in cultures. The significant amounts of phosphocholine in terminals (Table 1) could serve as a reservoir of readily available choline from which it could be released by the action of alkaline phosphatase, though it has to be remembered that the activity of the latter is negligible at physiological pH *in vitro* and also may well be on the outer surface of plasma membranes (Trams & Lauter, 1974). The interrelationship between the Na^+ -dependent high-affinity uptake system, the low-affinity uptake system for choline and the supply and utilization of this choline for the synthesis of acetyl-

choline and choline lipids merits further investigation.

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