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Phosphorylation of Choline and Ethanolamine in Ehrlich Ascites-Carcinoma Cells

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1. Ehrlich ascites-cell extracts convert choline and ethanolamine approximately equally well into their respective phosphoryl derivatives. 2. Choline is a potent inhibitor of ethanolamine phosphorylation, but ethanolamine has little effect on choline phosphorylation. 3. 2,3-Dimercaptopropanol, cysteine and Ca^{2+} inhibit ethanolamine phosphorylation, but have no detectable effect on choline phosphorylation. 4. Choline-phosphorylating activity in Ehrlich ascites-cell extracts is more stable during storage than ethanolamine-phosphorylating activity. 5. Choline phosphorylation is stimulated in the presence of benzoylcholine, succinylcholine, butyrylcholine and propionylcholine, whereas ethanolamine phosphorylation is inhibited. This relationship is reciprocal: the compounds causing the greatest stimulation of choline phosphorylation bring about the greatest inhibition of ethanolamine phosphorylation.

We have previously reported (Sung & Johnstone, 1965) that Ehrlich ascites-cell suspensions when incubated with choline accumulate much greater quantities of phosphorylcholine per unit weight of tissue than any other mouse or rat tissue examined.

The observation that ethanolamine is nearly as well esterified as choline at equimolar concentrations prompted us to examine some of the properties of the phosphorylating system because it appeared to be clearly different from that reported in other systems (Wittenberg & Kornberg, 1953; Ramasarma & Wetter, 1957; McCaman, 1962). For example, it has been demonstrated that partially purified choline kinase (EC 2.7.1.32) from yeast phosphorylates both choline and ethanolamine but that the K_m for the latter substrate is 500 times greater than that for choline (Wittenberg & Kornberg, 1953). The choline kinase from Polish rapeseed, however, has been reported to be inactive towards ethanolamine (Ramasarma & Wetter, 1957). McCaman (1962) reported that ethanolamine in great excess (260 times that of choline) causes a slight inhibition of choline phosphorylation in rabbit brain homogenates, but the extent of ethanolamine phosphorylation in these preparations was not shown.

In the present work we provide evidence that in Ehrlich ascites cells separate enzymes are responsible for the phosphorylation of choline and ethanolamine respectively. A preliminary report of these studies has been presented (Sung & Johnstone, 1966).

MATERIALS AND METHODS

Chemicals. [*Me*-^{14}C]Choline and [$^{14}C_2$]ethanolamine were purchased from The Radiochemical Centre, Amersham, Bucks., and were given routine examination on arrival for radiochemical purity by chromatography on Whatman no. 1 filter paper with butan-1-ol–acetic acid–water (4:1:1 or 20:6:17, by vol.) followed by radioautography with Kodak X-ray film.

All non-radioactive chemicals used were of A.R. grade and were used without further purification except ethanolamine, which was distilled before use.

Preparation of cell-free extracts of Ehrlich ascites-carcinoma cells. Ehrlich ascites cells, obtained as described by Coles & Johnstone (1962), were broken osmotically by suspending in 9 times their packed volume of ice-cold distilled water for 10min. in an ice bath. The cell suspension was then homogenized briefly with a Teflon homogenizer. The homogenate was centrifuged for 10min. at 20000*g* in a refrigerated International centrifuge or in a Sorvall RC-2B type centrifuge. A 0·5ml. sample of the supernatant was used per 1·6ml. of incubation medium.

Incubation procedure. All incubations were carried out at 37° in stoppered 25ml. Erlenmeyer flasks in a shaker bath. The standard incubation medium contained 0·2mM-[*Me*-^{14}C]choline or [$^{14}C_2$]ethanolamine, 2mM-ATP, 2·5mM-$MgSO_4$ and 20mM-sodium phosphate buffer, pH 8·0. After 5min. of thermal equilibration in the shaker bath, the reaction was initiated by adding either the cell extract or the radioactive solutions. An incubation period of 30min. was employed except when otherwise stated.

Analytical procedures. After incubation, the reactions were terminated by adding 38% trichloroacetic acid to a final concentration of 6%. The samples were kept for 30min. in an ice bath, centrifuged and the supernatants

were collected. The trichloroacetic acid extracts thus obtained were extracted three times with 5ml. of ether to remove the trichloroacetic acid. A 1ml. sample of this extract was passed through a 10mm.×10mm. column of Dowex 50 (H^+ form; 12% cross-linked; 200–400 mesh). The first 1ml. of water eluate was discarded, since no radioactivity was found therein. Another 10ml. of water was passed through the resin, and the water eluate was collected and counted. After elution with 10ml. of water, no further radioactivity could be eluted with water.

The components appearing in the water eluate of the resin after incubation with [*Me*-^{14}C]choline and [$^{14}C_2$]-ethanolamine were identified as phosphorylcholine or phosphorylethanolamine respectively by the following methods. The Ehrlich ascites cells were incubated with (1) [^{32}P]P_i+unlabelled choline; (2) [^{32}P]P_i+unlabelled ethanolamine; (3) [*Me*-^{14}C]choline; (4) [$^{14}C_2$]ethanolamine. After incubation, the acid-soluble extracts were processed as described above and the water eluates from the resins from each of the four incubations were subjected to paper chromatography in butan-1-ol–acetic acid–water (20:6:17 or 4:1:1, by vol.) followed by radioautography with Kodak X-ray film. In addition, a portion of the water eluate from (1) was pooled with a portion of the water eluate from (3) and a portion from (2) pooled with a portion from (4). The pooled extracts were chromatographed and radioautographed. Radioautography showed that ^{32}P-labelled and ^{14}C-labelled compounds coincided on the same spot. The water eluates from (1) and (2) were also mixed with authentic samples of phosphorylcholine and phosphorylethanolamine respectively, chromatographed and radioautographed. Subsequently the latter chromatograms were stained with a modified Dragendorff solution (Bregoff, Roberts & Delwiche, 1953) or I_2 vapour to detect the choline esters and with ninhydrin to detect the ethanolamine esters. These tests showed that the radioactive compounds obtained from incubations (1) and (3) coincided on the chromatogram with authentic phosphorylcholine. The radioactive compounds obtained from incubations (2) and (4) coincided with authentic phosphorylethanolamine. In addition, exposure of the water eluate from incubation (3) to a rat liver homogenate liberated a compound that had an R_F value identical with that of free choline.

RESULTS

Localization of the choline- and ethanolamine-phosphorylating enzymes of Ehrlich ascites cells. The initial experiments were undertaken to determine the intracellular localization of the enzyme or enzymes responsible for phosphorylation of choline and ethanolamine in Ehrlich ascites cells. The results obtained (Table 1) showed that the phosphorylating activity is associated primarily with the supernatant obtained after centrifugation at 100000*g* for 1hr.

Since most of the enzymic activity responsible for the phosphorylation of choline and ethanolamine was found in the supernatant fraction, cell-free extracts were used throughout this paper.

Effect of pH *on choline and ethanolamine phosphorylation in extracts of Ehrlich ascites cells.* Both choline and ethanolamine are phosphorylated at fairly high pH. Maximum phosphorylation is obtained in the range pH 8·5–10·5. Below pH 7·0

Table 1. *Intracellular localization of choline- and ethanolamine-phosphorylating activity in Ehrlich ascites cells*

Homogenates of the Ehrlich ascites cells were prepared in ice-cold 0·25M-sucrose. Fractionation of homogenates in 0·25M-sucrose was performed as described by Wilgram & Kennedy (1963). Incubations were carried out for 1hr. at 37° in 20mM-sodium phosphate buffer, pH 8·0. ^{14}C-labelled bases were present at concentrations of 0·2mM (specific activity 770 counts/min./mμmole and 800 counts/min./mμmole for [*Me*-^{14}C]choline and [$^{14}C_2$]ethanolamine respectively). ATP was present at 2mM, and Mg^{2+} at 2·5mM. Protein concentration was determined as described by Herscovics & Johnstone (1964).

Fraction	Sp. activity (mμmoles phosphorylated/mg. of protein/hr.) Choline	Ethanolamine
Nuclei	8·0	4·8
Mitochondria	Nil	Nil
Microsomes	6·7	5·3
Supernatant	33·0	26·0

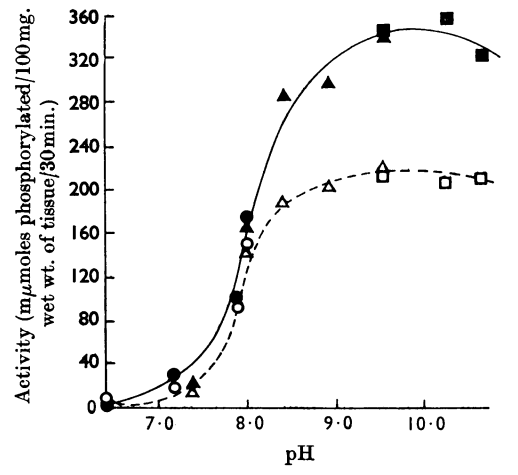

Fig. 1. Effect of pH on choline and ethanolamine phosphorylation in Ehrlich ascites cell-free extracts. Extracts of Ehrlich ascites cells (0·5ml.) were incubated for 30min. at 37° with 0·2mM-[*Me*-^{14}C]choline (specific activity 770 counts/min./mμmole) or [$^{14}C_2$]ethanolamine (specific activity 800 counts/min./mμmole). ATP was present at a concentration of 2mM and Mg^{2+} at a concentration of 2·5mM; 50mM-NaCl was present throughout. ●, ▲ and ■, Choline phosphorylation; ○, △ and □, ethanolamine phosphorylation. The buffer solutions (concentrations 10mM) used were: ● and ○, sodium phosphate; ▲ and △, glycylglycine; ■ and □, glycine. The final volume was 1·6ml. and the gas phase was air.

Table 2. *Phosphorylation of* [Me-^{14}C]*choline and* [$^{14}C_2$]*ethanolamine in different buffers and the effect of univalent cations*

The cell-free extract (1·0 ml.) was incubated for 1 hr. at 37°. The bases were present at a concentration of 0·2 mM.Specific activities were 770 counts/min./mμmole and 800 counts/min./mμmole for [*Me*-^{14}C]choline and [$^{14}C_2$] ethanolamine respectively. ATP was present at a concentration of 2 mM and Mg^{2+} at a concentration of 2·5 mM. Phosphate and glycylglycine buffers were present at a concentration of 20 mM, and tris buffer at 10 mM.

Buffer (pH 8·0)	Univalent cation present in the medium	Concn. of cation (mM)	Phosphorylation (mμmoles) Of choline	Of ethanolamine
Phosphate	Na^+	40	226·0	162·0
Tris	Na^+	0	151·5	73·3
Tris	Na^+	10	165·0	81·9
Tris	Na^+	40	218·0	102·0
Tris + phosphate	Na^+	40	240·0	136·0
Glycylglycine	Na^+	0	145·0	114·0
Glycylglycine	Na^+	10	167·0	132·0
Glycylglycine	Na^+	40	251·0	160·0
Glycylglycine	K^+	10	170·0	119·0
Glycylglycine	K^+	40	206·0	140·0
Glycylglycine	Li^+	10	161·0	122·0
Glycylglycine	Li^+	40	209·0	147·0
Glycylglycine	Cs^+	10	174·0	118·0
Glycylglycine	Cs^+	40	222·0	148·0

Table 3. *Effect of ATP and bivalent cations on phosphorylation of choline and ethanolamine*

The cell-free extract (0·5 ml.) was incubated for 30 min. with 20 mM-sodium phosphate buffer, pH 8·0. ATP was present at a concentration of 2 mM. The bivalent cations were present at a concentration of 2 mM. [*Me*-^{14}C]Choline and [$^{14}C_2$]ethanolamine were used at concentrations of 0·2 mM and their respective specific activities were 770 counts/min./mμmole and 800 counts/min./mμmole. The results given are the averages of six determinations. The ranges of values obtained are given in parentheses.

Additions	Phosphorylation (mμmoles) Of choline	Of ethanolamine
Nil	1·1 (0·9–1·2)	0·7 (0·6–0·9)
Mg^{2+} (−ATP)	1·7 (1·0–3·0)	1·1 (0·7–1·5)
Mg^{2+}	56·3 (54–59)	54·4 (50–60)
Ca^{2+}	6·3 (5·8–7·0)	1·9 (1·6–2·4)
Mn^{2+}	5·6 (4·8–6·5)	10·1 (9·7–10·5)
$Mg^{2+} + Ca^{2+}$	57·7 (53–63)	25·1 (21·6–29·0)
$Mg^{2+} + Mn^{2+}$	8·0 (7·5–8·6)	12·8 (12·5–13·0)
$Mn^{2+} + Ca^{2+}$	9·8 (9·5–10·0)	11·6 (11·3–12·0)
$Mg^{2+} + Ca^{2+} + Mn^{2+}$	11·5 (10·8–12·0)	14·9 (14·5–15·5)

little phosphorylation occurs and above pH 10·5 activity decreases, as shown in Fig. 1.

Phosphorylation of choline and ethanolamine in different buffers and the effect of univalent cations. The initial observations suggested that optimum phosphorylation takes place in a sodium phosphate buffer. The results in Table 2 show that in tris or glycylglycine buffer less phosphorylation is observed of both choline and ethanolamine than in a sodium phosphate buffer.

The decreased phosphorylation of choline in tris and glycylglycine buffers appears to be largely due to an absence of univalent cations since their addition to either of the latter buffers restores activity to the level observed in a phosphate buffer. However, ethanolamine phosphorylation is not fully restored by the addition of Na^+ to a tris buffer, although equal extents of ethanolamine phosphorylation are observed in glycylglycine buffer and phosphate buffer so long as equal concentrations of Na^+ is present. The results of a typical experiment are presented in Table 2.

The effect of Na^+ is not specific since a number of univalent cations, namely K^+, Li^+ and Cs^+, are able to increase the phosphorylation of both choline and ethanolamine.

Effects of ATP and bivalent cations. ATP and Mg^{2+} are essential cofactors for both choline and ethanolamine phosphorylation. The data in Table 3 show that in the absence of either ATP or Mg^{2+} negligible phosphorylation takes place. Neither Ca^{2+} nor Mn^{2+} substitutes for Mg^{2+}. However, Ca^{2+} inhibits ethanolamine phosphorylation, but not choline phosphorylation, when it is present in addition to Mg^{2+}. Mn^{2+} inhibits the phosphorylation of both choline and ethanolamine.

Action of choline on ethanolamine phosphorylation. The results in Table 4 show that choline is a potent inhibitor of ethanolamine phosphorylation in extracts of Ehrlich ascites cells. With choline at a

Table 4. *Choline and ethanolamine phosphorylation in a variety of mammalian tissues*

Tissue extracts were prepared in distilled water (1 g. of tissue + 9 vol. of water), the homogenates were centrifuged at 20000*g* for 10 min. A 0·5 ml. sample of supernatant was used/1·6 ml. of incubation medium. Incubations were carried out for 30 min. at 37° in the air. 0·2 mM-[*Me*-^{14}C]Choline (specific activity 770 counts/min./mμmole) or 0·2 mM-[$^{14}C_2$]ethanolamine (specific activity 800 counts/min./mμmole) was present. The buffer solution used was 20 mM-sodium phosphate, pH 8·0. ATP was present at 2 mM, Mg^{2+} at 2·5 mM. Positive values indicate stimulation, negative values inhibition. 'Nil' is used when the change was less than 10%.

Additions	Change of ethanolamine phosphorylation by choline (%)				
	Ehrlich ascites cell (mouse)	Liver (mouse)	Spleen (mouse)	Kidney (mouse)	Brain (rat)
Choline (0·05 mM)	−78	Nil	−44·4	−36·3	−32·0
Choline (0·2 mM)	−93	—	—	—	—
Choline (0·5 mM)	—	Nil	−45·0	−47·3	−64·0
Control ethanolamine phosphorylation (mμmoles)	77·3	43·3	5·4	5·5	3·5
	Change of choline phosphorylation by ethanolamine (%)				
Ethanolamine (0·05 mM)	Nil	Nil	Nil	+20·0	Nil
Ethanolamine (0·5 mM)	Nil	Nil	Nil	Nil	Nil
Ethanolamine (5·0 mM)	Nil	Nil	+22·0	Nil	+26·8
Ethanolamine (10·0 mM)	−16·0	Nil	Nil	Nil	+15·4
Control choline phosphorylation (mμmoles)	57·3	27·3	15·7	28·2	17·0

concentration one-quarter that of ethanolamine, 78% inhibition of ethanolamine phosphorylation is observed. Nearly complete inhibition is observed when the choline concentration used is equal to the ethanolamine concentration. Although choline inhibits ethanolamine phosphorylation in several mammalian tissues, such as mouse spleen, mouse kidney and rat brain, the effect is much less pronounced than in Ehrlich ascites cells. No significant inhibition of ethanolamine phosphorylation by choline is observed with mouse liver. Ethanolamine did not inhibit choline phosphorylation in any of the tissues examined, even when used at a concentration 30–50 times that of choline.

These results suggest either (*a*) that choline and ethanolamine are phosphorylated by separate enzymes or (*b*) that the K_m of ethanolamine is so large compared with the K_m for choline that reciprocal inhibitions of phosphorylation are not observed. The K_m for choline phosphorylation was determined to be about 0·12 mM and that for ethanolamine 0·2 mM. Therefore it does not appear that the lack of reciprocal inhibition is due to a relatively high K_m for ethanolamine and suggests that choline and ethanolamine are phosphorylated by separate enzymes.

Effects of thiol agents on the phosphorylation of choline and ethanolamine. Though it has been reported that choline kinase from yeast requires cysteine for optimum activity (Wittenberg & Kornberg, 1953), the lack of effect of monothiol or dithiol reagents on choline kinase activity has also been reported in other tissue preparations (Ramasarma & Wetter, 1957; McCaman, 1962). The results in Table 5 show that cysteine and 2,3-dimercaptopropanol (BAL) inhibit ethanolamine phosphorylation but are without effect on

Table 5. *Effect of thiol agents on the phosphorylation of* [*Me*-^{14}C]*choline and* [$^{14}C_2$]*ethanolamine*

The conditions of incubation were the same as in Table 1. 'Nil' is used when the inhibition was less than 5%. The results given are the averages of four determinations. The ranges of the inhibitory effects are given in parentheses.

Additions	Inhibition (%)	
	Of choline phosphorylation	Of ethanolamine phosphorylation
2,3-Dimercaptopropanol (1 mM)	Nil	30 (24–35)
Cysteine (1 mM)	Nil	17 (14–20)
Cysteine (5 mM)	12 (10–13)	38 (37–40)
Sodium arsenite (0·1 mM)	Nil	Nil
Sodium arsenite (1 mM)	Nil	Nil
p-Chloromercuribenzoate (0·2 mM)	Nil	Nil

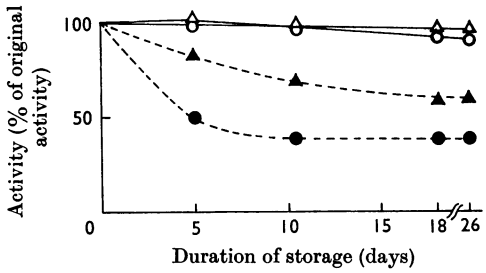

Fig. 2. Maintenance of phosphorylating activity to choline and ethanolamine in extracts of Ehrlich ascites cells. A portion of the extract was assayed immediately after preparation. The remainder of the extract was distributed in eight tubes, four of which were stored at 0–4° and the remaining four at −20°. At the times indicated, one tube from each of the stored samples was assayed for ethanolamine and choline phosphorylation. The incubation mixture contained 20mM-sodium phosphate buffer, pH 8; 2mM-ATP; 2·5mM-$MgSO_4$; 0·2mM-[*Me*-^{14}C]choline (specific activity 770 counts/min./mμmole) or 0·2mM-[$^{14}C^2$]ethanolamine (specific activity 800 counts/min./mμmole). The gas phase was air. A 0·5ml. sample of extract was used/1·6ml. of incubation medium. The incubation period was 30min. ▲, Ethanolamine stored at −20°; ●, ethanolamine stored at 0–4°; △, choline stored at −20°; ○, choline stored at 0–4°.

choline phosphorylation. Thiol-binding agents such as sodium arsenite and *p*-chloromercuribenzoate have no effect on either choline or ethanolamine phosphorylation.

Maintenance of choline- and ethanolamine-phosphorylating activities during storage. Some of the characteristics of choline and ethanolamine phosphorylation described above suggest that two enzymes are involved in the phosphorylation of choline and ethanolamine respectively. The results presented in Fig. 2 also support this conclusion. Storage of the cell extracts at −20° or at 0–4° results in an appreciable loss of ethanolamine-phosphorylating activity, whereas choline-phosphorylation remains unchanged (Fig. 2).

Effect of structural analogues of choline and ethanolamine on choline and ethanolamine phosphorylation. A variety of structural analogues of choline and ethanolamine, namely betaine, *NN*-dimethylglycine, *N*-methylglycine and several choline esters, were also examined as inhibitors of choline and ethanolamine phosphorylation. The results in Table 6 show that betaine, *NN*-dimethylglycine and *N*-methylglycine at concentrations of 0·5mM have no effect on either choline or

Table 6. *Effect of structural analogues of choline and ethanolamine on choline and ethanolamine phosphorylation*

Extracts of Ehrlich ascites cells (1ml.) were incubated for 30min. at 37° with 0·2mM-[*Me*-^{14}C]choline (specific activity 770 counts/min./mμmole) or 0·2mM-[$^{14}C_2$]ethanolamine (specific activity 800 counts/min./mμmole); 20mM-Sodium phosphate buffer, pH 8·0, was used. ATP was present at 2mM, Mg^{2+} at 2·5mM. The results given are the averages of at least three determinations. The ranges of change observed are given in parentheses. The control value for choline phosphorylation was 154·5mμmoles and that for ethanolamine 114·6mμmoles. Positive values indicate stimulation, negative values inhibition. 'Nil' is used when the change was less than 5%.

Additions	Change (%) Of choline phosphorylation	Of ethanolamine phosphorylation
Betaine (0·5mM)	Nil	Nil
NN-Dimethylglycine (0·5mM)	Nil	Nil
N-Methylglycine (0·5mM)	Nil	Nil
Phosphorylcholine (0·5mM)	Nil	−35 (31–47)
Phosphorylcholine (1·0mM)	−10 (0–17)	−60 (54–66)
Phosphorylethanolamine (0·5mM)	Nil	Nil
Phosphorylethanolamine (5·0mM)	Nil	−17 (15–18)
Succinylcholine chloride (0·05mM)	+61·0 (52–70)	−25·5 (24–26)
Succinylcholine chloride (0·5mM)	+77·0 (65–83)	−65·4 (63–67)
Butyrylcholine iodide (0·05mM)	+37·0 (36–38)	−18·5 (16–26)
Butyrylcholine iodide (0·5mM)	+59·0 (50–70)	−57·0 (51–61)
Butyrylthiocholine iodide (0·05mM)	+24·0 (20–26)	−27·0 (26–28)
Butyrylthiocholine iodide (0·5mM)	+15·0 (13–17)	−73·0 (71–75)
Propionylcholine iodide (0·05mM)	+15·0 (14–16)	Nil
Propionylcholine iodide (0·5mM)	+40·0 (35–46)	−39·4 (38–41)
Acetyl-β-methylcholine chloride (0·05mM)	+16·0 (10–22)	Nil
Acetyl-β-methylcholine chloride (0·5mM)	+27·0 (24–35)	−14·0 (10–18)
Acetylcholine (0·05mM)	+ 9·0 (6–12)	−10·0 (7–12)
Acetylcholine (0·5mM)	+32·0 (20–38)	−45·0 (44–48)
Methanesulphonylcholine chloride (0·05mM)	Nil	Nil
Methanesulphonylcholine chloride (0·5mM)	+23·0 (19–27)	− 7·0 (5–10)

ethanolamine phosphorylation. Phosphorylcholine inhibits ethanolamine phosphorylation but has much less effect on the phosphorylation of choline itself. Phosphorylethanolamine is ineffective as an inhibitor of choline phosphorylation, and inhibits slightly ethanolamine phosphorylation when higher concentrations are used. Several choline esters tested, namely benzoylcholine, succinylcholine, butyrylcholine, acetyl-β-methylcholine, acetylcholine and methanesulphonylcholine, stimulate choline phosphorylation and inhibit ethanolamine phosphorylation at concentrations between 0·05 and 0·5mM. It is apparent that the degree of stimulation is related to the nature of the acyl side chain of the ester; the bulkier the acyl group, the greater the stimulation. Thus benzoylcholine and succinylcholine bring about the greatest stimulation whereas acetylcholine and methanesulphonylcholine bring about the least stimulation. Butyrylcholine and propionylcholine occupy intermediate positions. It may also be seen that the choline esters with the greatest potentiating effect on choline phosphorylation are the most inhibitory to ethanolamine phosphorylation.

DISCUSSION

Wittenberg & Kornberg (1953) suggested that the choline kinase they purified from yeast was probably also responsible for the phosphorylation of ethanolamine. The work of Ramasarma & Wetter (1957), however, suggested that the choline kinase from Polish rapeseed was specific for choline. In the present work we have obtained evidence that two enzymes may be involved in the phosphorylation of choline and ethanolamine in Ehrlich ascites cells. Although a physical separation of the two activites was not obtained, several lines of evidence are consistent with the conclusion that separate enzymes are involved in the phosphorylation.

First, storage of tissue extracts either in the frozen state or at 0–4° results in an appreciable loss of ethanolamine-phosphorylating activity with no detectable change in choline phosphorylation.

Secondly, although both enzymes require the presence of univalent cations for optimum activity and Mg^{2+} is essential for both, there are marked differences in response to Ca^{2+}. In absence of Mg^{2+}, the phosphorylation of choline is stimulated to a greater extent than that of ethanolamine by Ca^{2+}. However, when Ca^{2+} is present in addition to Mg^{2+}, ethanolamine phosphorylation, but not choline phosphorylation, is markedly inhibited. On the other hand, the presence of Mn^{2+} in the absence of Mg^{2+} stimulates ethanolamine phosphorylation to a greater extent than choline phosphorylation. Mn^{2+}, when present in addition to Mg^{2+}, nearly completely inhibits (about 80%) the phosphorylation of both choline and ethanolamine.

Thirdly, cysteine and 2,3-dimercaptopropanol inhibit ethanolamine phosphorylation at concentrations that are without effect on choline phosphorylation. Neither activity appears to require enzyme thiol groups, since arsenite and *p*-chloromercuribenzoate are without any effect at concentrations that inhibit many thiol enzymes (Johnstone, 1963; Webb, 1966).

Fourthly, eight choline derivatives were examined that inhibited ethanolamine phosphorylation at concentrations between 0·05 and 0·5mM. At these concentrations the choline esters enhanced choline phosphorylation. There appears to be a reciprocal relationship between the extent of stimulation of choline phosphorylation and the inhibition of ethanolamine phosphorylation by several of the choline derivatives. Compounds capable of eliciting a high stimulation of choline phosphorylation also produce the greatest inhibition of ethanolamine phosphorylation. These data suggest either that (*a*) two enzymes are involved or that (*b*) there is an unusual type of allosteric inhibition of the same enzyme so that activity to one substrate is increased while that to the alternative substrate is decreased. In view of the other data presented, the former possibility appears to be the more likely. We considered the possibility that one enzyme may be a polymeric form of the other by examining the effects of dilution of the enzyme preparation on the two activities as had been done by Tomkins, Yielding, Talal & Curran (1963) for glutamate dehydrogenase and alanine dehydrogenase. A 10–20-fold dilution of the extract affected choline and ethanolamine phosphorylation in a parallel manner.

Tris buffer proved to be unsuitable for these studies since it was found to inhibit ethanolamine phosphorylation, but not choline phosphorylation (Table 2). This inhibition by tris no doubt accounts for the observations made by Crone (1966) that tris inhibits ethanolamine incorporation into phospholipids.

Ethanolamine phosphorylation was found to be markedly inhibited by low concentrations of choline in extracts of Ehrlich ascites cells. This phenomenon does not appear to be true in all mammalian tissues, since it was not observed in preparations of mouse liver (Table 4). Phosphorylcholine is also an inhibitor of ethanolamine phosphorylation, but not of choline phosphorylation, in Ehrlich ascites cells at the concentrations examined. The action of phosphorylcholine appears to be direct and not due to its hydrolysis to free choline, since no hydrolysis of phosphorylcholine was observed with extracts of Ehrlich ascites cells.

In summary, the data presented are consistent

with the conclusion that separate enzymes are involved in the phosphorylation of choline and ethanolamine by extracts of Ehrlich ascites cells. The high rates of phosphorylation of both bases no doubt account for the fact that Ehrlich ascites-cell suspensions accumulate greater amounts of phosphorylcholine and phosphorylethanolamine than do other tissues that we have examined (Sung & Johnstone, 1965; Johnstone, 1967).

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