BBA 65689

THE MECHANISM OF ACTION AND SOME PROPERTIES OF SERINE ETHANOLAMINE PHOSPHATE SYNTHETASE

A. K. ALLEN* AND H. ROSENBERG**

Department of Biochemistry, John Curtin School of Medical Research, Australian National University, Canberra (Australia)

(Received August 7th, 1967)

SUMMARY

1. Serine ethanolamine phosphate is synthesised in the microsomes of the chicken intestinal mucosa according to the reaction:

CDP-ethanolamine + L-serine ⇒ CMP + L-serine ethanolamine phosphate

2. In the intact microsomes CDP-ethanolamine participates in two other concomitant reactions: phosphatidylethanolamine biosynthesis and CDP-ethanolamine cleavage. Differential denaturation studies have now shown these three activities to be due to distinct enzymes.

3. Serine ethanolamine phosphate synthetase is absolutely specific for serine, and both stereoisomers are active.

4. In the microsomal system CDP-choline and CDP-serine could not replace CDP-ethanolamine as substrates for serine ethanolamine phosphate synthetase. On the other hand, its phosphonic analogue (CMP-aminoethylphosphonate) and CDP-2-amino-2-methylpropanol were used as substrates and formed the corresponding phosphodiesters with serine. The phosphonic analogue was also an effective substrate for phospholipid biosynthesis.

5. Bivalent cations are essential for activity. Mg^{2+} are most effective, Mn^{2+} and Co^{2+} are less so. Ca^{2+} are inhibitory.

6. Kinetic studies of product inhibition show that the reaction mechanism is ordered, CDP-ethanolamine being the first reactant. The apparent K_m values for the substrates CDP-ethanolamine and L-serine have been calculated to be 0.085 and 1 mM, respectively.

7. A mechanistic model for the reaction is suggested on the basis of these findings.

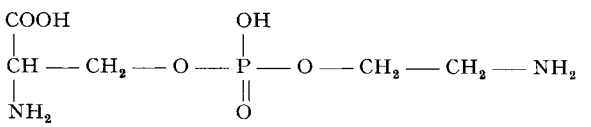
Abbreviation: SEP, serine ethanolamine phosphate.

* Australian National University Research Scholar.

** To whom correspondence should be addressed.

INTRODUCTION

L-Serine ethanolamine phosphate (L-SEP)



was first reported in extracts of the muscle of river turtle *Pseudemys elegans* by ROBERTS AND LOWE[1]. On the basis of degradative studies they suggested the above structure which was later confirmed by synthesis[2].

The distribution of L-SEP in representatives of 12 phyla was investigated in this laboratory[3] and it was found that the compound was confined to the phylum Chordata. Within this phylum it was shown to be present only in fish, amphibians, reptiles and birds, and absent from all of the mammals examined.

The first attempt to elucidate the biosynthesis of L-SEP was made by AYENGAR AND ROBERTS[4]. They found that the injection of ^{32}P-labeled P_i into turtles resulted in a rapid uptake of ^{32}P into ethanolamine phosphate, phospholipids and phosphoproteins, but only slow incorporation into SEP.

On the other hand, ROSENBERG AND ENNOR[3] showed that ^{32}P-labeled P_i injected into a chicken was rapidly incorporated into SEP of several organs. Since [^{32}P]SEP of highest radioactivity was found in the kidney, homogenates of this organ were used to demonstrate the incorporation of ^{32}P-labeled P_i into SEP *in vitro*. These authors[3] also showed that CTP stimulated the incorporation, and suggested that SEP might be synthesised in a manner similar to that of the phospholipids. Further evidence for this proposal came from the finding of ROSENBERG, ENNOR AND SUGAI[5], that a chicken kidney homogenate catalysed the transfer of [^{14}C]ethanolamine from CDP-ethanolamine to SEP, from which it was concluded that SEP synthesis proceeded according to the reaction:

$$\text{CDP-ethanolamine} + \text{L-serine} \rightarrow \text{SEP} + \text{CMP}$$

In a subsequent report ROSENBERG AND ENNOR[6] demonstrated the net synthesis of SEP by a preparation of microsomes from the mucosa of chicken gut. The advantage of this tissue over the kidney preparation lay in the fact that not only did the intestine have a relatively high biosynthetic activity, but it also contained much lower levels of a specific SEP diesterase, which hydrolyses SEP to serine and ethanolamine phosphate[7]. The synthesis of SEP was shown to have an obligatory requirement for Mg^{2+}. It was also observed that CDP-ethanolamine was involved in three reactions catalysed by the microsomal preparation, namely, the transfer of the ethanolamine phosphate moiety of CDP-ethanolamine to serine to produce SEP, to water to produce ethanolamine phosphate, and to endogenous 1,2-diglyceride to produce phosphatidylethanolamine. Such results suggested that the same enzyme may be responsible for these activities, and it was postulated that all reactions may occur *via* a common intermediate involving enzyme-bound ethanolamine phosphate. In an endeavour to gain further information about the nature of this complex, we attempted to obtain a soluble preparation so that the possibility of resolving the three activities could be investigated. These attempts have not been successful. However, in the course of

these experiments it was found that some of the activities could be selectively destroyed. It has now been shown conclusively, both by differential inactivation and in kinetic experiments with the particulate preparation, that SEP synthesis is distinct from the other two activities, and that the reaction mechanism does not involve a common enzyme–ethanolamine phosphate complex.

MATERIALS AND METHODS

CDP-ethanolamine and [β-^{32}P]CDP-ethanolamine were prepared either chemically as described elsewhere[6], or biosynthetically from [^{32}P]ethanolamine phosphate and CTP using the cytidyl transferase (EC 2.7.7.14) from rat liver (SCHNEIDER, FISCUS AND LAWLER[8]). [^{32}P]Ethanolamine phosphate was synthesised by a modification[7] of the method of CHRISTENSEN[9].

[β-^{32}P]CMP-aminoethyl phosphonate was prepared enzymically from CTP and [^{32}P]aminoethyl phosphonate. The enzyme source was a supernatant fraction from *Tetrahymena pyriformis* described by LIANG AND ROSENBERG[10]. The [^{32}P]aminoethyl phosphonate used in this preparation was also prepared biosynthetically[10]; it was completely free from [^{32}P]ethanolamine phosphate.

CDP-serine was a gift from Dr. Y. FURUKAWA of Osaka University. [β-^{32}P]CDP-2-amino-2-methylpropanol was prepared by the method of CHOJNACKI[11]. ^{32}P-Labelled P_i was supplied by the Australian Atomic Energy Commission. L-[3-^{14}C]serine and D-[3-^{14}C]serine were products of California Corporation for Biochemical Research. L-SEP was the natural product isolated from pooled chicken intestines and kidneys as described elsewhere[12]. bis-L-Serine phosphate was a gift from Dr. D. I. MAGRATH of this department. Hog kidney D-amino-acid oxidase, a gift from Mr. B. THORPE of this department, had been prepared according to the method of MASSEY, PALMER AND BENNETT[13] and taken to Stage 3 of the preparation. All other chemicals were analytical grade or the highest purity available.

Separation of compounds on paper

Electrophoresis on Whatman 3 MM paper was carried out at pH 2.0 in a cooled plate apparatus[14], for periods varying from 20 to 60 min, under a voltage gradient of 100 V/cm. The buffer used contained 12.5 ml of 98% formic acid and 43.5 ml of glacial acetic acid per l.

Ascending paper chromatography on Whatman 3 MM was carried out using a solvent of the composition: 95% ethanol–98% formic acid–water (7:1:2, by vol.).

Detection of compounds on paper

Nucleotides were detected by their absorption when viewed under an ultraviolet light. Ninhydrin-reacting compounds were detected by dipping the papers in 0.2% ninhydrin in acetone and heating for 10 min at 80°. Where the need to reisolate compounds from paper precluded the use of chemical reagents, radioactive bands were located by exposure to Kodak 'Blue Brand' X-ray film, for periods varying between 1 and 7 days.

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, using the 'Dioxan' scintillator of BRAY[15] for aqueous samples, and a 'Toluene' scintillator (containing 4.0 g 2,5-diphenyloxazole and 100 mg 1,4-bis-(5-phenyloxa-

zole-2)-benzene per l of toluene) for paper samples and lipids. pH was measured with a glass electrode. Protein was measured by the biuret method[16] with bovine serum albumin as a standard.

Enzyme preparation

Microsomes were prepared from chicken gut mucosa as previously described[6], and were stored at −10° in 0.25 M sucrose containing 1 mM EDTA. Batches of the microsomes were suspended in 8 M urea containing 10 mM EDTA and 0.1 M Tris–HCl buffer (pH 8.5). The suspension was incubated for 1 h at 37° and then dialysed overnight against several changes of 0.02 M imidazole–HCl buffer (pH 7.0), containing 1 mM EDTA. The suspension was then centrifuged at 78 000 × g for 1 h and the pellet resuspended in 0.25 M sucrose to a protein concentration of 10 mg/ml. Chloramphenicol (100 μg/ml) was added and the preparation was stored at 4°. Although the enzyme was stable to freezing, the preparation aggregated on thawing, making the withdrawal of homogeneous samples difficult. However, if stored at 4°, the enzyme preparation remained active and did not aggregate.

Assay systems

SEP synthesis. The assay depended on the estimation of [^{32}P]SEP derived from [β-^{32}P]CDP-ethanolamine of known specific radioactivity. This procedure allowed dilution of reaction products with 'carrier' SEP for convenient isolation. The complete reaction mixture contained: 100 mM imidazole–HCl buffer (pH 7.0), 10 mM $MgCl_2$, 10 mM L-serine, 1.0 mM [β-^{32}P]CDP-ethanolamine (0.1 μC/μmole approx.) and the microsomal preparation (about 1 mg protein). The final volume was 0.5 ml.

The reaction mixtures were usually incubated at 37° for 1 h. For kinetic investigations, to ensure that initial velocities were being measured, each assay was run for 2 time periods (15 and 30 min).

The reaction was stopped by heating the tubes in a boiling water bath for 2 min. They were then cooled in ice, 0.2 μmole of both SEP and ethanolamine phosphate were added and the suspension was centrifuged. The pellet was washed twice by resuspension in water and centrifugation. The combined supernatants were adjusted to pH 5.0 with acetic acid and applied to a column (1 cm × 3 cm) of Dowex 1-2X (200–400 mesh, acetate form). The column was washed with 50 ml of water, and the effluent, in which [^{32}P]SEP was the only radioactive compound, taken to dryness on a rotary evaporator. The concentrate was transferred quantitatively to a vial with 1 ml of water followed by 2 portions of 5 ml of Dioxan scintillator, and the radioactivity was determined in the scintillation counter. (Control experiments with standard samples of radioactive SEP which were put through this procedure, in the presence of boiled microsomal extracts, showed that recovery of SEP was complete.)

Assay of CDP-ethanolamine hydrolysis. This interfering activity results in the presence of [^{32}P]ethanolamine phosphate amongst the reaction products. Unlike SEP, ethanolamine phosphate is not removed from Dowex 1 (acetate form) with water, and this forms the basis of the assay. Thus, after SEP was removed from the resin with water, the column was eluted with 20 ml of 5% acetic acid. This eluate contained ethanolamine phosphate and CDP-ethanolamine as the only radioactive components. It was concentrated under reduced pressure, transferred quantitatively to paper and

subjected to chromatography. Ethanolamine phosphate, which was well separated from CDP-ethanolamine in the system used, was located with 0.01% ninhydrin. The corresponding areas were cut out and counted in Toluene scintillator.

Assay of phospholipid biosynthesis. When it was desirable to determine the incorporation of radioactivity from [^{32}P CDP-ethanolamine into microsomal phospholipid, the reaction was stopped with 5% (final concn.) trichloroacetic acid. The pellet was washed twice with water and extracted 3 times with chloroform–methanol mixtures (2:1, by vol.). The combined lipid extracts were placed in a vial, taken to dryness with a current of air and counted after the addition of 10 ml of the Toluene scintillator. Extraction and purification according to the method of KANFER AND KENNEDY[17] showed that all the radioactivity in this material was present in the phospholipids.

Analysis of data from kinetic experiments

The initial velocity data were plotted in double reciprocal form to check the linearity of the plots and any points which lay well off the line were discarded. The data were then analysed by means of the appropriate computer programme of CLELAND[18] with the use of an IBM 1620 computer. According to the type of plot, the SEQUEN, COMP or NONCOMP programmes were used (Eqns. 7, 8 or 10 of ref. 18) to obtain the best estimates of the kinetic constants, together with their standard errors. These values were used to draw the lines of the figures; the points represent the actual experimental values.

RESULTS

The distribution of serine ethanolamine phosphate synthetase in the tissues of the chicken

In a study of the distribution of SEP synthetase, microsomal preparations from 9 tissues (Table I) were assayed for this activity as well as for glyceride ethanol-

TABLE I

THE DISTRIBUTION OF SEP SYNTHETASE AND GLYCERIDE ETHANOLAMINE PHOSPHOTRANSFERASE IN THE TISSUES OF THE CHICKEN

In all tissues the source of enzyme was the resuspended microsomal fraction. For conditions of assay see MATERIALS AND METHODS.

Tissue	*mμmoles of product formed/h per mg microsomal protein*	
	SEP	*Phosphatidyl-ethanolamine*
Gut mucosa	15	18
Kidney	5	30
Pancreas	0.5	6
Liver	<0.1	37
Spleen	<0.1	10
Brain	<0.1	7
Oviduct	<0.1	0.5
Blood	<0.1	0.5
Heart	<0.1	<0.1

amine phosphotransferase. It is obvious that of the two activities the former has a much narrower distribution, and gut mucosa, which was used throughout this work, showed the highest activity.

Separation of SEP synthetase from nucleotide pyrophosphatase (EC 3.6.1.9) and diglyceride ethanolamine phosphotransferase (EC 2.7.8.1)

As mentioned in INTRODUCTION, the separation of SEP synthetase from the other two enzymes which use CDP-ethanolamine would be simplified if the complex could be solubilised.

In an attempt to obtain a soluble preparation of SEP synthetase we have used

TABLE II

DIFFERENTIAL INACTIVATION OF THE MICROSOMAL ENZYMES WHICH UTILIZE CDP-ETHANOLAMINE

Treatment	*Enzymic activity following treatment*		
	SEP synthetase	*Ethanolamine glyceride phosphotransferase*	*CDP-ethanolamine pyrophosphatase*
1% Nonidet P-40	Inactive	Inactive	Active
1% Triton X-100	Inactive	Inactive	Active
1% Digitonin	Active	Active	Active
Butanol extraction	Inactive	Inactive	Inactive
Acetone powder	Inactive	Inactive	Active
Incubation at 55° for 15 min	Inactive	—	Active
Incubation at 37° for 60 min in 8 M urea	Active	Inactive	Inactive

a variety of treatments (Table II), which resulted in differential denaturation of the three activities, but did not bring about the release of a truly soluble SEP synthetase. Treatment with digitonin did result in apparent solubilisation, but the resulting preparation, when subjected to freezing and thawing or dialysis, showed signs of aggregation. Such behaviour is characteristic of detergent-solubilised preparations (*cf.* NORDLIE AND ARION[19]). However, it can be seen that treatment with 8 M urea for 1 h, while not resulting in a soluble enzyme, did produce a preparation of SEP synthetase virtually free from the other two activities (Fig. 1). This treatment thus resulted in a preparation suitable for kinetic and other studies of SEP synthetase.

Substrate specificity

Amino acids. SEP synthetase displays a high degree of specificity towards serine. Thus, of a number of natural and synthetic hydroxyamino acids tested, only DL-α-methylserine could replace serine as a substrate. The following compounds could not replace L-serine in the reaction: *N*-acetyl-L-serine, L-homoserine, 4-hydroxy-L-proline, 5-hydroxy-DL-lysine, 3-hydroxy-DL-glutamic acid, L-threonine, ethanolamine and 3-hydroxypropionic acid. However, the addition at 10 mM concentration of L-threonine, L-homoserine or L-alanine produced between 27 and 34% inhibition of the reaction when tested in the standard assay. The apparent K_m for serine was calculated to be 1 mM (Fig. 2).

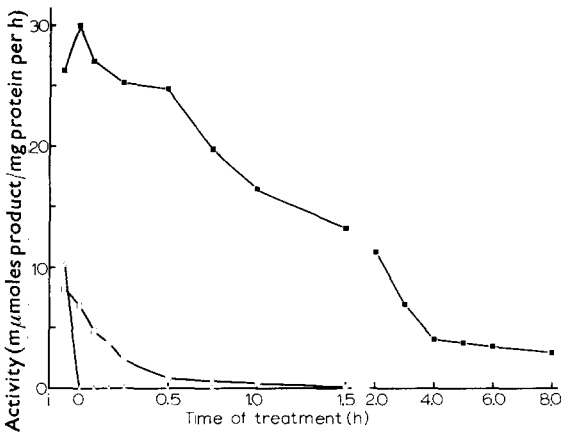


Fig. 1. The effect of urea on some synthetic activities of microsomes from chicken gut mucosa. The microsomal preparation was incubated at 37° in 0.1 M Tris buffer (pH 8.5) containing 8 M urea and 0.01 M EDTA. Samples were taken at the time intervals shown and immediately dialysed against cold water. The fully dialysed samples were assayed under standard conditions (see MATERIALS AND METHODS). The first point on each curve (marked i on the abscissa) represents initial activity before the addition of urea. Abscissa: time of incubation in urea; ordinate: activity (mμmoles of respective product formed per h of assay); ■, SEP production; △, ethanolamine phosphate production; □, phospholipid production.

Stereospecificity with respect to serine. The microsomal preparation catalysed the incorporation of radioactivity from D-[3-^{14}C]serine into SEP. This observation alone did not constitute sufficient proof of the incorporation of D-serine since, firstly, the D-[^{14}C]serine available contained some 10% L-[^{14}C]serine and, secondly, the result did not preclude racemisation (however unlikely) prior to incorporation. The direct demonstration, by a specific enzymatic method, that D-serine was present in SEP synthesised by the microsomal preparation (Table III), leaves no doubt that the enzyme lacks stereospecificity with respect to serine.

Nucleotides. To test CDP-choline and CDP-serine as analogues of CDP-ethanolamine in the system, 0.25 μmole of each nucleotide was incubated with 0.07 μmole

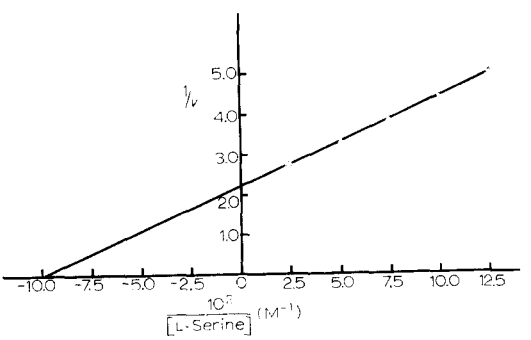


Fig. 2. The effect of variation of the concn. of L-serine on the rate of synthesis of SEP. CDP-ethanolamine concns. were held constant at 1.0 mM. v is in arbitrary units. Conditions of assay are described in MATERIALS AND METHODS.

TABLE III

DETERMINATION OF THE OPTICAL CONFIGURATION OF SERINE IN SEP

Microsomes were incubated for 5 h with CDP-ethanolamine and either D- or L-[3-^{14}C]serine under the conditions described in MATERIALS AND METHODS. The trichloroacetic acid-soluble materials were processed, and separated by paper chromatography (see MATERIALS AND METHODS). The L-SEP and (presumed) D-SEP were isolated and were each hydrolysed with the SEP-diesterase (which is not stereospecific) in 0.1 M Tris–HCl buffer (pH 9.0) for 5 h at 37°. Serine was isolated from the products of each reaction mixture and was incubated 4 h with D-amino acid oxidase in 0.1 M $Na_2P_4O_7$ buffer (pH 8.7). The reaction products were applied to Dowex-50 (H^+) ion-exchange resin which was washed and eluted as shown below, and the radioactivity in the effluents was measured.

	From L-*SEP*	*From* D-*SEP*
Radioactive products expected		
(a) After SEP diesterase	L-[^{14}C]Serine	D-[^{14}C]Serine
(b) After D-amino acid oxidase	L-[^{14}C]Serine (no oxidation)	[^{14}C]Hydroxy-pyruvate
Results		
counts/min applied to Dowex 50 (H^+)	4650	2800
counts/min in water effluent and wash	90	2300
counts/min eluted with NH_4OH	4390	460
Total recovery (counts/min)	4480	2760
D *Isomer (%)*	<1	82.5*

* This value was reduced to less than 1% if the D-amino-acid oxidase was denatured before incubation.

of L-[3-^{14}C]serine (200 000 counts/min) and the remaining components of the standard assay mixture for 1 h. The deproteinised supernatants were subjected to paper electrophoresis and autoradiographed. The only radioactive areas located on the electrophoretogram by this method were those corresponding to serine. In the case of the CDP-serine incubation, bis-L-serine phosphodiester was added to the deproteinised solution to aid the detection of any radioactive phosphodiester. The bis-L-serine-

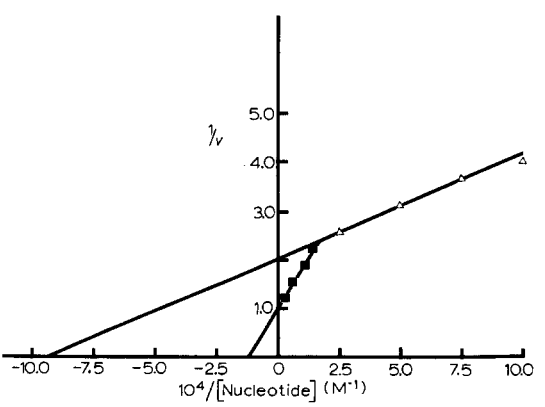


Fig. 3. The effect of variation of the concn. of CMP-aminoethyl phosphonate (△) and CDP-ethanolamine (■) on the synthesis of serine aminoethyl phosphonate and SEP, respectively. Conditions as described in MATERIALS AND METHODS. The same enzyme preparation was used for both determinations. The concn. of L-serine was 10 mM. v is in arbitrary units.

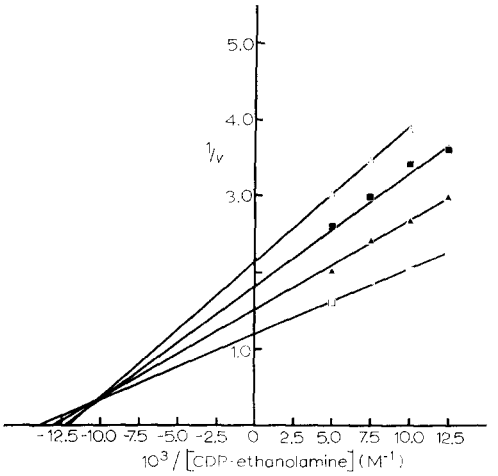


Fig. 4. The effect of L-serine concn. on the initial velocity of SEP synthesis with CDP-ethanolamine as the variable substrate. v is in arbitrary units. The concns. of L-serine were: 2 mM (□); 1 mM (▲); 0.66 mM (■); 0.5 mM (△).

phosphate band was located by the use of 0.01% ninhydrin reagent and the corresponding area of the paper was cut out and counted in Toluene scintillator. The radioactivity in this band did not exceed that of the control. In a similar experiment it was found that the microsomal preparation did not catalyse the production of [^{14}C]SEP from CDP-serine and [^{14}C]ethanolamine. On the other hand, when [β-^{32}P]-CDP-ethanolamine was replaced by [β-^{32}P]CDP-2-amino-2-methylpropanol in the standard assay, radioactivity was incorporated into a SEP analogue, and into lipid (*cf.* CHOJNACKI[11]).

CMP-aminoethyl phosphonate is the phosphonate analogue of CDP-ethanolamine, differing from it by the possession of a direct link between carbon and the β-phosphorus[10]. This compound was found to be an effective substitute for CDP-ethanolamine (Fig. 3). The apparent K_m values calculated for CMP-aminoethyl phosphonate and for CDP-ethanolamine were 11 μM and 85 μM, respectively. However, the value of apparent $v_{max.}$ for CMP-aminoethyl phosphonate is lower than that for CDP-ethanolamine. Proof of the incorporation of the aminoethyl phosphonate moiety of CMP-aminoethyl phosphonate into an analogue of SEP is given in Table IV. It rests upon the isolation of this compound after incubation under standard conditions (but with [β-^{32}P]CMP-aminoethyl phosphonate replacing CDP-ethanolamine) and its subsequent acid hydrolysis to yield serine and aminoethyl phosphonate. Under the same conditions SEP yields serine, ethanolamine and P_i. The appearance of P_i after incubation of SEP with SEP diesterase (Table IV) was due to traces of phosphomonoesterase present in the diesterase preparation.

The microsomal preparation also catalysed the biosynthesis of a phosphonic analogue of a phospholipid (presumably cephalin) when [β-^{32}P]CMP-aminoethyl phosphonate was used as a substrate. Subsequent hydrolysis of the microsomal lipid showed that the bulk of the radioactivity (6040 counts/min of a total 6099) was still associated with aminoethyl phosphonate.

TABLE IV

EVIDENCE FOR THE PRODUCTION OF A PHOSPHONIC ANALOGUE OF SEP

Incubations were carried out as described in MATERIALS AND METHODS, except that in one incubation CDP-ethanolamine was replaced by CMP-aminoethyl phosphonate. The reaction products were separated by paper electrophoresis, the product from each incubation was eluted from the paper and divided into two equal parts. One was incubated with SEP diesterase for 30 min at pH 9.0; the other was hydrolysed with 6 M HCl for 48 h at 110°. The treated samples were again subjected to paper electrophoresis and the radioactivity of appropriate areas of the electrophoretogram were determined after location by autoradiography.

	Reaction products			
	Serine aminoethyl phosphonate		*SEP*	
Method of cleavage of product	*SEP diesterase*	*6 M HCl*	*SEP diesterase*	*6 M HCl*
Counts/min in aminoethyl phosphonate	214	194	—	—
Counts/min in ethanolamine phosphate	—	—	300	2
Counts/min in inorganic phosphate	5	3	189	460

Metal-ion requirement for SEP synthetase

Metal ions are necessary for the activity of SEP synthetase (Table V); at 10 mM, Mg^{2+} are at least 10 times more effective than Mn^{2+} or Co^{2+}. On the other hand, these three metals are equally good activators of the ethanolamine glyceride phosphotransferase activity.

Inhibition of SEP synthetase by specific reagents

All the sulphydryl reagents investigated inhibited SEP synthetase; iodoacetate was less effective than iodoacetamide. This indicates that a free sulphydryl group is essential for activity and that its immediate ionic environment is such that it interferes with the approach of the charged iodoacetate molecule. The inhibition by *N*-acetylimidazole and diazotized sulphanilic acid would suggest that a tyrosine

TABLE V

THE EFFECT OF VARIOUS METAL IONS ON THE RATES OF SYNTHESIS OF SEP AND PHOSPHATIDYL ETHANOLAMINE BY A MICROSOMAL PREPARATION

See MATERIALS AND METHODS for assay conditions. The metal ions were present as their chloride salts at a concn. of 10 mM.

Ion	*mμmoles of product formed/h per mg of protein*	
	SEP	*Phospholipid*
Mg^{2+}	42	17.8
Mn^{2+}	4.2	21.7
Co^{2+}	2.4	20.3
Ca^{2+}	0.2	0.0
$Mg^{2+} + Ca^{2+}$	0.2	0.0
EDTA	0.0	0.0
None	0.4	0.0

TABLE VI

THE EFFECT OF SPECIFIC REAGENTS ON THE ACTIVITY OF SEP SYNTHETASE

For assay conditions see MATERIALS AND METHODS. Preincubations with these reagents were all conducted at 37° with the exception of the preincubation with diazotized sulphanilic acid which was at 0°.

Reagent	*Concn. (mM)*	*Pre-incubation time (min)*	*Per cent activity remaining*
p-Hydroxymercuribenzoate	0.1	30	2
Methylmercuric iodide	0.1	30	3
Methylmercuric bromide	0.1	30	6
Phenylmercuric acetate	0.1	30	2
N-Ethylmaleimide	0.2	30	25
Iodoacetamide	1	60	6
Iodoacetate	1	60	42
DFP	1	60	100
N-Acetylimidazole	10	60	8
Diazotized sulphanilic acid	0.2	30	36

residue is also involved. Since the enzyme is not affected by DFP it is unlikely that a serine residue is involved in the catalytic activity.

Reaction mechanism

If, as postulated[6], the enzyme reacted with CDP-ethanolamine to form an intermediate enzyme–ethanolamine phosphate complex with the release of CMP, it would be expected to catalyse a partial exchange reaction between [^{14}C]CMP and unlabelled CDP-ethanolamine. However, when [^{14}C]CMP was incubated with the enzyme preparation and unlabelled CDP-ethanolamine, no radioactivity (less than

TABLE VII

THE PRODUCT INHIBITION PATTERNS FOR ENZYMES WITH TWO SUBSTRATES (A AND B) AND TWO PRODUCTS (P AND Q) WHEN THE SUBSTRATE WHICH IS NOT VARIED IS AT NON-SATURATING CONCENTRATION*

See Fig. 5. NC, non-competitive; C, competitive.

Mechanism	*Product tested as inhibitor*	*Variable substrate*	
		A	*B*
Ordered	P	NC*	NC
	Q	C*	NC
Theorell–Chance	P	NC	C
	Q	C	NC
Rapid equilibrium random	P	C	C
	Q	C	C
Ping-Pong (non-sequential)	P	NC	C
	Q	C	NC

* After CLELAND[20].

0.1%) was incorporated into CDP-ethanolamine, showing that no exchange had taken place. Thus it is unlikely that the reaction is of the Ping-Pong type[20]. Kinetic studies were undertaken to gain further information about the reaction mechanism.

Initial velocity and product inhibition studies

The effect of the concentration of CDP-ethanolamine on the initial velocity of the reaction at different fixed concentrations of L-serine is illustrated (Fig. 4) in the form of a double reciprocal plot. The results indicate that the family of straight lines intersect to the left of the ordinate. Thus it may be concluded that the reaction mechanism is of the Sequential type so that both substrates must add to the enzyme

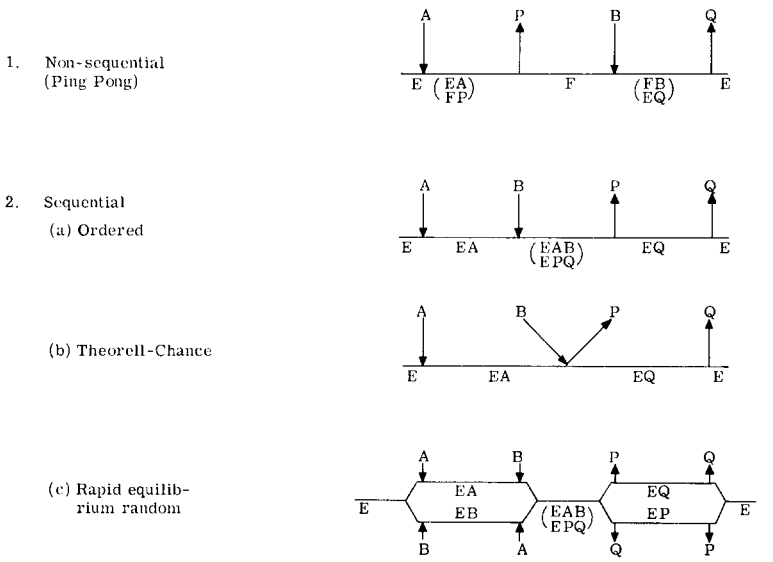


Fig. 5. Basic reaction mechanisms for enzyme with two substrates (A and B) and two products (P and Q) (after CLELAND[20]).

before the product is released. However, from the results of initial velocity studies, it is not possible to distinguish between the three possible Sequential mechanisms (see Fig. 5), but this can be done by means of product inhibition studies[20] (see Table VII).

The data from the product inhibition studies (Figs. 6–8) were analysed by computer (see MATERIALS AND METHODS) to determine whether a competitive or non-competitive inhibition pattern would give the best fit for the data. The following conclusions were arrived at.

CMP was found to act as a linear competitive inhibitor of the reaction with respect to CDP-ethanolamine (Fig. 6) and as a linear non-competitive inhibitor with respect to serine (Fig. 7). SEP was also a linear non-competitive inhibitor in relation to serine (Fig. 8). From these results, it may be concluded that the SEP synthetase reaction has an ordered mechanism.

Although complete inhibition studies were not attempted, it was found that

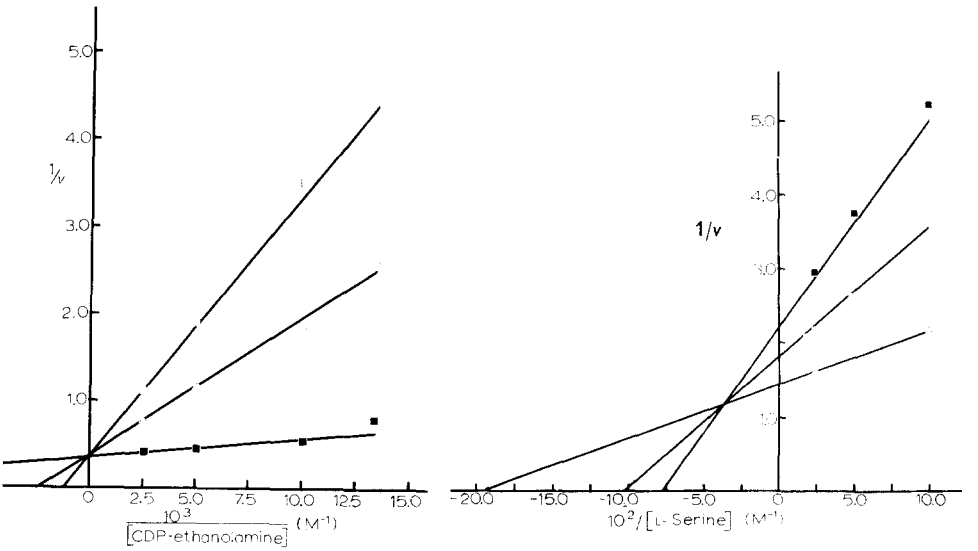


Fig. 6. Inhibition of SEP synthesis by CMP with CDP-ethanolamine as the variable substrate and L-serine concn. held constant at 2 mM. v is in arbitrary units. The concns. of CMP were: none (■); 0.2 mM (△); 0.4 mM (□).

Fig. 7. Inhibition of SEP synthesis by CMP with L-serine as the variable substrate and the CDP-ethanolamine concn. held constant at 0.2 mM. v is in arbitrary units. The concns. of CMP were: none (△); 0.1 mM (□); 0.2 mM (■).

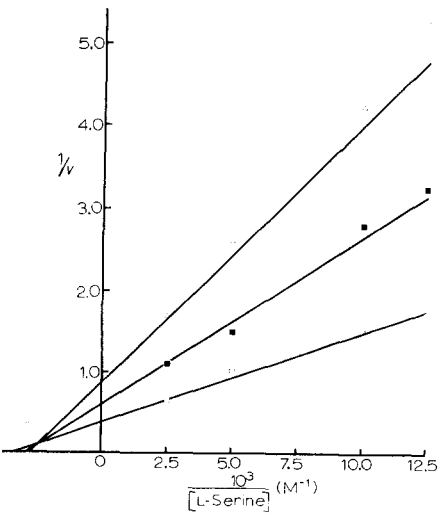


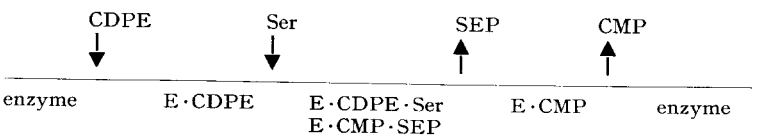
Fig. 8. Inhibition of SEP synthesis by SEP with L-serine as the variable substrate. v is in arbitrary units. The concn. of CDP-ethanolamine was held constant at 0.08 mM. The concns. of SEP were: none (□); 40 mM (■); 80 mM (△).

CTP was about twice as inhibitory as CMP when used at the same concentration. In preliminary studies CMP and CTP were found to inhibit the microsomal-stimulated incorporation of [β-^{32}P]CDP-ethanolamine into phosphatidylethanolamine to about the same extent as the incorporation into SEP.

DISCUSSION

The original hypothesis that one enzyme in the chicken gut microsomes was responsible for the synthesis of SEP and cephalin and for the hydrolytic breakdown of CDP-ethanolamine, *via* an enzyme–ethanolamine phosphate complex, must now be revised. The present results leave little doubt that these three activities are, in fact, distinct. Present kinetic studies clearly indicate that the reaction mechanism is not compatible with the existence of an active enzyme–ethanolamine phosphate complex. Furthermore, the various activities showed different stabilities under a variety of treatments. Thus butanol, detergent or acetone treatment abolished both SEP and cephalin synthesis, while the pyrophosphatase activity remained. On the other hand, urea-treated preparations synthesised SEP but lost the ability to synthesise cephalin and to cleave CDP-ethanolamine. The kinetic evidence obtained gave a clear indication of the mechanism of SEP synthesis. Thus, the double reciprocal plots of initial velocities *versus* CDP-ethanolamine concentration with variable, non-saturating concentrations of serine gave a series of converging straight lines, showing that the reaction was sequential. A non-sequential mechanism would have given parallel lines. The sequential nature of the mechanism was further confirmed by the failure to obtain enzyme-catalysed exchange between CMP and CDP-ethanolamine. This means that both CDP-ethanolamine and serine have to be combined with the enzyme before any product is released.

The inhibition of the reaction by CMP is competitive with respect to CDP-ethanolamine, as would be expected for any type of sequential (rather than non-sequential) mechanism for which CDP-ethanolamine was the first reactant (see Table VII). Inhibition of the reaction by CMP is non-competitive with respect to serine and this would make a rapid equilibrium random mechanism unlikely, unless a dead-end enzyme–CMP-serine complex were formed. The non-competitive inhibition by SEP with respect to serine is not consistent with either a rapid equilibrium random mechanism, a Theorell–Chance mechanism or an ordered reaction in which serine is the first reactant, but is consistent with an ordered sequential reaction mechanism in which CDP-ethanolamine is the first reactant. The proposed mechanism for this reaction can be illustrated as follows (E, enzyme; CDPE, CDP-ethanolamine):



As far as we are aware this is the first reported investigation of the reaction mechanism of an enzyme of the EC 2.7.8 group.

Although inhibition of the reaction by SEP is of theoretical importance in the determination of the mechanism of action of the enzyme, high concentrations of SEP

are needed before any inhibition is apparent and it is therefore unlikely that SEP biosynthesis *in vivo* is controlled by the levels of SEP normally found in the tissues (2 μmoles/g in the gut)[3]. However, the other product of the reaction, CMP, does inhibit the reaction at physiological concentrations and therefore could be a means of controlling the synthesis *in vivo*. The similar response of SEP synthetase and glyceride ethanolamine phosphotransferase to CMP and CTP is further evidence of the close metabolic association already noted[6] between these enzymes.

The specificity with respect to the terminal portion of the cytidine nucleotides is similar to that of transferases which catalyse phospholipid synthesis. Thus, CHOJNACKI[11] showed that a variety of cytidine diphosphate derivatives (containing unnatural bases) were suitable substrates for the biosynthesis of phospholipids; he concluded that the base specificity of these phosphotransferases (EC 2.7.8.1 and 2.7.8.2) is low. We have shown that CDP-2-amino-2-methylpropanol which is a substrate for the glyceride ethanolamine transferase[11] (EC 2.7.8.1) is also a substrate for SEP synthetase.

The synthesis of phosphonate analogues of both SEP and phosphatidyl ethanolamine from CMP-aminoethyl phosphonate is in accord with the work of KANDATSU, HORIGUCHI AND TAMARI[21] who reported that [^{32}P]aminoethyl phosphonate fed to rats was incorporated into their phospholipids. Thus avian, as well as mammalian, systems cannot reject aminoethyl phosphonate as a source of phospholipid base. In the protozoan *Tetrahymena pyriformis*, aminoethyl phosphonate is a normal constituent of the lipid[10].

The experimental findings reported here allow some conclusions to be drawn about the nature of the reactive site. Thus, the sequential addition of the two reactants demanded by the mechanism means that CDP-ethanolamine must be on the active site before serine can react with the enzyme. This may mean an effect of CDP-ethanolamine on the serine site or on the alignment. The enzyme is similar to phosphotransferases in that it has a requirement for divalent metal ions which may be satisfied by Mg^{2+}. One of the ways in which Mg^{2+} may function would involve binding to the acidic groups of the pyrophosphate of CDP-ethanolamine, with possible further co-ordination to the enzyme site.

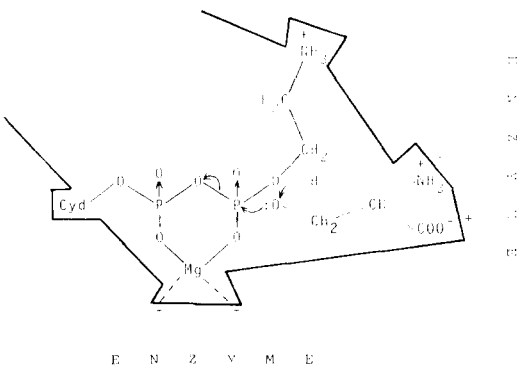


Fig. 9. A hypothetical model for the reactive site. The charged sites on the enzyme have been shown where a compatible charged group on one of the substrates was found to be essential. The electron shift which constitutes the reaction is shown by curved arrows.

The special alignment of the serine is important since homoserine does not react, possibly because of the increased length of the molecule. Steric hindrance in the region of the β-carbon of serine is not tolerated since L-threonine is not active. The charged groups of serine — the carboxyl and amino group — are both important in the reaction since ethanolamine, *N*-acetylserine and 3-hydroxypropionate are not active as substrates. Steric arrangements around the α-carbon of serine are less important since both L- and D-serine, as well as α-methylserine are active.

Although the specificity for the aliphatic base attached to the CDP is not strict, the lack of activity with CDP-serine suggests a requirement for a net positive charge on the terminal end. C-methylated derivatives of CDP-ethanolamine are active, but methylation of the nitrogen apparently introduces steric hindrance since CDP-choline, although it possesses a positive charge on the nitrogen, is not a substrate.

These observations have been combined into a hypothetical model shown in Fig. 9. For the sake of clarity the serine and CDP-ethanolamine molecules have been shown adjacent. It is suggested that the reaction takes place through a nucleophilic attack by the hydroxyl oxygen of serine on the β-phosphorus.

The function of SEP is as yet unknown, but there is a rapid turnover of this compound, and an association with phospholipid metabolism seems likely[6]. Further studies are at present being pursued in an attempt to determine the role of this compound in avian metabolism.

ACKNOWLEDGEMENTS

We wish to thank Dr. Y. FURUKAWA of Osaka University for the gift of CDP-serine, Dr. D. I. MAGRATH of this department for bis-L-serine phosphodiester and Mr. B. THORPE of this department for the D-amino-acid oxidase preparation.

Our thanks are also due to Dr. J. F. MORRISON for helpful discussions in the design and interpretation of the kinetic experiments.

REFERENCES

1 E. ROBERTS AND I. P. LOWE, *J. Biol. Chem.*, 211 (1954) 1.
2 E. E. JONES AND D. J. LIPKIN, *J. Am. Chem. Soc.*, 78 (1956) 2408.
3 H. ROSENBERG AND A. H. ENNOR, *J. Biochem. Tokyo*, 50 (1961) 81.
4 P. AYENGAR AND E. ROBERTS, *Proc. Soc. Exptl. Biol. Med.*, 103 (1960) 811.
5 H. ROSENBERG, A. H. ENNOR AND S. SUGAI, *Nature*, 203 (1964) 410.
6 H. ROSENBERG AND A. H. ENNOR, *Biochim. Biophys. Acta*, 115 (1966) 23.
7 D. D. HAGERMAN, H. ROSENBERG, A. H. ENNOR, P. SCHIFF AND S. INOUE, *J. Biol. Chem.*, 240 (1965) 1108.
8 W. C. SCHNEIDER, W. G. FISCUS AND J. A. B. LAWLER, *Anal. Biochem.*, 14 (1966) 121.
9 H. N. CHRISTENSEN, *J. Biol. Chem.*, 135 (1940) 399.
10 C. R. LIANG AND H. ROSENBERG, *Biochim. Biophys. Acta*, 125 (1966) 548.
11 T. CHOJNACKI, *Acta Biochim. Polon.*, 11 (1964) 11.
12 A. H. ENNOR, H. ROSENBERG, R. J. ROSSITER, I. M. BEATTY AND T. GAFFNEY, *Biochem. J* 75 (1960) 179.
13 V. MASSEY, G. PALMER AND R. BENNETT, *Biochim. Biophys. Acta*, 48 (1961) 1.
14 G. N. ATFIELD AND C. J. O. R. MORRIS, *Biochem. J.*, 81 (1961) 606.
15 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
16 A. G. GORNALL, C. J. BARDAWILL AND M. M. DAVID, *J. Biol. Chem.*, 177 (1949) 751.
17 J. KANFER AND E. P. KENNEDY, *J. Biol. Chem.*, 238 (1963) 2919.
18 W. W. CLELAND, *Nature*, 198 (1963) 463.
19 R. C. NORDLIE AND W. J. ARION, *J. Biol. Chem.*, 239 (1964) 1680.
20 W. W. CLELAND, *Biochim. Biophys. Acta*, 67 (1963) 104.
21 M. KANDATSU, M. HORIGUCHI AND M. TAMARI, *Agr. Biol. Chem. Tokyo*, 29 (1965) 779.