Enzymatic synthesis of cytidine diphosphate diglyceride

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ABSTRACT Evidence is presented for the enzymatic formation of cytidine diphosphate diglyceride in microsomal preparations from guinea pig liver according to the reaction:

CTP + phosphatidic acid $\rightleftharpoons$ CDP-diglyceride + P—O—P.

Conditions have been found in which the incorporation of labeled CTP into CDP-diglyceride is almost entirely dependent upon added phosphatidic acid. The incorporation of CMP into lipid is very slight. A substantial net synthesis of CDP-diglyceride takes place under these conditions. Some properties of the enzyme system are described.

KEY WORDS cytidine nucleotide · CDP-diglyceride · enzymatic · biosynthesis · phosphatidic acid · microsomes · guinea pig · liver · dependence · physicochemical dispersion state

Cytidine diphosphate diglycerides (CDP-diglycerides) are lipid-soluble nucleotides that may be regarded as activated forms of phosphatidic acids. CDP-diglycerides have been identified as precursors of phosphatidyl inositol (1, 2) and phosphatidyl glycerol (3) in animal tissues, and of phosphatidyl serine and phosphatidyl ethanolamine in bacterial systems (4) and thus play a central role in the biogenesis of glycerophosphatides.

In a previous publication from this laboratory (2), evidence was presented for the enzymatic formation of CDP-diglyceride according to the reaction:

CTP + phosphatidic acid $\rightleftharpoons$ CDP-diglyceride + P—O—P (I).

The suggestion (1) that CDP-diglyceride might be formed from CDP-choline and phosphatidic acid appears not to have been substantiated by later work.

In a recent publication, Petzold and Agranoff (5) reported that phosphatidic acid did not stimulate the formation of CDP-diglyceride in an enzyme system from guinea pig liver. These workers suggested that CDP-diglyceride was formed by a reaction between CMP and phosphatidyl inositol, i.e., a reversal of the synthesis of phosphatidyl inositol.

CDP-diglyceride + inositol $\rightleftharpoons$ CMP + phosphatidyl inositol (II).

However, reaction II cannot account for the net synthesis of CDP-diglyceride, since phosphatidyl inositol itself arises from CDP-diglyceride in the only known pathway for its biosynthesis.

In the present paper, we wish to report a further study of the enzymatic synthesis of CDP-diglyceride from CTP and phosphatidic acid in enzyme preparations from guinea pig liver. We have been able to find conditions in which a substantial net synthesis of CDP-diglyceride takes place, which is almost entirely dependent upon added phosphatidic acid. This and other evidence support the view that the synthesis of CDP-diglyceride in this tissue takes place by reaction I.

MATERIALS AND METHODS

Materials

Adenine and cytidine nucleotides were products of the Pabst Research Laboratories (Milwaukee, Wis.) and Schwarz Bio Research Inc. (Orangeburg, N.Y.). CTP labeled in the pyrimidine moiety with ^{3}H was the Schwarz product. Synthetic cytidine diphosphate dipalmitin was prepared by a modification of the method of Agranoff and Suomi (6).

Phosphatidic acid was prepared from egg lecithin by a modification of the method of Kates (7) and was stored as the neutral Tris [tris(hydroxymethyl) aminomethane]

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salt. One such preparation gave a single spot on chromatography on thin layers of silica gel in the system chloroform–pyridine—formic acid (50:20:7 by volume), with an R_f of 0.76 closely similar to that of synthetic dipalmitoyl phosphatidic acid. A second preparation was contaminated with significant amounts of lysophosphatidic acid, but its behavior in the enzyme system for the synthesis of CDP-diglyceride was not detectably different from that of the pure material.

Preparation of Enzyme

For experiments on the intracellular localization of the enzyme that catalyzes the synthesis of CDP-diglyceride, the procedure of Wilgram and Kennedy (8) for the fractionation of tissue was used. For subsequent studies, an enzyme fraction consisting principally of microsomes was isolated as follows. Fresh guinea pig livers were homogenized in 4 volumes of cold 0.25 M sucrose containing 10^{-2} M 2-mercaptoethanol and 10^{-3} M EDTA. Whole cells and nuclei were removed by centrifugation at 700 × *g* for 10 min, and mitochondria were removed by centrifugation at 12,000 × *g* for 12 min. The supernatant fraction was then centrifuged at 40,000 × *g* for 100 min and the pellet ("microsomes") washed by suspension in the same medium used for recentrifugation. The final washed pellet was suspended in cold medium in a final volume of 1 ml per g of original tissue, and stored at −15°C. The particles maintained activity during several months of storage.

Assay

The conversion of radioactive CTP, labeled with tritium in the cytidine moiety, to a radioactive lipid was measured as follows. The enzymatic reaction, usually in a final volume of 0.5 ml, was carried out in a conical-tipped vessel of 40 ml capacity, fitted with a ground-glass stopper. The reaction was stopped by the addition of 5 ml of methanol containing 0.1 N HCl. After about 10 min, chloroform (10 ml) was added and the single phase system mixed thoroughly. An aqueous solution of 2 M KCl (20 ml) was then added, and the tightly stoppered vessels were shaken vigorously for 5 min. The phases were allowed to separate, and the upper aqueous phase was drawn off through a capillary. The chloroform was washed twice more with 2 M KCl in the same manner. An aliquot was then dried in a scintillation vial, 10 ml of Buhler's solution (9) was added, and the radioactivity determined in a Packard Liquid Scintillation Counter.

RESULTS

Requirement for Phosphatidic Acid for the Conversion of CTP to CDP-Diglyceride

Preparations of microsomes from guinea pig liver catalyze the conversion of CTP, labeled in the cytidine moiety, to a lipid form (CDP-diglyceride) in the presence of cofactors needed for the generation of phosphatidic acid (Fig. 1). This finding confirms the previous report of Paulus and Kennedy (2). The system for generating phosphatidic acid may be completely replaced by phosphatidic acid prepared from egg yolk lecithin (Fig. 2). Indeed, synthesis of CDP-diglyceride is much higher in the latter case.

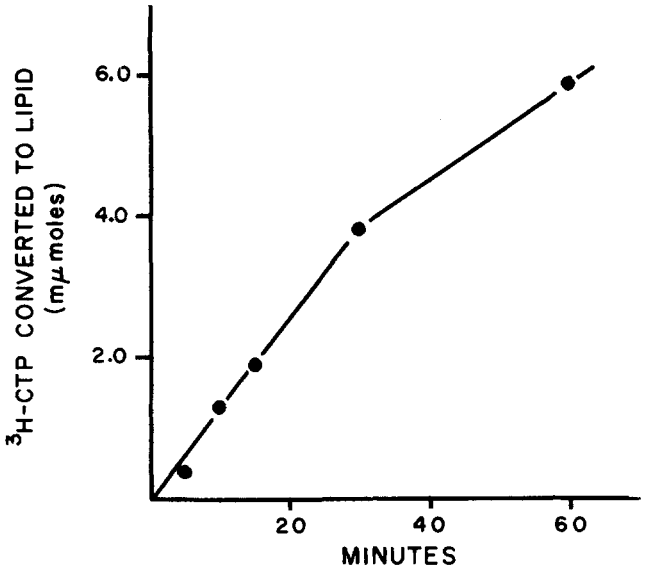

FIG. 1. Conversion of ^{3}H-labeled CTP to lipid, catalyzed by microsomes supplemented with cofactors needed for the generation of phosphatidic acid. The system contained guinea pig liver microsomes (3.4 mg of protein), phosphate buffer of pH 7.4 (0.1 M), DL-α glycerophosphate (2 mM), ^{3}H-CTP (2 mM), CoA (2 mM), sodium oleate (0.2 mM), ATP (4 mM), $MgCl_2$ (2 mM), and $MnCl_2$ (2 mM). The final volume of the system was 0.5 ml. The tubes were incubated at 37°C for the times indicated. The conversion of ^{3}H-CTP to lipid (CDP-diglyceride) was measured as indicated in the text under Materials and Methods.

The dependence of the reaction upon the concentration of added phosphatidic acid is shown in Fig. 3. Very little synthesis is observed in the absence of added phosphatidic acid. The apparent K_m is about 2.5 mM for this concentration of CTP (2 mM).

Fully saturated phosphatidic acids, such as synthetic dipalmitoyl L-α-glycerophosphate, are much less active in this reaction; this is presumably because the saturated compound is more difficult to disperse in the reaction mixture, even in the presence of detergent.

Specificity for CTP

The incorporation of cytidine nucleotides into lipid under the conditions described is almost entirely specific for CTP (Table 1). CMP does not substitute for CTP in the reaction. Addition of unlabeled CMP to the reaction had no effect on the incorporation of labeled CTP. In brief incubations, ATP had no effect on the reaction; in

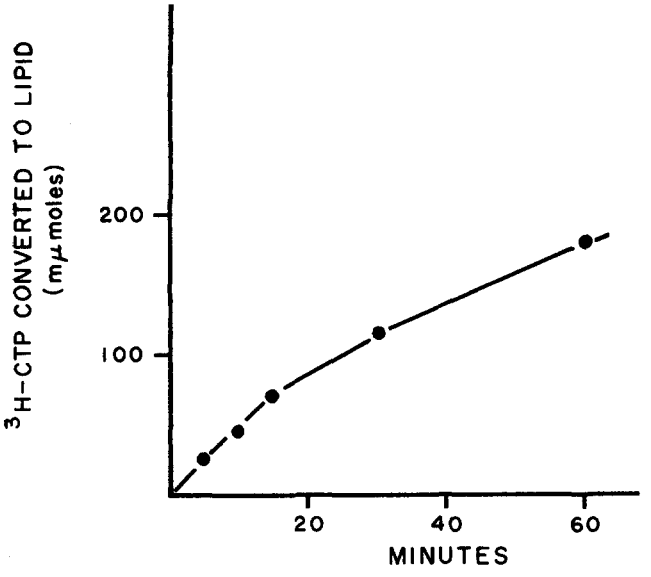

FIG. 2. Rate of conversion of labeled CTP to lipid in the presence of added phosphatidic acid. The system contained guinea pig liver microsomes (ca. 3 mg of protein), Tris buffer of pH 7.4 (0.1 M), phosphatidic acid prepared from egg lecithin (4 mM), ATP (6 mM), ^{3}H-CTP (2 mM), and $MgCl_2$ (40 mM). The $MgCl_2$ was added last. The final volume of the system was 0.5 ml. The tubes were incubated at 37 °C for the times shown.

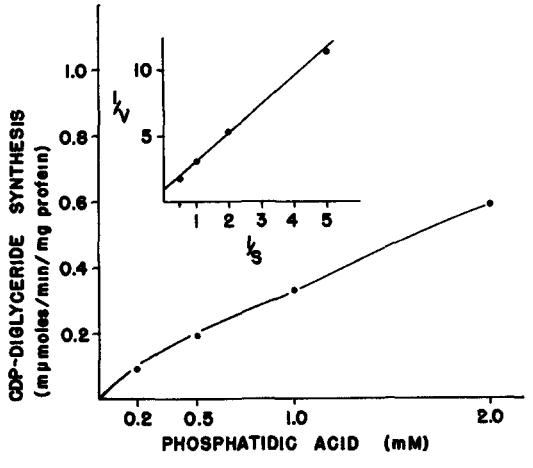

FIG. 3. Effect of varying phosphatidic acid concentration on rate of synthesis of CDP-diglyceride. The conditions of the experiment were similar to those of Fig. 2, except that the phosphatidic acid concentration was varied as shown. The time of incubation was 10 min at 37 °C.

experiments allowed to proceed for 1–2 hr ATP enhanced the incorporation of CTP into lipid by 10–20%, presumably by preventing the breakdown of CTP by microsomal phosphatases. For this reason, ATP was routinely added to incubations longer than 15 min.

Affinity for CTP

The affinity of the enzyme for CTP is shown in Fig. 4. The apparent K_m is approximately 1 mM when phosphatidic acid is 2 mM.

TABLE 1 ACTIVITY OF CTP AND OF CMP IN FORMATION OF CYTIDINE-CONTAINING LIPID

Nucleotide Added	Cytidine Nucleotide Converted to Lipid
^{3}H-CTP (2 mM)	20
^{3}H-CTP (2 mM) + unlabeled CMP (2 mM)	20
^{3}H-CMP (2 mM)	1.5
^{3}H-CMP (2 mM) + unlabeled CTP (2 mM)	0.6

Each tube contained guinea pig liver microsomes (1.2 mg of protein), Tris buffer of pH 7.4 (0.1 M), phosphatidic acid derived from egg lecithin (2 mM), $MgCl_2$ (20 mM), and added nucleotides as indicated. The tubes were incubated at 37 °C for 60 min.

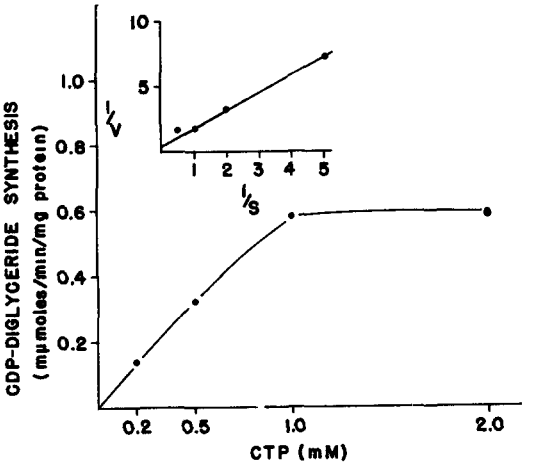

FIG. 4. Effect of varying CTP concentration on rate of synthesis of CDP-diglyceride. The conditions of the experiment were similar to those of Fig. 3, except that the phosphatidic acid concentration was held constant at 2 mM, while the CTP concentration was varied as shown. The time of incubation was 10 min at 37 °C.

Identification of Product

Evidence that the lipid product of the reaction between CTP and phosphatidic acid is CDP-diglyceride has been previously presented by Paulus and Kennedy (2). Further evidence for this was obtained in the present study by thin-layer chromatography, and by direct enzymatic assay of the product.

An aliquot of the ^{3}H-labeled lipid, obtained in an experiment similar to that in Fig. 2, was applied to a silica gel thin-layer plate along with synthetic CDP-dipalmitin as carrier. The chromatogram was developed in the system chloroform–methanol–acetic acid–water 50:24:4:8 and the spots were located under UV light after they were sprayed with Rhodamine G. The spot corresponding to the marker CDP-dipalmitin was scraped directly into a counting vial and counted in a thixotropic gel mixture. Eighty-three per cent of the applied counts were recovered in the CDP-dipalmitin spot.

TABLE 2 NET ENZYMATIC SYNTHESIS OF CDP-DIGLYCERIDE

Time of Incubation	^{3}H-CTP Converted to Lipid	CDP-Diglyceride Found by Enzymatic Assay
min	*mμmoles*	
0	3	0
120	168	150

The reaction mixture used for the enzymatic synthesis of CDP-diglyceride was the same as that in the experiment of Fig. 2. At the end of the experiment, the labeled lipids were extracted and washed as described under Materials and Methods. An aliquot was taken for counting, to determine the conversion of ^{3}H-CTP to lipid, and the remainder of the washed chloroform solution was taken to dryness under a stream of nitrogen. The dried residue was suspended in an aqueous solution of the nonionic detergent Triton X 100 and assayed for its content of CDP-diglyceride, by means of the partially purified enzyme from *E. coli* described in the text. Synthetic CDP-dipalmitin was used as standard in this assay.

Enzymatic Net Synthesis of CDP-Diglyceride

Further evidence as to the nature of the product was obtained in experiments designed to demonstrate net synthesis of CDP-diglyceride. Use was made of a partially purified enzyme preparation from *E. coli* that catalyzes the following reaction:

CDP-diglyceride + L-α-GP ⇌

phosphatidyl glycerophosphate + CMP (III).

Reaction III is completely dependent upon added CDP-diglyceride and can thus be used for a convenient and highly sensitive assay for this nucleotide (Chang, Y. Y., and E. P. Kennedy, unpublished observations). In the experiment shown in Table 2, the conversion of labeled CTP to radioactive liquid was compared with the net synthesis of CDP-diglyceride, as measured by direct assay. No significant amount of CDP-diglyceride was detected in the "zero time" control while an increment of 150 mμmoles was found as a result of the action of the enzyme. This value is 91% of that calculated on the basis of incorporation of radioactivity from ^{3}H-CTP and leaves no doubt that a substantial net synthesis of enzymatically active CDP-diglyceride is catalyzed by the enzyme system.

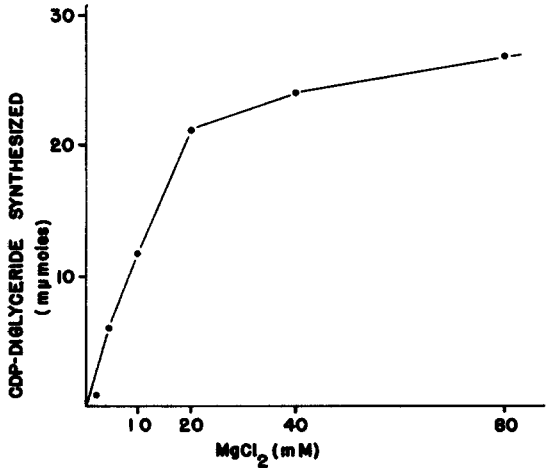

FIG. 5. Effect of varying Mg^{++} concentration on synthesis of CDP-diglyceride. The conditions of the experiment were similar to those of Fig. 2, except that the concentration of added $MgCl_2$ was varied as indicated.

Cation Requirement

The synthesis of CDP-diglyceride showed an absolute dependence upon added divalent cations. Mg^{++} was most effective (Fig. 5); Mn^{++} could be substituted for Mg^{++} but under optimal conditions gave only 50% of the rate of incorporation observed with Mg^{++}.

Because of the insolubility of the Mg salts of phosphatidic acid, we routinely added the cation to the incubation mixture after the enzyme preparation. The importance of the order of addition was demonstrated in the experiment shown in Table 3. Addition of the particulate enzyme preparation to the reaction mixture as little as 15 sec before the Mg^{++} gave high levels of activity, and more prolonged prior incubation of enzyme and substrate gave no greater activity. However, when the enzyme was added immediately after the cation, only 10% of the activity was observed. An insoluble precipitate was observed when the Mg^{++} was added first.

pH Optimum

The enzyme is active in the pH range 6–8, with an optimum at about 7.5.

Effect of Enzyme Concentration

Anomalous results were observed when the amount of enzyme added to the system was varied over a wide range. A typical experiment is shown in Fig. 6. At very low enzyme concentrations, little activity is observed, while the addition of larger amounts leads to a linear increment in the amount of product. In other experi-

TABLE 3 EFFECT OF ORDER OF ADDITION OF Mg^{++} ON ACTIVITY

Order of Addition of Components	CDP-Diglyceride Synthesized
	mμmoles
A. 1. All other components	
2. Mg^{++}	4.5
3. Enzyme (30 sec after Mg^{++})	
B. 1. All other components	
2. Enzyme	51.6
3. Mg^{++} (15 sec after enzyme)	

The composition of the enzyme system was the same as in Fig. 2. The final volume was 0.5 ml. The enzyme (0.2 ml of guinea pig liver microsomes containing 6 mg of protein) was added either before or after the $MgCl_2$ (final concentration of 20 mM) as indicated. The tubes were incubated at 37°C for 15 min.

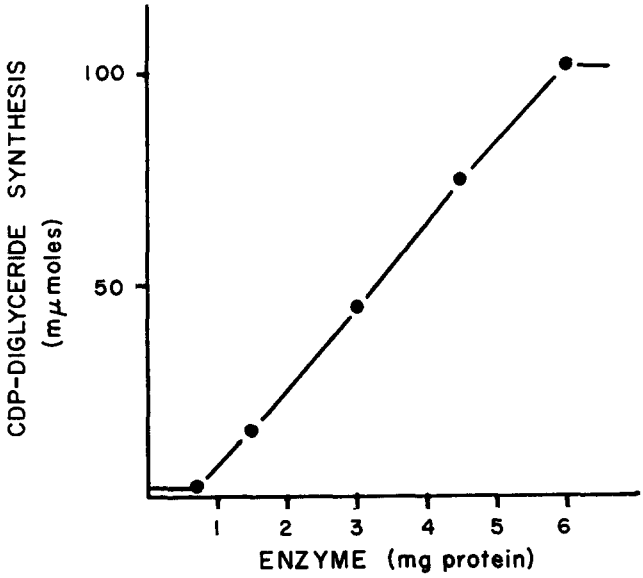

FIG. 6. Effects of varying enzyme concentration. The conditions of the experiment were similar to those of Fig. 2, except that the amount of microsomal enzyme added was varied as shown.

ments (data not shown) some inhibition at very high enzyme concentrations was noted.

These results may perhaps be explained in light of the findings on the importance of the order of adding the reagents to the system (Table 3). Presumably the stabilization of the magnesium–phosphatidic acid complex by the lipoprotein of the microsomal enzyme preparation is needed for activity, and at very low concentrations of enzyme this effect may not be obtained.

Effects of Inorganic Pyrophosphate

Pyrophosphate in a concentration of 4 mM was found to inhibit by 90% the incorporation of labeled CTP into CDP-diglyceride. Numerous experiments were done with pyrophosphate, in an attempt to demonstrate the reversal of reaction I, without consistent results. It was possible to demonstrate readily a CTP-dependent pyrophosphate exchange reaction in which labeled pyrophosphate was incorporated into nucleotide. However, when synthetic CDP-diglyceride was incubated with ^{32}P-pyrophosphate, the incorporation of radioactivity into the charcoal-adsorbable nucleotide fraction was small and not readily reproducible.

TABLE 4 INTRACELLULAR LOCALIZATION OF ENZYME IN GUINEA PIG LIVER

Fraction	^{3}H-CTP Converted to Lipid
	mμmoles
Nuclei, unbroken cells	28.2
Mitochondria	1.4
"Intermediate"	2.6
Microsomes	51.8
Supernatant fraction	0

Fresh guinea pig liver, 2 g, was fractionated essentially according to the method of Wilgram and Kennedy (8). Each fraction was made up to a final volume of 10 ml, except for the supernatant fraction (22.6 ml). The assay was carried out as described in the experiment of Fig. 2, except that 0.3 mg of the detergent trimethyl octadecyl ammonium chloride was included in the incubation mixture. The volume of each fraction assayed was 0.4 ml.

TABLE 5 ACTIVITY OF HOMOGENATES OF VARIOUS TISSUES OF THE GUINEA PIG IN THE CONVERSION OF ^{3}H-CTP TO LIPID

Tissue	^{3}H-CTP Converted to Lipid
	mμmoles
Liver	74.3
Intestinal mucosa	25.4
Kidney	9.3
Brain	8.4
Heart	2.4

Tissues were removed immediately after the guinea pig was killed and were homogenized in 8.5% (w/v) sucrose containing 0.01 M mercaptoethanol and 1 mM EDTA, 4 ml/g of fresh tissue. The incubation mixture for the assay contained 0.5 ml of homogenate, Tris buffer of pH 7.4 (0.1 M), ^{3}H-CTP (1 mM), ATP (5mM), $MgCl_2$ (20 mM), and trimethyl octadecyl ammonium chloride (0.5 mg) in a final volume of 1.0 ml. The incubation was carried out for 30 min at 37°C.

Intracellular Distribution in Liver

As demonstrated in Table 4, the microsomal fraction showed the greatest activity in the synthesis of CDP-diglyceride.

Distribution in Tissues of the Guinea Pig

A survey of guinea pig tissue homogenates demonstrated activity in each tissue tested, with liver and gut mucosa the most active (Table 5).

DISCUSSION

The results reported in this paper leave little doubt that the net synthesis of CDP-diglyceride may take place by the reaction shown in equation I. That the incorporation of labeled CMP into CDP-diglyceride may also take place via equation II seems likely from the results of Paulus and Kennedy (2), who presented evidence suggesting that the enzymatic synthesis of phosphatidyl inositol may be reversible. The latter reaction, however, as indicated above, would not lead to a net synthesis of CDP-diglyceride. It is noteworthy that in the enzyme system studied in this paper CMP is a much less efficient precursor of CDP-diglyceride than is CTP (Table 1).

There is increasing evidence that the physicochemical state of substrate and of enzyme is of crucial importance in determining the activity of systems involving lipid intermediates. Three properties of the enzyme system

described here may be significant in this regard. First, it appears that the order of addition of the reagents to the enzyme system may determine the catalytic activity (Table 3). Secondly, the failure of synthetic saturated phosphatidic acids to substitute for the naturally occurring unsaturated compounds may be related to the striking differences in the ease of dispersion of the two kinds of substrate, rather than to a strict chemical specificity in the true sense of the word. Finally, the disproportionately low activity observed with very small amounts of microsomes (Fig. 6) may be a reflection of the physical state of the system when the content of lipoprotein derived from the enzyme preparation falls below a crucial level. Some combination of these factors may account, in part at least, for the rather different observations of Petzold and Agranoff (5) in their study of the enzymatic formation of CDP-diglyceride.

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