Biosynthesis of phosphatidylcholine by enzyme preparations from spinach leaves

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ABSTRACT The enzymic incorporation of choline-1,2-^{14}C from CDP-choline-1,2-^{14}C into phosphatidylcholine by spinach leaf preparations was characterized.

The enzyme catalyzing the incorporation, choline phosphotransferase, had a pH optimum of about 8.0 and required either Mn^{2+} or Mg^{2+} as cofactor. The saturation concentration of Mn^{2+} was 0.3 mM and that for Mg^{2+} was 13 mM. The K_m for CDP-choline was 10 μM. The choline phosphotransferase was inhibited by sulfhydryl reagents. The enzyme was inactivated at 30°C, but this inactivation could be prevented by dithiothreitol and Mn^{2+}. Preincubation of the enzyme with Mn^{2+} prevented inhibition by sulfhydryl reagents. The incorporation of diglyceride-U-^{14}C into phosphatidylcholine was also studied. The enzyme did not show any diglyceride specificity when exogenous diglyceride was added, indicating that fatty acid distribution in phosphatidylcholine of spinach is not controlled by choline phosphotransferase.

SUPPLEMENTARY KEY WORDS diglyceride molecular species · CDP-choline · sulfhydryl inhibitors · subcellular distribution

The role of CDP-choline (cytidine-5′-diphosphate choline) in the synthesis of phosphatidylcholine was shown by Kennedy and Weiss in 1955 in rat liver (1). Later Wilgram and Kennedy showed that CDP-choline-1,2-diglyceride-choline phosphotransferase (EC 2.7.8.2) (choline phosphotransferase) was located in the microsomal fraction of rat liver (2). Choline phosphotransferase in rat liver also shows a lack of specificity for diglycerides (3), indicating that this step of PC synthesis does not play a role in determining fatty acid distribution in PC.

The synthesis of glycerides and phosphatidic acid has been demonstrated in spinach leaves (4, 5). The enzyme activity was found to be primarily in the microsomal fraction. However, the enzymic synthesis of phosphatidylcholine in plants has not been reported previously.

This report is concerned with some of the properties of choline phosphotransferase of spinach leaves and the specificity of this enzyme for diglycerides. It is part of a series of studies designed to elucidate the structures of phosphatidylcholine in plants, with a view to assessing the factors responsible for the fatty acid composition and positional distribution (6, 7).

MATERIALS AND METHODS

Preparation of Subcellular Fractions

Spinach leaves were washed, deveined, and cut into small pieces prior to preparation of the homogenate. In a typical preparation, 170 g of chopped spinach leaves was homogenized with 250 ml of 0.5 M sucrose, 10 mM with respect to Tris–HCl, pH 8.0, in a Waring Blendor at 4°C for 3–4 sec. In experiments where diglyceride stimulation was to be demonstrated or where radioactive diglyceride was used as substrate, the homogenizing medium was also made 1 mM with respect to EDTA, pH 8.0, to inhibit phospholipase D activity.

The homogenate was then filtered through four layers of cheesecloth and was separated into subcellular fractions (Fig. 1). For most preparations, however, the microsomal fraction was used as enzyme source. This fraction was usually prepared by centrifuging the

Abbreviations: CDP-choline, cytidine-5′-diphosphate choline; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; PC, phosphatidylcholine; POPOP, 1,4-bis-2-(5-phenyloxazolyl)-benzene; PPO, 2,5-diphenyloxazole.

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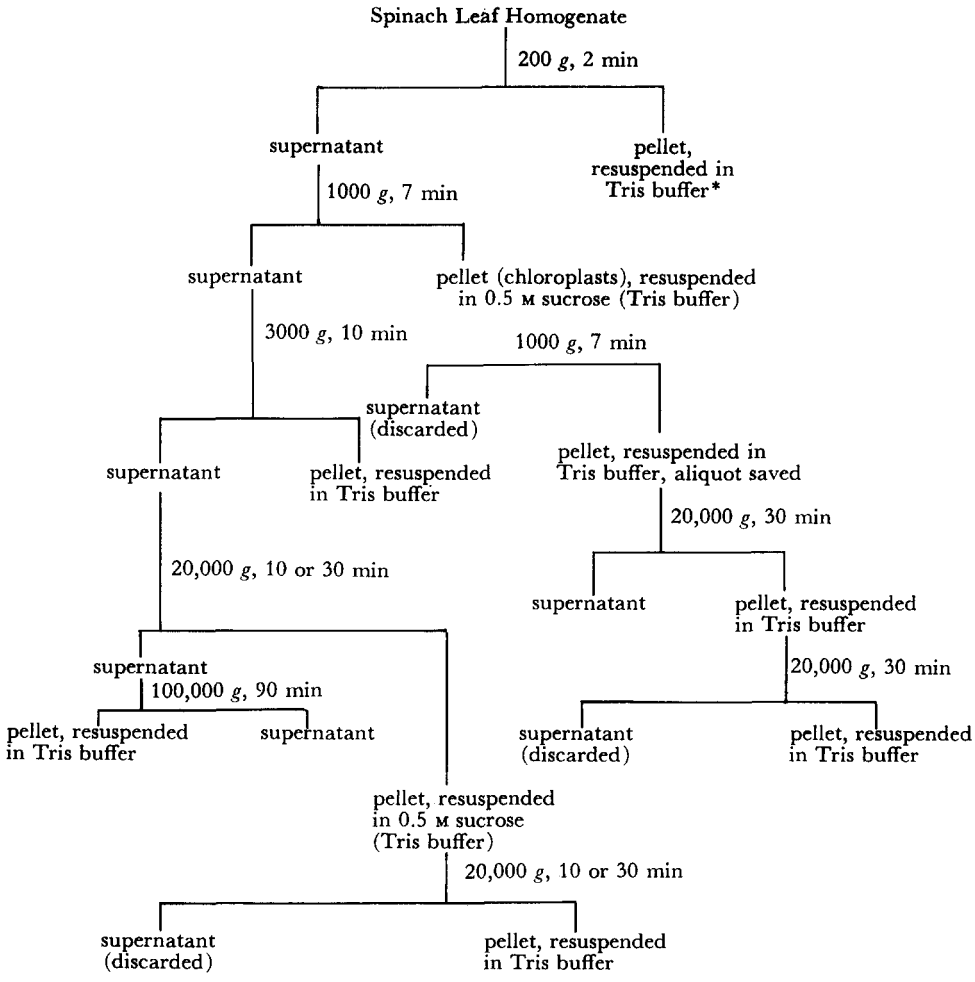

FIG. 1. Fractionation of the spinach leaf homogenate.

filtered homogenate at 20,000 *g* for 30 min and then centrifuging the resulting supernatant at 100,000 *g* for 90 min. All pellets were resuspended in 10 mM Tris–HCl, pH 8.0. Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (8). Enzyme assays were carried out on the same day as the preparation of the subcellular fractions.

Preparation of Diglycerides

Radioactive diglycerides were prepared from radioactive PC, which was isolated from an ethanol–ether extract of *Chlorella vulgaris*-U-^{14}C (Amersham/Searle Corp., Des Plaines, Ill.) by silicic acid column chromatography as described previously (6). The PC from the silicic acid column was purified further by thin-layer chromatography on Silica Gel G (E. Merck A.G., Darmstadt, Germany), using chloroform–methanol–water 70:30:4. Purity was checked by two-dimensional thin-layer chromatography (9). The spot corresponding to PC contained 86% of the radioactivity; phosphatidylethanolamine, digalactosyl diglyceride, monogalactosyl diglyceride, and phosphatidylglycerol also contained small amounts of radioactivity. The PC was then digested with phospholipase C as described previously (6). The incubation mixture was dried under nitrogen, taken up in chloroform, and chromatographed on Silica Gel G thin-layer plates with hexane–diethyl ether–acetic acid 70:30:2. The 1,2-diglyceride area was scraped and eluted with 100 ml of methanol. 85% of the radioactivity of this preparation migrated with 1,2-diglyceride on thin-layer plates. The major impurities were fatty acid, triglyceride, and 1,3-diglyceride, which accounted for 12% of the radioactivity. These impurities may have come from breakdown of the diglyceride during chromatography because if the purified 1,2-diglyceride spot was scraped, eluted, and rechromatographed, the three impurities and the 1,2-diglyceride had the same ratios as in the previous chromatogram.

Unlabeled diglyceride was prepared by phospholipase C degradation of approximately 100 mg of PC (6) which had been isolated from egg yolk by cold acetone precipitation and chromatography on silicic acid (10). The

resulting diglyceride was then purified on a silicic acid column (2.5 × 80 cm) with hexane–diethyl ether 4:1 as solvent. Purity of the diglyceride was checked by thin-layer chromatography on Silica Gel G plates developed in hexane–diethyl ether–acetic acid 70:30:2. One spot that migrated exactly like standard 1,2-diglyceride was detected. Detection was by spraying the plate with a 1% solution of iodine in methanol. The diglyceride was then applied to Silica Gel G silver nitrate-impregnated thin-layer plates which were developed with chloroform–methanol 92:8. The diglyceride fractions were then detected, eluted, and purified as previously described (6). The fatty acid analyses of the diglyceride fractions were carried out as described before (6).

When diglycerides, labeled or unlabeled, were to be used in enzymic assays they were added to vials as a chloroform solution. The chloroform was then evaporated with a stream of nitrogen, 0.01% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.) was added, and the mixture was sonicated using a Branson sonicator (Branson Instruments Co., Danbury, Conn.). The concentration of diglyceride and the fatty acid composition were then determined by gas–liquid chromatographic analysis using an internal standard (6). The preparations of synthetic diglyceride, dipalmitin, distearin, and diolein, were made and analyzed in the same way.

Reaction Mixtures

For the measurement of choline incorporation into PC, cytidine-5′-diphosphate choline-1,2-^{14}C, purchased from Tracerlab, Waltham, Mass., was used. The CDP-choline-1,2-^{14}C was checked for purity by paper chromatography (11) and was found to be 99% pure.

A typical incubation mixture for determining the incorporation of radioactivity from CDP-choline-1,2-^{14}C into PC contained 20 μmoles of dithiothreitol, 5 μmoles of $MnCl_2$, 80 μmoles of Tris–HCl, pH 8.0, 1.6 nmoles of CDP-choline-1,2-^{14}C (33,100 dpm), and 0.2 ml of enzyme preparation with a protein concentration of 1 mg/ml. The final incubation volume was 1.60 ml. When diglyceride was added to stimulate incorporation of CDP-choline-1,2-^{14}C into PC, Triton X-100 at a final concentration of 0.02% was found to be best for emulsifying the added diglyceride, which had already been sonicated in 0.01% Triton X-100. Before the addition of the enzyme preparation, the reaction mixture was preincubated at 30°C for 5 min. Incubation was then carried out for 10–20 min at 30°C. Incubations were terminated by the rapid addition of 6.0 ml of methanol–chloroform 2:1. A monophasic system resulted, and the lipid fraction was extracted according to the method of Bligh and Dyer (12). A blank that contained all the constituents of the reaction mixture except the enzymic preparation was also run.

Originally, incorporation into PC was determined by chromatographing an aliquot of the chloroform layer on a Silica Gel G thin-layer plate developed in chloroform–methanol–water 70:30:4. However, 97% of the radioactivity that was found in the chloroform layer was found consistently in the PC isolated by chromatography with the microsomal fraction as enzyme source. Therefore, routinely, incorporation of choline into phosphatidylcholine was determined by simply counting an aliquot of the chloroform layer.

The incubation mixture for the incorporation of radioactive diglyceride into PC consisted of 20 μmoles of DTT, 50 μmoles of $MnCl_2$, 80 μmoles of Tris–HCl, pH 8.0, 0.4 ml (11,600 dpm) of diglyceride-U-^{14}C previously sonicated in 0.01% Triton X-100, 10 μmoles of CDP-choline (Sigma Chemical Co.), and 0.08 ml of microsomal fraction with a protein concentration of 2 mg/ml. Incorporation of radioactive diglyceride into triglyceride was determined after chromatography on thin-layer plates developed in hexane–diethyl ether–acetic acid 70:30:2. The triglyceride area was scraped off and counted in the scintillation counter.

Scintillation Counting

The liquid scintillation counter used was a 720 series Liquid Scintillation System manufactured by the Nuclear-Chicago Corp., Des Plaines, Ill. When radioactivity was to be determined in spots on thin-layer chromatograms, the spots were scraped off and placed in scintillation vials after the iodine used for detection had sublimed off. 10 ml of counting solution (0.5% PPO and 0.03% POPOP in toluene) was added, and then 0.2 ml of 1 M Hyamine hydroxide in methanol (Amersham/Searle) was added to solubilize the compounds. Normally, either chloroform or water-soluble samples were dried under nitrogen in scintillation vials and then were counted after addition of the counting solution.

RESULTS

Subcellular Distribution of Phosphatidylcholine-synthesizing Activity

The data in Table 1 show the incorporation of radioactivity from CDP-choline-1,2-^{14}C into PC by subcellular fractions of spinach leaves. The highest activities of incorporation on the basis of protein content were found in the microsomes (100,000 *g* pellet) and in the 3,000 and 20,000 *g* pellets, both of which most likely correspond to the mitochondrial fraction. It is doubtful that the activity in these two fractions was due entirely to microsomal contamination; if it was, the activity on the basis of protein of the 20,000 *g* pellet would be expected to be markedly higher than that of the 3,000 *g* pellet. It

TABLE 1 INCORPORATION OF CDP-CHOLINE-1,2-^{14}C INTO PHOSPHATIDYLCHOLINE: SUBCELLULAR DISTRIBUTION OF ENZYMIC ACTIVITY

	Volume	Protein Concn.	Total Protein	Incorporation	Total Incorporation
	ml	*mg/ml*	*mg*	*dpm/mg protein (pmole/mg protein)*	*dpm*
Homogenate	161	3.5	560 (100%)	19,300 (76)	1,080 × 10^4 (100%)
200 *g* pellet	7.1	3.6	26 (4.3%)	7,080 (28)	18.4 × 10^4 (1.7%)
1000 *g* pellet (chloroplasts)	11.2	5.2	58 (9.8%)	3,440 (14)	20.6 × 10^4 (2.0%)
3000 *g* pellet	4.0	2.5	10 (1.6%)	38,800 (150)	38.8 × 10^4 (3.6%)
20,000 *g* pellet	7.3	1.4	10 (1.6%)	42,700 (170)	42.7 × 10^4 (4.0%)
100,000 *g* pellet (microsomes)	5.0	5.7	29 (4.9%)	200,000 (790)	580 × 10^4 (53.6%)
100,000 *g* supernatant	143	3.3	470 (77%)	1,720 (6.8)	80.9 × 10^4 (7.4%)
Recovery of protein and activity			(109%)		(74%)

Incubation mixtures consisted of: 80 μmoles of Tris–HCl, pH 8.0; 5 μmoles of DTT; 1.5 nmoles of CDP-choline-1,2-^{14}C (3.8 × 10^5 dpm); 2 μmoles of $MnCl_2$; and 0.2 ml of each subcellular fraction equivalent to 200 μg of protein added in a final incubation volume of 1.60 ml. Incubation was for 10 min at 30°C. All pellets were resuspended in 0.01 M Tris–HCl, pH 8.0. Incorporation into PC was determined after thin-layer chromatography on Silica Gel G with chloroform–methanol–water 70:30:4 as solvent.

is notable that there was very little or no enzymic activity in the chloroplasts. The low amount of activity present in this fraction may be attributable to some contamination from the 3,000 *g* pellet, although the chloroplast pellet was washed (Fig. 1).

The low recovery of enzymic activity may have been due perhaps to a lack of available endogenous diglyceride in several of the subcellular fractions or to resuspension of the pellets in dilute buffer. It cannot have been due to aging of the fractions during the period of isolation, since incubations with all of the preparations were carried out at the same time. In some experiments the chloroplast fraction was broken by osmotic shock and separated into fragment and supernatant fractions (Fig. 1). The recovery of activity in these fractions was only 25% of that in the chloroplasts, and the activity on a protein basis was lower in both fractions as compared with the chloroplast fraction.

Time Course of Incorporation

The incorporation of radioactivity from CDP-choline-1,2-^{14}C was linear as a function of time up to 15 min. There was a marked decrease in rate after 75 min. Because of the decrease in rate with longer incubations, the incubation time was limited to 10 min in most experiments.

Effect of Protein Concentration on Choline Incorporation

The increase in incorporation of choline-1,2-^{14}C into PC was proportional to the increase in microsomal protein added up to 4.0 mg.

Usually, the protein added in subsequent experiments was below 1 mg for an incubation volume of 1.60 ml.

Effect of CDP-choline Concentration on Choline Incorporation into PC

Fig. 2 shows the incorporation of choline-1,2-^{14}C into PC as a function of the CDP-choline added. The apparent K_m of CDP-choline calculated from these data was about 10 μM, which is much lower than the values of 0.2 mM (13) found for guinea pig liver and 0.16 mM found for rat liver (14).

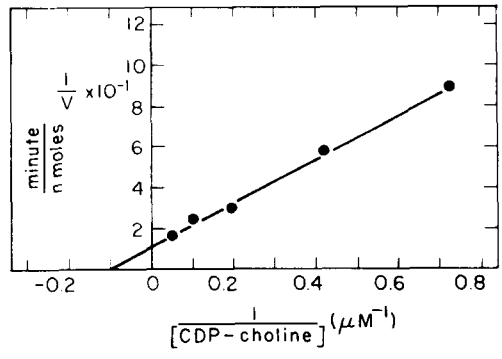

FIG. 2. Lineweaver-Burk plot of data from a CDP-choline concentration experiment. Incubation mixture: 70 μmoles of Tris–HCl, pH 7.5; 5 μmoles of DTT; different amounts of CDP-choline-1,2-^{14}C and unlabeled CDP-choline; 20 μmoles of $MgSO_4$; and 0.10 ml of microsomal preparation (250 μg of protein) in 0.1 M Tris–HCl, pH 7.5. Final incubation volume 1.60 ml. Incubation was for 10 min at 30°C. Experimental points are the averages of duplicate determinations.

Metal Requirement

Figs. 3 and 4 show the effects of Mg^{2+} and Mn^{2+} concentrations on the incorporation of choline-1,2-^{14}C into PC. Optimal activity with Mg^{2+} was reached at about 13 mM, while optimal activity with Mn^{2+} was reached at about 0.3 mM. These results contrast with those obtained with guinea pig liver, where optimal concentrations for both Mn^{2+} and Mg^{2+} were 2 mM, with equal stimulation by the two ions (13). The results do, however, resemble the results obtained with *Tetrahymena pyriformis*, where the optimum concentration for Mn^{2+} was lower than that for Mg^{2+} (15).

The nonlinear increase in incorporation at low metal ion concentrations in both figures may have been due to nonspecific binding of the metal ions, causing a decrease

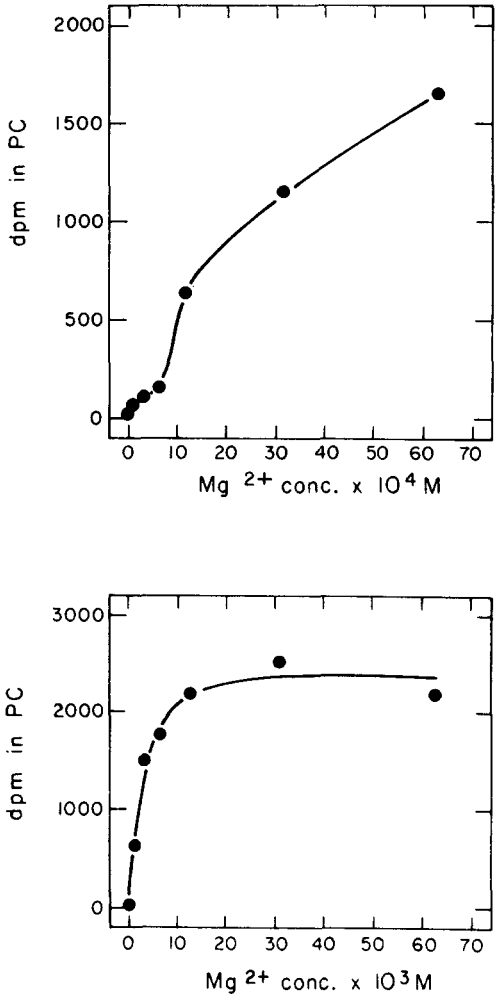

FIG. 3. Effect of Mg^{2+} concentration on incorporation of radioactivity from CDP-choline-1,2-^{14}C into phosphatidylcholine. Incubation mixture: 80 μmoles of Tris–HCl, pH 7.5; 5 μmoles of DTT; 16 nmoles of CDP-choline-1,2-^{14}C (82,000 dpm); different amounts of $MgCl_2$; and 0.10 ml of microsomal preparation (500 μg of protein) in 0.01 M Tris–HCl, pH 7.5. Incubation was for 10 min at 30°C. Final incubation volume was 1.60 ml. Experimental points are the averages of duplicate determinations.

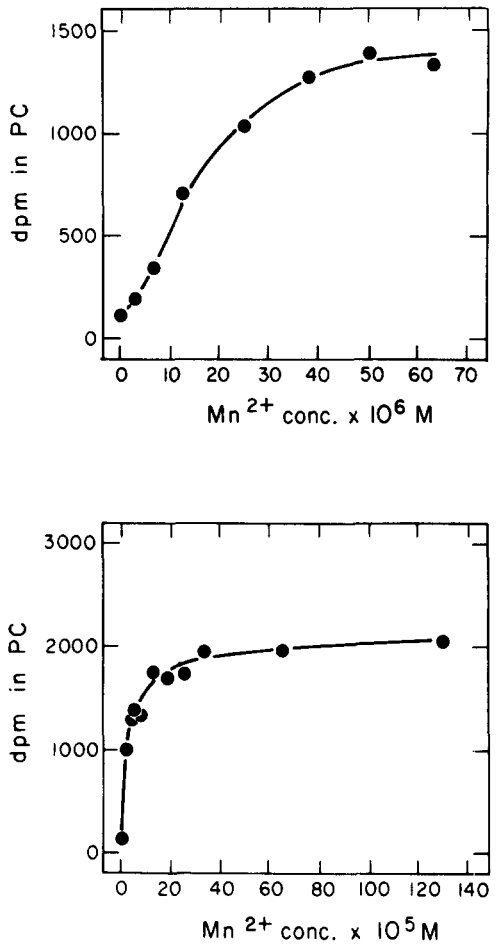

FIG. 4. Effect of Mn^{2+} concentration on incorporation of radioactivity from CDP-choline-1,2-^{14}C into phosphatidylcholine. Incubation mixture: 70 μmoles of Tris–HCl, pH 7.5; 5 μmoles of DTT; 16 nmoles of CDP-choline-1,2-^{14}C (82,000 dpm); different amounts of $MnCl_2$; and 0.10 ml of microsomal preparation (500 μg of protein) in 0.01 M Tris–HCl, pH 7.5. Incubation was for 10 min at 30°C. Final incubation volume was 1.60 ml. Experimental points are the averages of duplicate determinations.

in the actual concentration of the metal ion. The data show that the maximal rates obtainable in the presence of saturating amounts of either Mg^{2+} or Mn^{2+} are the same.

pH Dependence

The dependence of incorporation of choline-1,2-^{14}C into PC on pH is shown in Fig. 5. The concentration of Mg^{2+} was 100 times the concentration of Mn^{2+}. The microsomal preparation used was the same for both pH curves. The optimal pH for Mn^{2+} was 8.0 and for Mg^{2+} 7.5. The pH optimum with chicken liver particles was pH 8.0 with Tris buffer and Mg^{2+} as metal cofactor (16).

Inhibition of Choline Phosphotransferase

Table 2 shows the effect of sulfhydryl inhibitors on the incorporation of choline-1,2-^{14}C from CDP-choline-1,2-^{14}C into phosphatidylcholine. All three inhibitors

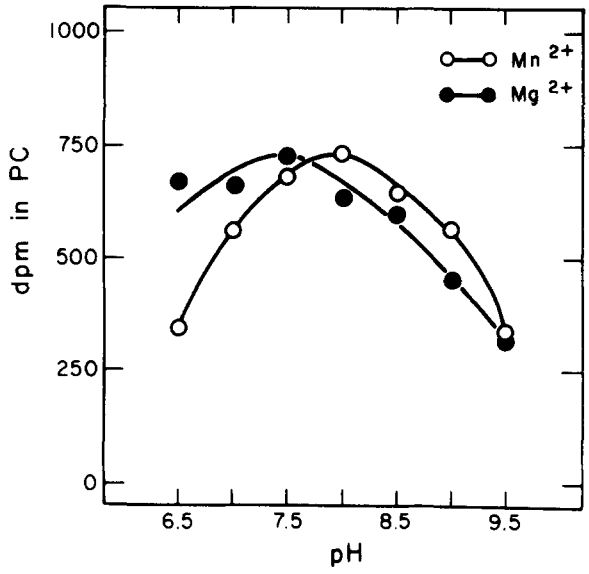

FIG. 5. Dependence of choline phosphotransferase on pH. Incubation mixture: 80 μmoles of Tris–HCl, different values of pH; 5 μmoles of DTT; 16 nmoles of CDP-choline-1,2-^{14}C (41,000 dpm); 50 μmoles of $MgCl_2$ or 0.5 μmole of $MnCl_2$; 0.10 ml of microsomal preparation (500 μg of protein) in 0.01 M Tris–HCl, pH 8.5. Incubation was for 10 min at 30°C. Final incubation volume was 1.60 ml. Experimental points are the averages of duplicate determinations.

were effective at 1.0 mM, with *p*-hydroxymercuribenzoate being the most effective at all concentrations tested. The absence of DTT from the reaction mixture during preincubation and incubation caused a significant loss of enzymic activity. This implies that some portion of the enzyme catalyzing transfer of choline to phosphatidylcholine may have a free sulfhydryl group.

Table 3 shows the protective effect of Mn^{2+} on the enzyme catalyzing choline transfer. Again, the loss of activity can be seen if DTT is left out of the reaction mixture. The protective effect of Mn^{2+} is illustrated by the reduction in incorporation from 720 dpm with Mn^{2+} present in the preincubation to 250 dpm when Mn^{2+} is present only during the final incubation with CDP-choline. The degree of inhibition by sulfhydryl reagents was increased when Mn^{2+} was not present during the preincubation. With Mn^{2+} present the percentages of control were 32%, 76%, and 32%, and without Mn^{2+} present the percentages of control were 14%, 93%, and 12% for *N*-ethylmaleimide, iodoacetamide, and *p*-hydroxymercuribenzoate, respectively. It is apparent from these data that Mn^{2+} stabilizes or activates the enzyme in some manner, possibly by protecting sulfhydryl groups, which otherwise react with sulfhydryl inhibitors.

Stimulation of Choline Phosphotransferase by Added Diglyceride

Table 4 illustrates the effect of diglyceride addition on the incorporation of choline-1,2-^{14}C into PC. The reaction mixtures were 0.02% with respect to Triton X-100. To further increase the degree of stimulation by exogenous diglyceride, the protein was decreased from 600 μg for each reaction mixture (1.60 ml volume) to 40 μg. This was done to lower the amount of endogenous acceptor,

TABLE 2 INHIBITION OF CHOLINE PHOSPHOTRANSFERASE BY SULFHYDRYL INHIBITORS

Inhibitor*	DTT	dpm	Inhibition + DTT	Inhibition − DTT
			% of control	
0	+	2640	100	176
0	−	1500	57	100
1 mM *N*-ethylmaleimide	−	360	14	24
0.1 mM *N*-ethylmaleimide	−	980	37	66
0.01 mM *N*-ethylmaleimide	−	1520	58	102
1 mM iodoacetamide	−	710	27	47
0.1 mM iodoacetamide	−	1340	51	90
0.01 mM iodoacetamide	−	1430	54	95
1 mM *p*-hydroxymercuribenzoate	−	27	1	2
0.1 mM *p*-hydroxymercuribenzoate	−	54	2	4
0.01 mM *p*-hydroxymercuribenzoate	−	450	17	30

Incubation mixture: 70 μmoles of Tris–HCl, pH 8.0; plus or minus 5 μmoles of DTT; 16 nmoles of CDP-choline-1,2-^{14}C (82,000 dpm); 0.5 μmole of $MnCl_2$; and 0.10 ml of microsomal preparation in 0.1 M Tris–HCl, pH 8.0 (500 μg of protein). Final incubation volume was 1.60 ml. There was a preincubation of 10 min at 30°C in which all constituents except the CDP-choline were added. The inhibitor was also present during this preincubation. After the preincubation the CDP-choline-1,2-^{14}C was added and the incubation of 10 min at 30°C was started.

* Final concentration.

TABLE 3 EFFECT OF METAL ION ON THE INCORPORATION OF RADIOACTIVITY FROM CDP-CHOLINE-1,2-^{14}C INTO PHOSPHATIDYLCHOLINE WITH SULFHYDRYL BINDING AGENTS PRESENT

Mn^{2+} in Preincubation	DTT	Inhibitor Concentration	dpm
+	+	0	1940
+	−	0	720
−	−	0	250
+	−	1 mM *N*-ethylmaleimide	230
−	−	1 mM *N*-ethylmaleimide	36
+	−	1 mM iodoacetamide	550
−	−	1 mM iodoacetamide	230
+	−	0.1 mM *p*-hydroxymercuribenzoate	230
−	−	0.1 mM *p*-hydroxymercuribenzoate	30

Incubation mixture: 0.70 μmoles of Tris–HCl, pH 8.0; plus or minus 5 μmoles of DTT; 16 nmoles of CDP-choline-1,2-^{14}C (83,000 dpm); 0.5 μmole of $MnCl_2$; and 0.10 ml of microsomal suspension in 0.1 M Tris–HCl, pH 8.0 (500 μg of protein). The final incubation volume was 1.60 ml. There was a preincubation of 10 min at 30°C in which all constituents of the reaction mixture were added except the inhibitors and CDP-choline-1,2-^{14}C; Mn^{2+} was added in the preincubation as shown in the table. After this preincubation, inhibitor was added as shown and the preincubation was continued for 10 min at 30°C. After this preincubation, Mn^{2+} was added to the remaining tubes and then CDP-choline-1,2-^{14}C was added. Incubation was for 10 min at 30°C.

TABLE 4 STIMULATION OF INCORPORATION OF RADIOACTIVITY FROM CDP-CHOLINE-1,2-^{14}C INTO PHOSPHATIDYLCHOLINE BY ADDITION OF DIGLYCERIDE

Experiment			Experiment			Diglyceride Addition (220 μg each)
1	2	3	1	2	3	
	dpm			*% of control*		
38	88	162				None
46	88	144	121	100	89	Dipalmitin
51	87	157	134	99	97	Distearin
66	115	240	174	131	147	Diolein
82	163	366	216	185	226	Egg PC diglyceride 1
81	154	303	213	175	187	Egg PC diglyceride 2
94	128	354	248	145	218	Egg PC diglyceride 3
88	136	472	232	155	291	Egg PC diglyceride 4

Incubation mixture: 80 μmoles of Tris–HCl, pH 8.0; 2 μmoles of DTT; 1.6 nmoles of CDP-choline-1,2-^{14}C (33,100 dpm); 5 μmoles of $MnCl_2$; 0.10 ml of 0.32% Triton X-100; and 0.04 ml of microsomal preparation in 0.01 M Tris–HCl, pH 8.0 (40 μg of protein). Incubation volume was 1.60 ml. Incubation for 30 min at 30°C. The homogenizing medium for the spinach leaves contained 1 mM EDTA, pH 8.0, as did the resuspended microsomal fraction. The diglycerides were dispersed (sonicated), and concentrations were determined by gas–liquid chromatography as described in Methods.

which could cancel any stimulation of incorporation by added diglyceride.

In Table 5 the fatty acid composition of the diglyceride fractions 1–4 from egg PC (see Methods) are shown. All the diglycerides except dipalmitin and distearin consistently stimulated incorporation (Table 4). Besides a possible enzyme–substrate specificity, saturated diglycerides might not have been stimulatory because the sonicates produced were not as homogeneous as those of the unsaturated diglycerides. Although the enzymic activity did not show any specificity of incorporation of the various diglycerides from egg PC, all these diglycerides showed a greater stimulation of incorporation than diolein. However, the lower stimulation by diolein may have been due to the fact that the diolein is a mixture of 1,2-diacyl-*sn*-glycerol and 2,3-diacyl-*sn*-glycerol because it was chemically synthesized. In several additional experiments with egg PC diglycerides, the average stimulation by 100 μg of diglyceride was 140%, and by 200 μg of diglyceride, 192%. For the synthetic diolein, stimulations by 100 μg and 200 μg were 130% and 151%, respectively; this also indicates that the stimulation by the synthetic diolein may have been due only to the 1,2-diacyl-*sn*-glycerol.

TABLE 5 FATTY ACID ANALYSIS OF THE DIGLYCERIDES DERIVED FROM EGG PHOSPHATIDYLCHOLINE USED IN TABLE 4

Fraction	16:0	18:0	18:1	18:2	20:4
			mole %		
1	24	11	17	35	13
2	33	14	6	46	—
3	34	10	47	8	—
4	39	10	30	—	—

The phospholipase C incubation and the separation of diglycerides into molecular species are described in Methods. The concentrations of the diglycerides were determined using an internal standard of methyl behenate.

Incorporation of Diglyceride-U-^{14}C into PC

Table 6 shows the incorporation of ^{14}C-labeled diglyceride into lipid. This observation further demonstrates that PC is being synthesized by choline phosphotransferase. The incorporation was stimulated by the addition of Mn^{2+} and unlabeled CDP-choline. Incorporation into triglyceride was surprising, since neither ATP nor CoA was added to the incubation mixture; yet an activation of fatty acids is implicated, since incorporation was markedly lowered when Mn^{2+} was left out of the incubation mixture. Incorporation of radioactivity into other compounds was occasionally noticed. These included monoglyceride and free fatty acid, products of hydrolysis, and also some monogalactosyl diglyceride. The radioactivity in these compounds was 15–30% of the activity of phosphatidylcholine.

TABLE 6 INCORPORATION OF DIGLYCERIDE-U-^{14}C INTO PHOSPHATIDYLCHOLINE AND TRIGLYCERIDE

Sample	Reaction Conditions		Incorporation	
	CDP-choline	Mn^{2+}	PC	Triglyceride
			dpm	
1	+	+	306	1536
2	—	+	200	1425
3	+	—	99	254

Incubation mixture: 80 μmoles of Tris–HCl, pH 8.0; 5 μmoles of DTT; plus or minus 0.1 ml of unlabeled CDP-choline (10 nmoles); plus or minus 5 μmoles of $MnCl_2$; 0.50 ml of diglyceride-U-^{14}C in 0.01% Triton X-100 (11,600 dpm); and 0.08 ml of microsomal suspension in 0.01 M Tris–HCl, pH 8.0 (160 μg of protein). The final incubation volume was 1.60 ml. Incubation was for 20 min at 30°C. 1.0 mM EDTA was used as in Table 4.

DISCUSSION

In general, the enzymic reaction in spinach leaves transferring phosphorylcholine from CDP-choline to diglyceride appears to be similar to that of animal systems with regard to pH optimum, metal requirement, and K_m for CDP-choline. However, the specificity of the reaction with various nucleotide diphosphate derivatives of choline has not been studied for spinach, so that it cannot be said with certainty that the reaction is absolutely specific for CDP-choline. Also, the metal requirement of the enzymic reaction in spinach leaves closely resembles that for *Tetrahymena pyriformis*, where Mn^{2+} showed a lower optimal concentration than did Mg^{2+} (15).

The low stimulation of diglyceride utilization by added CDP-choline (Table 6) is somewhat surprising, since this latter molecule would not be expected to be in any but minute amounts in isolated microsomes. In Table 6 the molar amount of PC synthesized was not determined because of the unknown specific activity of the diglyceride-U-^{14}C. It may be that such a minute amount of PC was made that any endogenous CDP-choline was sufficient for some synthesis.

With reference to Table 1, it is notable that there is very little incorporation of choline-1,2-^{14}C by the chloroplasts although the percentage of phosphatidylcholine in the chloroplasts has been found to be 39% of the total found in spinach leaves (16).

The subcellular distribution of choline phosphotransferase is similar to that of animal systems, with the microsomal fraction having the highest specific activity (17). However, in *Tetrahymena pyriformis* the highest specific activity was found in the mitochondrial fraction (15). Cheniae (4) has found that the microsomal fractions had the highest specific activities for incorporating glycerophosphate-1,3-^{14}C into lipid. The specific activity of incorporation of glycerol by microsomes into lipid was 25 times that of the chloroplasts (4), and the specific activity of choline incorporation by microsomes was about 30 times as great as that of the chloroplasts (Table 1). Therefore, there is the possibility that the PC found in chloroplasts is not synthesized there but rather is synthesized in the endoplasmic reticulum and is then transported intact to chloroplasts, perhaps by a mechanism similar to that demonstrated in animals for the incorporation of PC into mitochondria (18, 19). Indeed, such a mechanism has already been reported for exchange between microsomes and mitochondria from potato tuber (20). A similar type of mechanism may hold true for the transport of synthesized fatty acid from the chloroplasts to the endoplasmic reticulum in spinach (21), although in other plants phosphatidylcholine has been implicated in fatty acid synthesis and especially polyunsaturated fatty acid synthesis (22).

The lack of specificity toward diglycerides in the synthesis of phosphatidylcholine in spinach agrees with the results with phosphatidylcholine synthesis in animals (3) but is different from the results with the synthesis of galactolipids in spinach (23), where there was a marked diglyceride specificity.

This work was supported in part by NIH Training Grant 5-TO1-ES84 from the National Institute of Environmental Health Sciences, and in part by Grant AP 00071 from the Air Pollution Control Office, Environmental Protection Agency.

Manuscript received 28 August 1970; accepted 11 February 1971.

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