Enzymatic synthesis and decarboxylation of phosphatidylserine in *Tetrahymena pyriformis*

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ABSTRACT Cell-free extracts of the protozoan *Tetrahymena pyriformis* have been found to catalyze the following reactions:

Phosphatidylethanolamine + L-Serine ⇌ Phosphatidylserine + Ethanolamine

Phosphatidylserine → Phosphatidylethanolamine + CO_2

Net: L-Serine → Ethanolamine + CO_2

The biosynthesis of phosphatidylserine in this organism resembles that found in higher animals. In contrast, phosphatidylserine is formed in bacteria in a reaction between CDP-diglyceride and L-serine. A detailed description is presented of the enzyme that catalyzes the synthesis of phosphatidylserine in *Tetrahymena* by the exchange reaction, together with some properties of the phosphatidylserine decarboxylase in this organism.

SUPPLEMENTARY KEY WORDS biosynthesis · exchange enzyme · mechanism · phosphatidylethanolamine · phosphatidylserine decarboxylase · phospholipids

THE *de novo* formation of the phosphodiester bond of phospholipids always involves cytidine coenzymes (1, 2). However, the pathway for the formation of serine- and ethanolamine-containing phospholipids differs in animal and bacterial systems. In animal tissues, CDP-ethanolamine is an obligatory intermediate in the formation of phosphatidylethanolamine via reaction (*1*) which leads to the net formation of the phosphodiester bond.

(*1*) CDP-ethanolamine + α,β-Diglyceride→Phosphatidylethanolamine + CMP

The incorporation of serine into phospholipid in animal tissues (3) takes place via an exchange reaction:

(*2*) Phosphatidylethanolamine + L-Serine ⇌ Phosphatidylserine + Ethanolamine

This is the only known pathway for serine incorporation into phospholipid in animal tissues.

In contrast, bacteria produce phosphatidylserine directly from CDP-diglyceride and L-serine (4):

(*3*) CDP-diglyceride + L-Serine → Phosphatidylserine + CMP

In both animal and bacterial systems, the decarboxylation (3, 5, 6) of serine takes place at the lipid level:

(*4*) Phosphatidylserine → Phosphatidylethanolamine + CO_2

In animal tissues, reactions (*2*) and (*4*) comprise the "decarboxylation cycle" discussed by Kennedy (2) and illustrated in Fig. 1. This cycle leads to the net production of ethanolamine and CO_2.

Since these two distinct pathways exist for animal and bacterial systems, it was of interest to determine the pathway in protozoa. In this paper, we shall present evidence for the occurrence of enzymes catalyzing reactions (*2*) and (*4*) in the protozoan *Tetrahymena pyriformis*. Reaction (*3*) has not been detected in *Tetrahymena*, nor has it been demonstrated in any animal tissue. The exchange enzyme catalyzing reaction (*2*) in cell-free preparations of *Tetrahymena* will be described in some detail; this enzyme has similar properties to the analogous enzyme found in rat liver (3, 7) and the housefly, *Musca domestica* (8).

This study of the pathway for phosphatidylserine and phosphatidylethanolamine biosynthesis in *Tetrahymena* has one further purpose. *Tetrahymena* contains considerable amounts of the phosphono analogue of phosphatidylethanolamine. The natural occurrence of the

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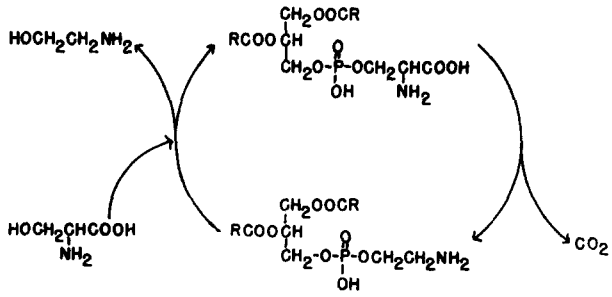

FIG. 1. Decarboxylation cycle in animal tissues.

phosphonolipids was first demonstrated by Horiguchi and Kandatsu in 1959 (9). Since then, the biochemistry of these lipids in *Tetrahymena*, several marine invertebrates, and higher animals has been the object of considerable study (10, 11). The mechanism of the biosynthesis of the phosphonolipids has not yet been elucidated. However, available evidence suggests that the synthesis of the C-P bond occurs at the lipid level (12–14). The pathways for the biosynthesis of the ethanolamine- and serine-containing lipids in this organism are thus of special interest. Glucose-^{14}C and related compounds are the precursors of the polar head-groups of the phosphonolipids (13, 15–17), and it has been suggested that phosphoenolpyruvate or oxaloacetate may be intermediates. Since it is possible that these compounds may be incorporated into phospholipids by way of an exchange reaction, we have tested phosphoenolpyruvate and oxaloacetate for their ability to displace ethanolamine from labeled phosphatidylethanolamine in cell-free extracts under conditions in which the exchange enzyme is active. Neither compound appears to be a substrate for this enzyme.

MATERIALS AND METHODS

Culture Conditions

A culture of *Tetrahymena pyriformis W* (Strain 10542) was obtained from the American Type Culture Collection (Rockville, Md.). The growth medium consisted of 0.5% proteose peptone (Difco Inc., Findlay, Ohio), 0.5% bactotryptone (Difco Inc.), and 0.02% KH_2PO_4 (Mallinckrodt Chemical Works, St. Louis, Mo.). The pH was adjusted to 7.1 with KOH. Stock cultures were grown at room temperature and were transferred weekly. Cultures were examined periodically using a phase-contrast microscope; growth was monitored at 650 nm. Under these conditions, mid-exponential phase was reached in 2–3 days.

Cell-Free Extracts

Cultures were grown as above using either (*a*) 500 ml of medium in a 2 liter DeLong culture flask equipped with a magnetic stirrer and maintained at 28–30°C in a water bath or (*b*) 14 liters of medium (containing 0.5 ml of silicone antifoam) in a Microferm Laboratory Fermentor (New Brunswick Scientific Co., New Brunswick, N. J.), equipped with mechanical stirring at 125 rpm, aeration at 4 psi, and maintained at 29°C. In the latter case, 1 liter of log-phase cells was used as innoculum, and the cultures were harvested in log phase (about 24 hr) as follows: the culture was cooled with a circulating ethylene glycol bath to about 2°C, and the cells were collected in a Sorvall centrifuge equipped with a Szent-Gyorgyi Blum continuous flow system (15,000 *g*, 0°C, 150 ml/min flow rate). The cells were washed once with 1.2 liter of buffer containing 20 mM imidazole-HCl, pH 7.1, 0.25 M sucrose, and 5 mM mercaptoethanol, and the cells were collected by centrifugation (15,000 *g*, 0°C, 15 min).

The washed cells were taken up in 125 ml of the same buffer and disrupted with a Potter–Elvejhem homogenizer (60 strokes, 0°C, 25-ml batches). Samples were checked microscopically to insure complete cell breakage. The broken cells were centrifuged (10,000 *g*, 0°C, 30 min), and the pellet was washed with 150 ml of buffer and respun. The washed pellet was taken up in 30 ml of 0.25 M sucrose, frozen in methyl Cellosolve or dry ice, and lyophilized. The resulting lyophilized powder was stored in the cold and retained full enzymatic activity for at least 6 months. A 14 liter preparation harvested in early log phase according to the above procedure usually yielded about 3.0 g of lyophilized powder containing about 0.5–0.8 g of protein (about 60% of the protein in the unwashed homogenate) as determined by the method of Lowry, Rosebrough, Farr, and Randall (18). About 90% of the enzymatic activity appeared in the 10,000 *g* pellet; the other 10% of the enzymatic activity could be recovered from the pellet sedimenting between 10,000 *g* and 105,000 *g*.

Sucrose Density Gradient Centrifugation

Early log-phase cultures were grown in 500-ml batches, harvested by centrifugation (15,000 *g*, 0°C, 15 min), washed with 0.2 M NaCl, disrupted with the Potter–Elvejhem homogenizer in buffer (20 mM imidazole-HCl, pH 7.1, 0.25 M sucrose), and centrifuged (10,000 *g*, 0°C, 30 min). The pellet was taken up in a small amount of the same buffer, and a sample (1.0 ml) was applied to a sucrose gradient which contained 20 mM imidazole-HCl, pH 7.1, and consisted of a 0.5 ml cushion of d 1.32, and 3.6 ml of a linear gradient of d 1.14–1.27 in a cellulose nitrate tube of capacity 5.2 ml. The tube was centrifuged in the SW 39 rotor of a Beckman Model L ultracentrifuge (100,000 *g*, 0°C, 150 min). After centrifugation, the contents were collected in 3-drop samples (approximately 0.2 ml each) by puncturing the bottom of the tube.

Assay of the Exchange Enzyme

The following procedure (19, 20) was used for assay of the exchange enzyme: assays were carried out in conical-tipped vessels (40 ml capacity) using a final incubation volume of 0.4 ml. The standard assay system consisted of 50 mM imidazole-HCl, final pH 7.7, 1.5 mM L-serine-3-^{14}C at 10^6 cpm/μmole, and 20 mM $CaCl_2$. Incubations were conducted at 22°C for 10 min. For "zero-time" controls, methanol was added at this point. The vessels containing the assay reagents and radioactive label were equilibrated in a water bath at the appropriate temperature; then cell-free preparations of *Tetrahymena* (lyophilized powder resuspended in water and subjected to five strokes with the Potter–Elvejhem homogenizer) were added to initiate the reaction. The final concentration of protein used was 3.7 mg/ml as determined by the method of Lowry et al. (18). In some experiments, a different preparation of lyophilized enzyme, which gave a final concentration of 2.8 mg/ml of protein, was used; this will be referred to as "preparation B." Reactions were stopped by the addition of 5 ml of methanol.

To determine the amount of radioactive phospholipid produced, chloroform (10 ml) was added to each vessel, and the water-soluble components were extracted twice with 20 ml of 2 M $MgCl_2$ and once with 20 ml of water. Aliquots of the resulting chloroform phase were dried in the presence of 100 mg of Triton X-100 and then resuspended in 1 ml of water and counted in 10 ml of 2:1 Patterson–Greene (21) counting solution. This assay was always performed in duplicate; the standard deviation of all of the duplicates of the phospholipid assays reported herein was ±0.25 nmoles of phospholipid. The averages of the duplicate values are reported; the average of the zero-time duplicates have been subtracted in all cases.

Assay of the Decarboxylase

The decarboxylation of phosphatidylserine was measured directly using dipalmitoyl-L-α-glycerophosphoryl-L-serine-1-^{14}C prepared according to the methods of Baer and Maurukas (22). The assay (3) was carried out in a 25 ml Erlenmeyer flask containing a center well in which was placed a piece of fluted filter paper (approximately 1 × $^3/_4$ in.) impregnated with 0.1 ml of 2 N KOH to collect the $^{14}CO_2$. The assay system contained 50 mM imidazole-HCl, final pH 7.7, 0.2 mM phosphatidyl-L-serine-1-^{14}C at 5 × 10^5 cpm/μmole, 4 mM of EDTA, and 2.0 mg/ml of Triton X-100 in a final volume of 0.4 ml. Incubations were conducted at 22°C for 10 min. For "zero-time" controls, H_2SO_4 was added at this point. To start the reaction, enzyme preparation B (lyophilized powder resuspended in water and subjected to five strokes with the Potter–Elvejhem homogenizer) was added to the medium giving a final concentration of 2.8 mg of protein per ml. The flask was stoppered with a rubber serum cap and shaken by gentle rocking in a thermostatted water bath. Reactions were stopped with 0.5 ml of 0.5 N H_2SO_4 added by syringe through the cap to the reaction mixture. Shaking was then continued for an additional 30 min. The KOH-impregnated paper was counted in 1 ml of water and 10 ml of 2:1 Patterson–Greene counting solution. As with the phospholipid assay, the average of duplicate determinations (SD ±0.2 nmoles of $^{14}CO_2$) is reported, and zero-time values have been subtracted.

Preparation of Phosphatidylethanolamine-1,2-^{14}C

Phosphatidylethanolamine-1,2-^{14}C was generated *in situ* from endogenous phospholipid and ethanolamine-1,2-^{14}C by carrying out the exchange reaction on a large scale using the same conditions as in the assay for the exchange enzyme except that the final volume was 3.6 ml, ethanolamine-1,2-^{14}C at 10^6 cpm/μmole was the labeled precursor, incubation was for 40 min, and enzyme preparation B was used. The incubation medium was centrifuged (20,000 *g*, 0°C, 5 min), and the pellet was washed twice with 5 ml of buffer containing 50 mM imidazole-HCl, pH 7.8, and 20 mM $CaCl_2$. The final pellet was resuspended in 3.6 ml of the buffer and subjected to five strokes with the Potter–Elvejhem homogenizer. Portions of these preparations were then immediately incubated with appropriate unlabeled substrates for the exchange enzyme to determine the loss of labeled ethanolamine from lipid. After incubation, the labeled phospholipid remaining was determined as in the assay for the exchange enzyme. The above procedure usually incorporated about 200 nmoles of ethanolamine-1,2-^{14}C of which about 55% was recovered in the final washed preparation.

Mild Alkaline Hydrolysis

In order to distinguish between ether and acyl phospholipids, a mild alkaline hydrolysis procedure (23) was used in conjunction with several of the assays. Acyl phospholipids are rapidly hydrolyzed in mild alkali giving water-soluble hydrolysis products, while ether phospholipids are not labile in mild alkali and, therefore, remain soluble in nonaqueous solvents. The acyl phospholipids are thus alkali-labile, and the ether phospholipids are alkali-stable. Methanol (0.5 ml) and 4 N NaOH (0.05 ml) were added to the lipids in 2.0 ml of chloroform. The sample was mixed and allowed to stand 5 min. The hydrolysis was stopped with the addition of 0.5 ml of 0.44 N HCl, and the resulting solution was mixed and centrifuged at low speed for 5 min; the aqueous and chloroform layers could then be separated with a capillary pipette. The aqueous layer contained the alkali-labile hydrolysis products, and the chloroform layer contained the alkali-stable phospholipid. If the separate

fractions were to be counted, they were evaporated to dryness in the presence of 100 mg of Triton X-100, taken up in 1.0 ml of H_2O, and then counted in 10 ml of 2:1 Patterson–Greene counting solution.

Lipid Phosphorus

Phosphorus was determined by the method of Bartlett (24) on the lyophilized powder after lipid extraction and the mild alkaline hydrolysis procedure described above. Enzyme preparation B contained about 0.35 μmoles of lipid-phosphorus per mg of protein which consisted of about 0.25 μmoles of alkali-labile lipid-phosphorus per mg of protein, and about 0.10 μmoles of alkali-stable lipid-phosphorus per mg of protein. According to Aalbers and Bieber (25), the procedure of Bartlett (24) would not have detected any phosphonolipid which might have been present in the preparation.

Ninhydrin-Positive Lipid

It was desirable to determine the total amount of phosphatidylethanolamine available for the exchange reaction. Since it will be shown later that the products of the exchange reaction under the assay conditions employed here are only acyl phospholipids and not ether phospholipids, the amount of ninhydrin-positive material in alkali-labile extracts from the mild alkaline hydrolysis was determined. For this purpose the aqueous fraction after mild alkaline hydrolysis was evaporated to dryness, taken up in water, neutralized to pH 5 with KOH, and its content of ninhydrin-reactive base was determined by the procedure of Cocking and Yemm (26). It was assumed that all of the ninhydrin-positive material represented phosphatidylethanolamine since phosphatidylserine is not present in this organism (27–29) in detectable amounts. A ninhydrin determination on the same alkali-labile fraction used in the phosphorus determination reported above, gave 0.11 μmoles of ethanolamine lipid per mg of protein. The ninhydrin-positive ethanolamine represents the ethanolamine moiety of both acyl phospholipids and any acyl phosphonolipids which may be present.

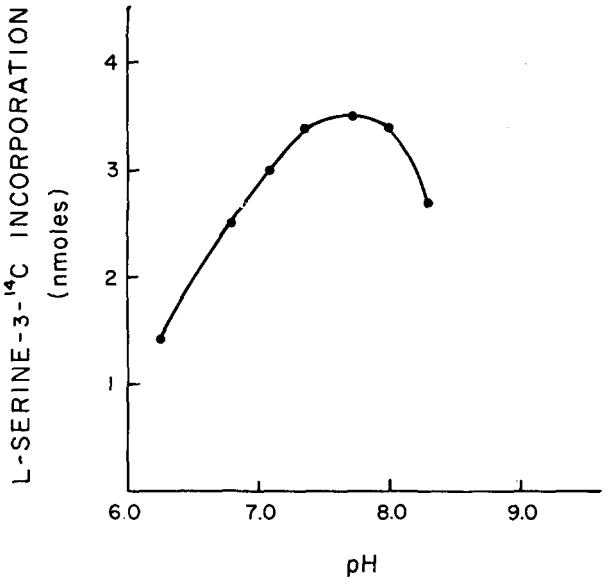

FIG. 2. pH dependence. The standard assay for the exchange enzyme was used except that the concentration of L-serine-3-^{14}C was 0.5 mM. The final pH was determined with a pH meter on identical solutions containing unlabeled L-serine.

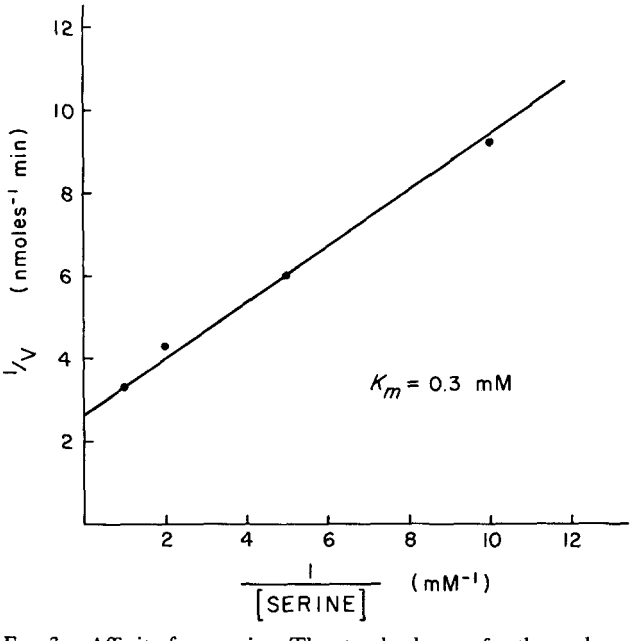

FIG. 3. Affinity for L-serine. The standard assay for the exchange enzyme was used.

EXPERIMENTAL RESULTS

pH Optimum

The pH optimum for the exchange reaction is 7.7 in imidazole buffer as shown in Fig. 2.

Affinity for L-Serine and for Ethanolamine

The concentrations of L-serine and ethanolamine as substrates for the exchange reaction were varied in the experiments shown in Figs. 3 and 4, respectively. The K_m for L-serine is about 0.3 mM; for ethanolamine, the K_m is about 0.1 mM. When assays for ethanolamine and L-serine incorporation were carried out using the same enzyme preparation, under identical conditions, and with saturating amounts of substrate, the maximum rate of incorporation of ethanolamine-1,2-^{14}C was twice as great as L-serine-3-^{14}C. Choline-methyl-^{3}H was not a substrate for the exchange reaction.

D-Serine as Substrate

In experiments with the particulate, lyophilized enzyme, there was much less incorporation of D-serine in the ex-

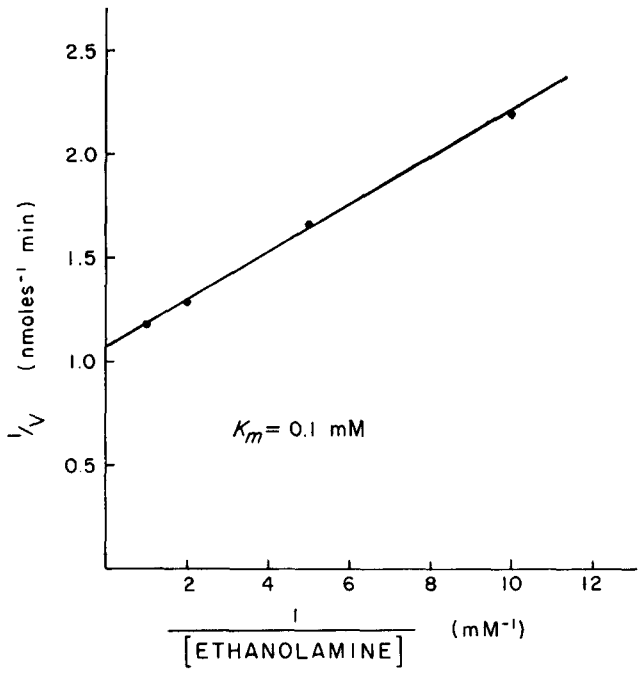

FIG. 4. Affinity for ethanolamine. The standard assay for the exchange enzyme was used except that ethanolamine-1,2-^{14}C at 10^6 cpm/μmole was employed instead of L-serine-3-^{14}C.

change reaction than L-serine as shown in Table 1; thus, in those experiments, in which DL-serine was used as substrate, the incorporation of the D-serine was negligible. The observed incorporation of D-serine itself may be due to a racemase in the preparation.

Ca^{++} Dependence

The K_m for Ca^{++} in the exchange reaction is about 0.9 mM as shown in Fig. 5. Ca^{++} (5 mM) was found to be at least 10 times more effective in catalysis than 5 mM Mg^{++}, Mn^{++}, or Ba^{++}.

Proportionality with Added Enzyme

Incorporation of L-serine into phospholipid is directly proportional to protein under the assay conditions used as shown in Fig. 6.

TABLE 1 EFFECT OF D-SERINE AS SUBSTRATE FOR THE EXCHANGE ENZYME

Substrate (0.5 mM)	Incorporation
	nmoles
L-serine-3-^{14}C	9.6
L-serine-3-^{14}C + 5 mM D-serine	7.5
D-serine-3-^{14}C	1.0
D-serine-3-^{14}C + 5 mM L-serine	0.08

The assay for the exchange enzyme was conducted using a 0.2 ml system and the following deviations from the standard conditions: 0.1 M imidazole-HCl, final pH 7.1, 0.5 mM L-serine-3-^{14}C or D-serine-3-^{14}C at 10^6 cpm/μmole, and 20 mM $CaCl_2$. Incubations were conducted at 29°C for 60 min. A fresh 10,000 *g* particulate fraction was used as enzyme.

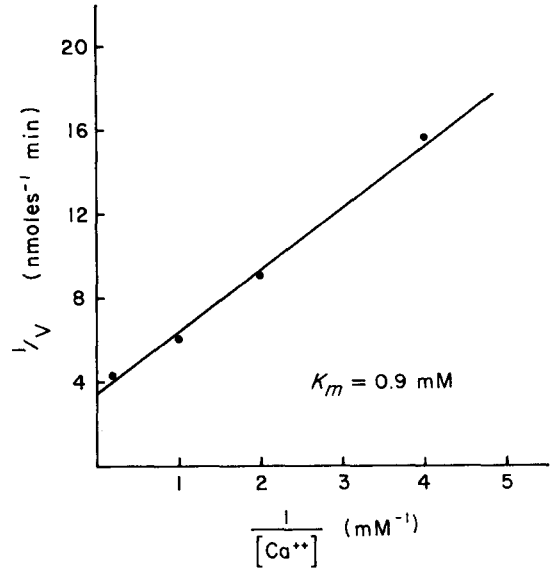

FIG. 5. Ca^{++} dependence. The standard assay for the exchange enzyme was used except that the lyophilized powder was washed with 0.1 mM EDTA (pH 7.1), and sucrose (final concentration, 25 mM) was added back to the final incubation mixture.

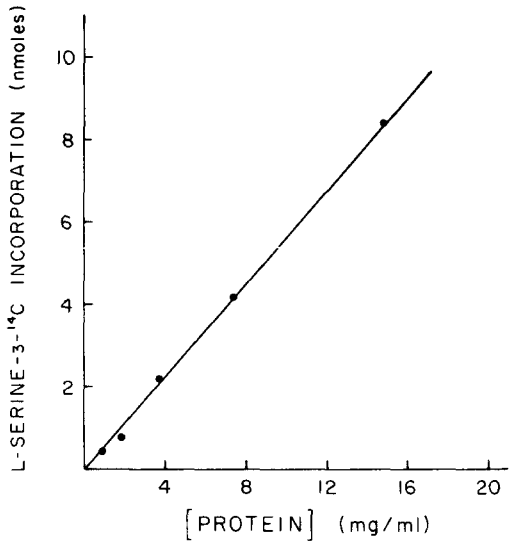

FIG. 6. Proportionality to added enzyme. The standard assay for the exchange enzyme was used except that the lyophilized powder was washed with water and the final incubations were made 25 mM in sucrose. The amount of phospholipid formed at 3.7 mg/ml of protein is 2.2 nmoles with or without 25 mM sucrose which is normally added to the incubation mixtures along with the protein from the lyophilized powder preparations.

Time Course

The time course of incorporation of L-serine is shown in Fig. 7. For both L-serine and ethanolamine, the rate of incorporation falls rapidly with time, and more rapidly at higher temperatures. However, at 22°C, the incorporation is approximately proportional to time for the first 10 min, the time used for most incubations re-

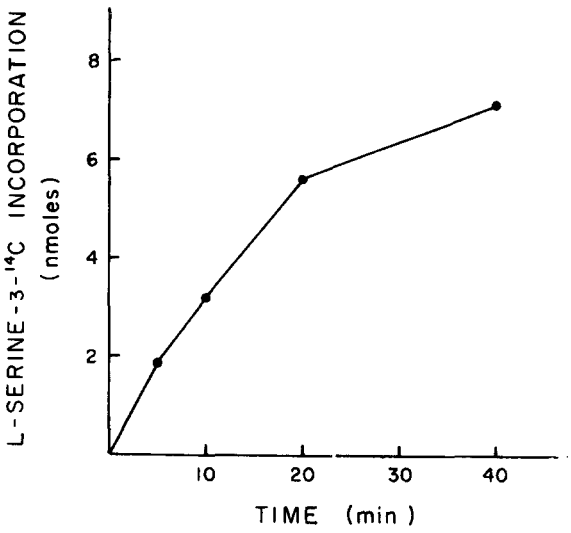

FIG. 7. Time course. The standard assay for the exchange enzyme was used.

ported herein. The lack of linearity at longer times will be considered further in the Discussion.

Temperature Dependence and Heat Inactivation

The time course for L-serine incorporation at 37°C falls off more rapidly than at 22°C. Although the initial rate of reaction is probably greater at 37°C, the enzyme is inactivated at this temperature as is shown by heat treatment of the enzyme before assay. (See experiment A of Table 2.) Ca^{++}, however, protects the enzyme somewhat against this inactivation as shown by experiments A and B. L-Serine, mercaptoethanol, and Tris are not effective as protective agents (experiment B).

Inhibition by Tris Buffer

Previous studies of the exchange enzyme in rat liver (3, 7) and the housefly (8) were performed using Tris buffer; Crone (30) recently found that high concentrations of Tris inhibit the exchange enzyme. We find that Tris gives a slight inhibition; for example, with 0.2 mM L-serine-3-^{14}C, from 4 mM to 24 mM Tris, the incorporation of radioactive L-serine is reduced by about 20%. The inhibition by Tris is thus important only at high concentrations.

Added Phospholipid, Detergents, Sonication

Enzyme preparation B contains about 0.25 μmoles of alkali-labile phospholipid per mg of protein. Of this phospholipid there are about 0.11 μmoles of alkali-labile ethanolamine phospholipid per mg of protein (possibly including some phosphono analogue) as described in Materials and Methods. When 1.1 mg of this protein was used in the assay, the production of labeled lipid leveled off at about 22 nmoles (0.02 μmoles/mg of protein) at 40 min. Clearly, all of the endogenous phosphatidylethanolamine was not used up. Neither sonication of the *Tetrahymena* preparations nor the addition of detergents during the assay stimulated the exchange enzyme to use additional endogenous phospholipid.

TABLE 2 HEAT INACTIVATION AND Ca^{++} PROTECTION

Heat Treatment (37°C, 15 min)	Additions (During Heating)	L-Serine-3-^{14}C Incorporation A	B
		nmoles	
−	—	9.9	13.7
+	—	4.7	
+	$CaCl_2$	7.9	8.0
+	L-Serine-3-^{14}C		2.1
+	Mercaptoethanol		0.8
+	Tris		2.2

The assay for the exchange enzyme was conducted using the conditions described in Table 1 with L-serine-3-^{14}C. Experiments A and B each employed different fresh preparations of enzyme.

Attempts were therefore made to stimulate the reaction by adding additional amounts of the particulate fraction of *Tetrahymena* which is itself a rich source of phospholipids. The data in Table 3 shows that the addition of phospholipid-containing particulate fraction from *Tetrahymena* (previously treated to inactivate enzymes) does not stimulate the exchange reaction, nor does added phosphatidylethanolamine obtained from the following sources: (*a*) synthetic, Mann Research Labs. Inc., New York; (*b*) from *Escherichia coli*, courtesy of N. Z. Stanacev; (*c*) phosphatidylethanolamine dialyzed from sodium deoxycholate–butanol according to the procedure of Fleischer, Klouwen, and Carpenter (31); and (*d*) phosphatidylethanolamine sonicated with several detergents.

Products of the Exchange Reaction

The products of the exchange reaction were subjected to mild alkaline hydrolysis (23), and the alkali-labile and alkali-stable fractions were separated and counted as shown in Table 4. In both a short- and a long-term in-

TABLE 3 FAILURE OF ADDED *Tetrahymena* LIPID TO STIMULATE EXCHANGE

Unheated Enzyme	Heated Particulate Fraction (60°C, 8 min)	Ethanolamine-1,2-^{14}C Incorporation
		nmoles
+	−	12.1
−	+	0.0
+	+	12.1

The standard assay for the exchange enzyme was used except that 0.5 mM ethanolamine-1,2-^{14}C at 10^6 cpm/μmole was used instead of L-serine-3-^{14}C, the incubation time was 45 min, and enzyme preparation B was used at a final concentration of 1.4 mg/ml.

TABLE 4 PRODUCTS OF THE EXCHANGE REACTION

Time	Alkali-Labile	Alkali-Stable	% Alkali-Labile
min	*nmoles*	*nmoles*	
10	10.7	0.22	98
40	16.9	0.38	98

The standard assay for the exchange enzyme was used except that 0.5 mM ethanolamine-1,2-^{14}C at 10^6 cpm/μmole was used instead of L-serine-3-^{14}C, and enzyme preparation B was employed.

cubation, the phospholipids formed were at least 98% alkali-labile, indicating that under these conditions the phospholipids formed contain acyl groups and not ether functions (27). In another experiment, the alkali-labile fraction was subjected to paper chromatographic analysis according to Baer, Sarma, Robinson, and Sastry (32) which distinguishes between glycerophosphorylethanolamine and its phosphono analogue. On descending paper chromatography (Whatman filter paper No. 43, 24 hr) with *n*-butanol–glacial acetic acid–water 5:3:1 (v/v/v), the alkali-labile hydrolysis product ran as a single peak with authentic glycerylphosphorylethanolamine.

Decarboxylase

The decarboxylation of the phosphatidylserine formed by the exchange enzyme was evident in the experiment described in Fig. 8 where DL-serine-1-^{14}C was used as substrate. Hydroxylamine, which inhibits the decarboxylase, was added to one set of tubes and not to the other; the difference in labeled phospholipid formed between the two tubes indicates the amount of decarboxylation which occurs. The decarboxylase was also demonstrated directly using the decarboxylase assay and phosphatidylserine-1-^{14}C as substrate as shown in Table 5. The activity of the decarboxylase is increased in the presence of Triton X-100 as is the similar enzyme obtained from *E. coli* (2, 4) and rat liver (2, 3). The presence of an active decarboxylase explains the reports (27–29) that the phospholipids of *Tetrahymena* are largely phosphatidylethanolamine and phosphatidylcholine with little or no phosphatidylserine.

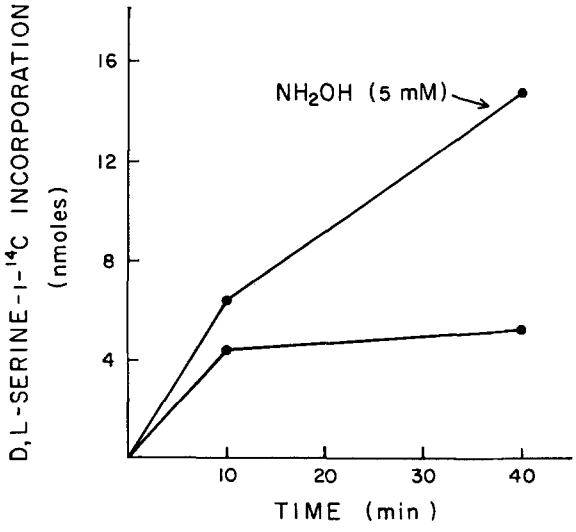

FIG. 8. Activity of decarboxylase. The standard assay for the exchange enzyme was used except that 3 mM DL-serine-1-^{14}C (10^5 cpm/μmole) was employed instead of L-serine-3-^{14}C, and enzyme preparation B was used.

Displacement of Ethanolamine-1,2-^{14}C from Phosphatidylethanolamine-1,2-^{14}C by L-Serine

It has been assumed that the observed incorporation of labeled L-serine or ethanolamine involves a displacement of the base from the endogenous phosphatidylethanolamine present in the particulate preparations of *Tetrahymena* added during the incubations. A direct demonstration of this displacement would be to add phosphatidylethanolamine labeled in the ethanolamine moiety to the incubation medium and to observe the displacement of labeled ethanolamine from this phosphatidylethanolamine by unlabeled L-serine. However, as shown above, phosphatidylethanolamine added to the particulate fraction is not used as a substrate by this enzyme. Therefore, in order to demonstrate this displacement reaction, endogenous phospholipid was labeled with ethanolamine-1,2-^{14}C *in situ* using the exchange enzyme as described in Materials and Methods. The resulting endogenous phosphatidylethanolamine-1,2-^{14}C was then used as a substrate in a reaction mixture containing unlabeled L-serine. The displacement of the ethanolamine-1,2-^{14}C could be assayed as loss of labeled phospholipid. The experiment reported in Table 6 shows that L-serine is effective at this displacement. This experiment also shows that there is a small, but measurable breakdown of phospholipid in the absence of L-serine under the conditions of the assay. Similarly, oxaloacetate (Table 6) and phosphoenolpyruvate (not shown) do not displace labeled ethanolamine and, therefore, are presumably not substrates for the exchange reaction.

TABLE 5 DECARBOXYLATION OF PHOSPHATIDYL-L-SERINE-1-^{14}C

Triton X-100	Phosphatidylserine-1-^{14}C Decarboxylation
mg/ml	*nmoles*
None	3.5
0.2	6.3
2.0	14.2

$^{14}CO_2$ was determined according to the assay for the decarboxylase given in Materials and Methods except that the Triton X-100 concentration was varied as shown in the table, and the incubation time was 15 min.

TABLE 6 DISPLACEMENT OF ETHANOLAMINE-1,2-^{14}C FROM PHOSPHATIDYLETHANOLAMINE-1,2-^{14}C BY L-SERINE

Additions	Phosphatidylethanolamine-1,2-^{14}C Remaining	Ethanolamine-1,2-^{14}C Displaced	% Displaced
	cpm	*cpm*	*cpm*
Zero-time	14,767	—	—
None	13,415	1352	9
1.5 mM L-serine	9,434	5333	36
15 mM oxaloacetate	14,690	77	0.5

The preparation of a particulate containing phosphatidylethanolamine-1,2-^{14}C is described in Materials and Methods. The assay for the exchange enzyme in this preparation was conducted according to the standard assay, but in a 0.47 ml system containing 43 mM imidazole-HCl, final pH 7.7, 17 mM $CaCl_2$, and the additions noted in the table. Incubations were conducted at 22°C for 20 min. To initiate reaction, the particulate fraction containing the protein and the phosphatidylethanolamine-1,2-^{14}C, was added to the incubation medium.

In the experiment described in Table 6, 36% of the labeled phosphatidylethanolamine was displaced in the exchange reaction under conditions in which only a small amount of L-serine would be expected to be incorporated into phospholipid. In an experiment essentially similar to that of Table 6, the particulate enzyme preparation was labeled by a preliminary incubation with radioactive ethanolamine as described, and a control was included in which the enzyme preparation was incubated with unlabeled ethanolamine, but was otherwise treated identically. Subsequent incubation of the unlabeled preparation with labeled L-serine resulted in 8 nmoles incorporation under conditions in which 50% of the labeled ethanolamine was displaced. This suggests that the total pool available for the exchange reaction in this experiment was about 16 nmoles of phosphatidylethanolamine, which is much less than the total amount of phosphatidylethanolamine present in the sample.

Intracellular Localization of the Exchange and Decarboxylase Enzymes

Using a modification of the procedure of Müller, Hogg, and de Duve (33) for the subcellular fractionation of *Tetrahymena*, the 10,000 *g* pellet was subjected to sucrose density gradient centrifugation as described in Materials and Methods. The protein distribution in a typical experiment is shown in Fig. 9*a*. Müller et al. (33) used the whole homogenate in their experiments; in the experiments reported herein, only the 10,000 *g* particulate fraction (about 60% of the total protein) was used in the density gradients. The protein distributions reported here agree closely with those of the above workers, except that theirs contained a large amount of protein in the region above d 1.14 because of the inclusion of the whole homogenate.

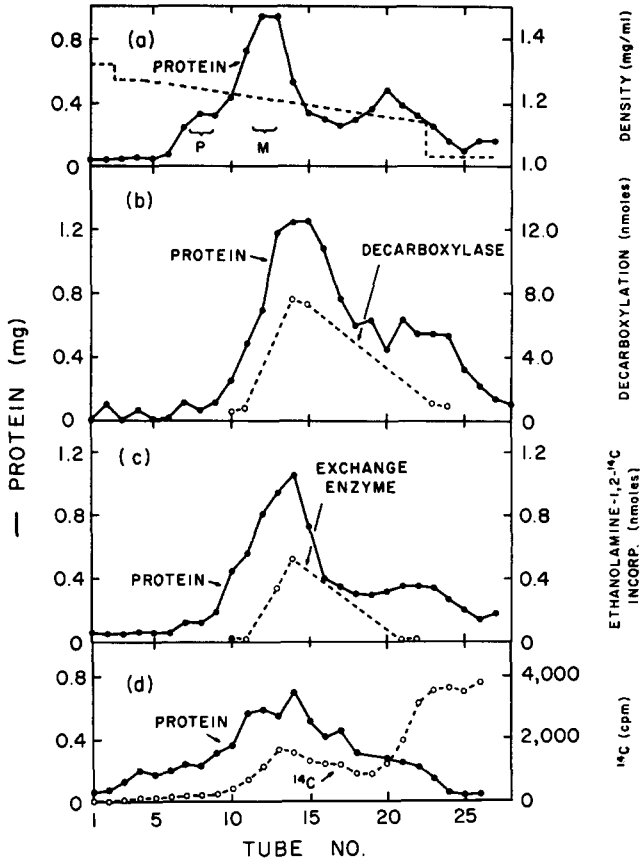

FIG. 9. Sucrose density gradient centrifugation. (*a*) Distribution of protein and density of sucrose. The peroxisomes (*P*) and mitochondria (*M*) were assigned on the basis of the sucrose density from Müller, Hogg, and de Duve (33). (*b*) Distribution of protein and assay for the decarboxylase. The standard assay for the decarboxylase was used. (*c*) Distribution of protein and assay for the exchange enzyme. The standard assay for the exchange enzyme was employed except that ethanolamine-1,2-^{14}C (10^6 cpm/mole) was used as substrate. (*d*) Distribution of protein and radioactivity of particulate fraction preincubated with ethanolamine-1,2-^{14}C. A 10,000 *g* particulate fraction was incubated 40 min with ethanolamine-1,2-^{14}C under the standard conditions of the exchange enzyme assay in order to generate phosphatidylethanolamine-1,2-^{14}C *in situ*. After incubation, the suspension was centrifuged (40,000 *g*, 0°C, 15 min) and washed once with buffer containing 20 mM imidazole-HCl, pH 7.1, 0.25 M sucrose, 0.1 mM EDTA, and 0.5 mM unlabeled ethanolamine. The washed 10,000 *g* particulate fraction containing the phosphatidylethanolamine-1,2-^{14}C was then subjected to the sucrose density gradient centrifugation giving the distribution of radioactivity shown.

One sample, centrifuged for 900 min instead of 150 min, gave the same pattern which confirms that the gradient is essentially at equilibrium after 150 min. In a similar experiment, several fractions were assayed for the decarboxylase, and the activities are shown in Fig. 9*b*. Assays for the exchange enzyme in another experiment showed a large loss of activity relative to the starting material, but with the activity distribution shown in Fig. 9*c*.

In order to examine the exchange enzyme more carefully, a 10,000 *g* particulate fraction was incubated with ethanolamine-1,2-^{14}C in order to label the phosphatidylethanolamine in the preparation, and this particulate fraction was then subjected to sucrose density gradient centrifugation with the resulting distribution of label shown in Fig. 9*d*. The distribution of label generally follows the activity of the exchange enzyme in Fig. 9*c*, but with a very large amount of label appearing in the low-density region. This latter accumulation of label presumably represents phosphatidylethanolamine-1,2-^{14}C liberated from the particulate fraction and (or) the products of its breakdown during the time of the fractionation. From the preliminary experiments reported here, it thus appears that both the decarboxylase and the exchange enzyme in *Tetrahymena* are localized in the same particulate fraction which may be mitochondrial. In rat liver in contrast, the decarboxylase is localized in the mitochondria, but the exchange enzyme is localized in the microsomal fraction.[1]

DISCUSSION

The incorporation of L-serine into phospholipid in animal tissue has been postulated to occur by two different mechanisms: an exchange reaction (1, 3) as was shown in reaction (*2*) or the reverse action of a phospholipase (7, 34) as shown in the following reaction:

(*5*) Phosphatidylethanolamine + H_2O ⇌ Phosphatidic Acid + Ethanolamine

The data in Table 6 shows that the release of ethanolamine from phosphatidylethanolamine is almost completely dependent upon the presence of L-serine. Thus, the incorporation of L-serine into phospholipid probably occurs by an exchange reaction; the dependence on L-serine for the release of ethanolamine from phosphatidylethanolamine shown in Table 6 is not consistent with a mechanism involving the reverse action of a phospholipase.

The main anomaly in the data presented here is the time course of the reaction (Fig. 7). The production of labeled phospholipid falls off rapidly with time in an excess of substrate, yet the displacement of labeled ethanolamine by L-serine (Table 6) shows that the exchange enzyme is still quite active even after the initial 40 min incubation. Thus, the fall in phospholipid production at 40 min cannot be due solely to inactivation of the exchange enzyme. The data in Table 6 also show that the breakdown of the endogenous phosphatidylethanolamine during the incubations by processes other than the exchange enzyme would not be sufficient to hydrolyze enough of the endogenous phospholipid substrate to cause the rapid fall in phospholipid production with time as illustrated in Fig. 7. This finding coupled with the fact that it has not been possible to stimulate the exchange reaction with sonication, detergents, or the addition of phosphatidylethanolamine suggests that only a part of the phosphatidylethanolamine pool is available to the exchange enzyme. Possibly some structural requirement in terms of lipoprotein interactions is a necessary requirement for catalysis by the exchange enzyme. This point is under current investigation.

[1] Dennis, E. A., and E. P. Kennedy. Manuscript in preparation.

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REFERENCES

1. Kennedy, E. P. 1961. *Fed. Proc.* **20:** 934.
2. Kennedy, E. P. 1962. The Harvey Lectures, Series 57. Academic Press Inc., New York. 143–171.
3. Borkenhagen, L. F., E. P. Kennedy, and L. Fielding. 1961. *J. Biol. Chem.* **236:** PC28.
4. Kanfer, J., and E. P. Kennedy. 1964. *J. Biol. Chem.* **239:** 1720.
5. Wilson, J. D., K. D. Gibson, and S. Udenfriend. 1960. *J. Biol. Chem.* **235:** 3539.
6. Bremer, J., P. H. Figard, and D. M. Greenberg. 1960. *Biochim. Biophys. Acta.* **43:** 477.
7. Hübscher, G. 1962. *Biochim. Biophys. Acta.* **57:** 555.
8. Crone, H. D. 1967. *Biochem. J.* **104:** 695.
9. Horiguchi, M., and M. Kandatsu. 1959. *Nature (London).* **184:** 901.
10. Quin, L. D. 1967. *In* Topics in Phosphorus Chemistry. M. Grayson and E. J. Griffith, editors. John Wiley & Sons, Inc., New York. **4:** 23.
11. Kittredge, J. S., and E. Roberts. 1969. *Science (Washington).* **164:** 37.
12. Rosenberg, H. 1964. *Nature (London).* **203:** 299.
13. Liang, C. R., and H. Rosenberg. 1968. *Biochim. Biophys. Acta.* **156:** 437.
14. Thompson, G. A., Jr. 1969. *Biochim. Biophys. Acta.* **176:** 330.
15. Trebst, A., and F. Geike. 1967. *Z. Naturforsch.* **22b:** 989.
16. Warren, W. A. 1968. *Biochim. Biophys. Acta.* **156:** 340.
17. Horiguchi, M., J. S. Kittredge, and E. Roberts. 1968. *Biochim. Biophys. Acta.* **165:** 164.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. *J. Biol. Chem.* **193:** 265.
19. Weiss, S. B., S. W. Smith, and E. P. Kennedy. 1958. *J. Biol. Chem.* **231:** 53.
20. Chang, Y. Y., and E. P. Kennedy. 1967. *J. Lipid Res.* **8:** 447.
21. Patterson, M. S., and R. C. Greene. 1965. *Anal. Chem.* **37:** 854.
22. Baer, E., and J. Maurukas. 1955. *J. Biol. Chem.* **212:** 25.
23. Tarlov, A. R., and E. P. Kennedy. 1965. *J. Biol. Chem.* **240:** 49.

24. Bartlett, G. R. 1959. *J. Biol. Chem.* **234:** 466.
25. Aalbers, J. A., and L. L. Bieber. 1968. *Anal. Biochem.* **24:** 443.
26. Cocking, E. C., and E. W. Yemm. 1954. *Biochem. J.* **58:** xii.
27. Thompson, G. A., Jr. 1967. *Biochemistry.* **6:** 2015.
28. Erwin, J., and K. Bloch. 1963. *J. Biol. Chem.* **238:** 1618.
29. Dawson, R. M. C., and P. Kemp. 1967. *Biochem. J.* **105:** 837.
30. Crone, H. D. 1966. *Biochem. J.* **100:** 12.
31. Fleischer, S., H. Klouwen, and E. Carpenter. 1961. *Biochem. Biophys. Res. Commun.* **5:** 378.
32. Baer, E., G. R. Sarma, R. Robinson, and P. S. Sastry. 1967. *Can. J. Biochem.* **45:** 1783.
33. Müller, M., J. F. Hogg, and C. de Duve. 1968. *J. Biol. Chem.* **243:** 5385.
34. Dils, R. R., and G. Hübscher. 1961. *Biochim. Biophys. Acta.* **46:** 505.