senger RNA suggests that the messenger-RNA content of the ribosome may differ in different parts of the log phase. Such a condition could also lead to a variation of the polysome content of the cell, and these questions are being investigated. Differences in the transfer enzyme content of the ribosomes do not appear to be the explanation because 100,000 × *g* supernatant fluid, which contains these enzymes, was used in the incubation mixture.

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Cyclopropane Fatty Acid Synthetase: Partial Purification and Properties*

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The cyclopropane fatty acid synthetase from *Clostridium butyricum* has been partially purified. The enzyme system transfers the methyl group of *S*-adenosylmethionine to a monounsaturated fatty acid chain of phosphatidylethanolamine to form a cyclopropane–fatty acid chain. The enzyme system is stimulated by anionic surfactants and inhibited by cationic and neutral surfactants. The Michaelis constants for phosphatidylethanolamine and *S*-adenosylmethionine are 5.3×10^{-4} M and 5.7×10^{-5} M, respectively. The Arrhenius plot for the enzyme reaction is discontinuous over the temperature range 0–40°. The heat of activation for the enzyme reaction is lowered by the addition of the anionic surfactant sodium lauryl sulfate.

The occurrence in lactobacilli of a 19-carbon fatty acid containing a cyclopropane ring was first demonstrated by Hofmann and co-workers (Hofmann *et al.*, 1952), who characterized the compound as *cis*-11,12-methyleneoctadecanoic acid (Hoffmann *et al.*, 1952). A 17-carbon cyclopropane fatty acid from *Escherichia coli* was described by Dauchy and Asselineau (1960) and characterized as *cis*-9,10-methylenehexadecanoic acid by Kaneshiro and Marr (1961). The methylene carbon of lactobacillic acid has been shown to be derived from the methyl group of methionine (Liu and Hofmann, 1962), and several workers have demonstrated the incorporation of methionine methyl groups into cyclopropane fatty acids (O'Leary, 1959, 1962; Chalk and Kodicek, 1961; Law *et al.*, 1963). Zalkin and Law (1962) and Zalkin *et al.* (1963) have recently demon-

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strated the enzymatic synthesis of cyclopropane fatty acids in extracts of the Gram-negative aerobe, *Serratia marcescens*, and the Gram-positive anaerobe, *Clostridium butyricum*. The formation of the cyclopropane fatty acids occurred by addition of the methyl group of *S*-adenosylmethionine to the unsaturated fatty acid moiety of a phospholipid. The phospholipid substrate was tentatively identified as phosphatidylethanolamine.

In the system described by Zalkin *et al.* (1963) the phospholipid was an effective substrate only if it could be dispersed as a clear micellar suspension. Bangham and Dawson (1959, 1962) have suggested that the surface charge on phospholipid micelles is an important factor in enzyme-micelle interactions. The effectiveness of dispersions of phospholipids as substrates for a lecithinase from *Penicillium notatum* and *Clostridium perfringens* α-toxin could be correlated with the electrophoretic mobility and thus with the zeta potential of the substrate micelles. Certain detergents (amphipathic agents) altered the micellar charge and concurrently altered the substrate efficiency of the phospholipid micelles.

It was of considerable interest therefore, to determine the effect of several different amphipathic ions on the cyclopropane–fatty acid–synthetase system.

This report describes a method for obtaining a partially purified preparation of the cyclopropane–fatty acid–synthetase system from *Cl. butyricum*, studies on its kinetic parameters, its substrate requirements, and finally the effect of amphipathic compounds on its activity.

Experimental

Materials

DEAE-cellulose was obtained from Carl Schleicher and Schuell Co., Keene, N. H. Hypatite C and Unisil silicic acid were products of the Clarkson Chemical Co., Williamsport, Pa. [*methyl*-^{14}C]-*S*-Adenosylmethionine with a specific activity of 13.78 mc/mmole was purchased from Tracerlab, Waltham, Mass. *S*-Adenosylmethionine was prepared from activated baker's yeast according to Schlenk *et al.* (1959) and purified by the method of Mudd (1959).

Cutscum was obtained from the Fisher Scientific Co., Fairlawn, N.J. Hexadecyltrimethylammonium bromide was obtained from Fluka A. G., Chemische Fabrik, Buchs, Switzerland. Octadec-9-enylphosphate (oleyl acid phosphate) was a gift of the Hooker Chemical Corp., Niagara Falls, N.Y. Dihexadecylphosphate (dicetylphosphate) was obtained from Krishell Laboratories Inc., Portland, Ore. Sodium oleate was the product of J. T. Baker Chemical Co., Phillipsburg, N.J. Sodium lauryl sulfate was obtained from Sigma Chemical Co., St. Louis, Mo. The sodium salt of cholic acid was obtained from Matheson, Coleman and Bell, Norwood, Ohio. The California Corp. for Biochemical Research, Los Angeles, Calif., was the source of the sodium salts of deoxycholic acid and DL-α-tocopheryl phosphate. Tween 80 was obtained from the Atlas Powder Co., Wilmington, Del.

Asolectin was obtained from Associated Concentrates, Inc., Long Island, N. Y. Phosphatidylinositol and phosphatidylserine were purchased fron L. Light and Co., Colnbrook, England. Standard fatty acid methyl esters were obtained from Applied Science Laboratories, State College, Pa.

Deionized water was used for all experiments described.

Methods

Azotobacter agilis Phosphatidylethanolamine.—*Azotobacter agilis* cells were grown aerobically on Burk's synthetic medium (Wilson and Knight, 1952) and harvested at the end of exponential growth. The bacterial lipids were extracted and washed by the method of Folch *et al.* (1957). The solution containing the lipids was evaporated to dryness under reduced pressure with a rotary evaporator (Swissco Instruments, Greenville, Ill.) at 30–40°. The residue was redissolved in benzene and lyophilized to give a light-tan powder, which was stored at −20°.

This mixture of lipids was chromatographed on DEAE-cellulose and silicic acid as described by Rouser *et al.* (1961). The phosphatidylethanolamine obtained was chromatographed on thin layers of silicic acid by the techniques of Wagner *et al.* (1961). The resulting chromatograms revealed only one component. Aqueous dispersions of the crude and purified phospholipid preparations were obtained by the dialysis technique of Fleischer and Klouwen (1961). Zalkin *et al.* (1963) reported that purified phosphatidylethanolamine could not be dispersed by this technique. Careful attention to the instructions of Fleischer and Klouwen (1961) permitted dispersion of this phospholipid; however, it was not possible to obtain solutions of as high a concentration as could be obtained with other pure phospholipids or with crude preparations.

The fatty acids of the crude and purified phosphatidylethanolamine preparations were examined. The phospholipid was hydrolyzed in 50% aqueous methanol (*v*/*v*) and 8% KOH (w/v). The mixture was acidified with 6 N HCl and the fatty acids were extracted with diethyl ether. The methyl esters of the extracted fatty acids were made with freshly distilled diazomethane, and then subjected to gas-liquid chromatography. A Research Specialties instrument (Richmond, Calif.) equipped with an ionization detector and a 183-cm (6-foot) column packed with 10% diethylene glycol succinate on Chromosorb W was used for the analysis. The column was operated at 165–185° with the carrier gas, argon, flowing at a rate of 40–70 ml/minute. The fatty acid patterns obtained were similar to those reported by Kaneshiro and Marr (1962). Approximately 60% of the total fatty acids consisted of hexadecenoic and octadecenoic acids. Palmitic acid was the other major component.

Vegetable Phosphatidylcholine.—Asolectin was used as the source of phosphatidylcholine. The crude phospholipid mixture was chromatographed on deactivated alumina as described by Hanahan *et al* (1951). The fractions containing phosphatidylcholine were evaporated to dryness under reduced pressure with the aid of a rotary evaporator. The residue was redissolved in chloroform and to the solution thus obtained 20 volumes of cold acetone were added. The phospholipid precipitate was collected and rechromatographed on a silicic acid–silicate column by the method of Rouser *et al.* (1961). Thin-layer chromatography of the purified phosphatidylcholine on silicic acid revealed only one component in the solvent systems of Wagner *et al.* (1961). The methyl esters of the fatty acids of the purified phosphatidylcholine were examined by gas-liquid chromatography. The major components were palmitic, stearic, octadecenoic, octadecadienoic, and octadecatrienoic acids. Approximately 60% of the total fatty acids was octadecadienoic acid.

Assay of Protein Concentration.—Protein concentration was determined either by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard, or by the method of Warburg and Christian (1941). The two methods were in fair agreement in the absence of high concentrations of nucleic acids.

Assay of Enzyme Activity.—The catalytic activity of the cyclopropane fatty acid—synthesizing system was

determined by the method of Zalkin *et al.* (1963). The lipid substrate routinely used was an aqueous dispersion of the phospholipids extracted from *A. agilis* cells. All incubations were carried out at 30° for 30 minutes unless otherwise indicated. The assay conditions were such that no more than 15% of the substrate reacted during the assay. The amount of substrate which reacted under these conditions was a linear function of time. One enzyme unit is defined as that quantity of enzyme which transfers the methyl group from 1 mμmole of *S*-adenosylmethionine to the phospholipid substrate in 1 hour at 30°.

Preparation of Clostridium butyricum Extracts.—*Clostridium butyricum* (ATCC 6015) cells were grown at 30° under anaerobic conditions in the medium described by Wolfe and O'Kane (1953). The cells were harvested in the exponential phase of growth with a Sharples centrifuge, washed once with 0.1 M Tris-acetate buffer at *p*H 7.0, and stored at −20°.

A 30% suspension of cells was prepared in 0.1 M Tris-acetate buffer at *p*H 7.0. To each 100 ml of cell suspension was added 60 ml of acid-washed glass beads (120–130 μ in diameter; Minnesota Mining and Manufacturing Co.). The mixture was ground in an Eppenbach colloid mill (Model QV-6, Gifford-Wood Co., Hudson, N.Y.); the clearance between the rotor and stator was 0.64 mm (0.025 in.) (O'Connor *et al.*, 1960). The temperature of the mixture was maintained between 10 and 15° during the grinding by circulating ice water through the cooling coils of the mill. Whole cells, glass beads, and large particulate matter were removed from the resulting mixture by centrifugation for 30 minutes at 6000 × *g* in a Servall centrifuge. The supernatant solution was recentrifuged for 60 minutes at 100,000 × *g* in a Spinco Model L centrifuge. The clear supernatant solution obtained was titrated with a 0.5 M solution of K_2HPO_4 to *p*H 7.0 and stored in small batches at −20° until required. The frozen supernatant solution maintained its full catalytic activity for several months.

Purification of the Enzyme.—DEAE-CELLULOSE FRACTIONATION.—A volume of 50 ml of the *Clostridium butyricum* extract was dialyzed for 15 hours against 1 liter of 0.01 M KPO_4 buffer at *p*H 7.0. The dialyzed solution was chromatographed on a 30 × 2.5-cm column of DEAE-cellulose; the column had been packed under gravity and equilibrated with 0.01 M KPO_4 solution at *p*H 7.0. The protein which was not adsorbed to the DEAE-cellulose was eluted with 500 ml of the buffer which had been used to equilibrate the column. The enzyme was then eluted from the column with 0.1 M KPO_4 buffer at *p*H 7.0; fractions of 50 ml were collected and separately assayed for enzyme activity and protein concentration. The fractions which contained protein of the highest specific activity were pooled and then diluted with an equal volume of water.

HYPATITE C FRACTIONATION.—The diluted enzyme solution obtained from the DEAE-cellulose column was further fractionated on a column of Hypatite C. The Hypatite C column (2.5 × 10 cm) was packed under gravity and equilibrated with 0.01 M KPO_4 buffer at *p*H 7.0. The protein which was not adsorbed on the column was eluted with 0.01 M KPO_4 buffer at *p*H 7.0. The column was developed in a discontinuous manner with increasing concentrations of KPO_4 buffer at *p*H 7.0. Three fractions each of 50 ml were collected for each of the following concentrations of phosphate buffer: 0.10, 0.15, 0.20, and 0.30 M, respectively. The fractions were analyzed for enzyme and protein concentrations.

Concentration of Enzyme.—The catalytically active fractions could not be concentrated without considerable loss of activity. Partial recovery of the active protein and some degree of concentration could be achieved by dialysis of the dilute enzyme solution with small quantities of Hypatite C against 0.01 M KPO_4 buffer at *p*H 7.0. The enzyme was adsorbed on the Hypatite C and could be eluted with small volumes of 0.3 M KPO_4 at *p*H 7.0.

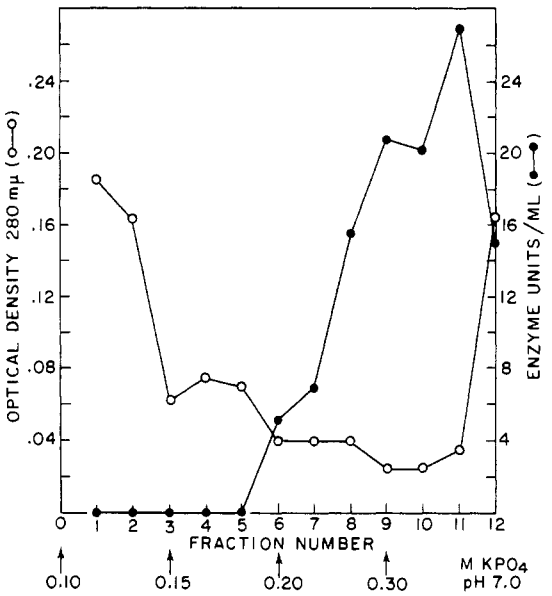

FIG. 1.—Hypatite C fractionation of cyclopropane–fatty acid synthetase. Simultaneous patterns of optical density at 280 mμ, and enzyme activity per ml against fraction number are presented. The arrows indicate points at which the molarity of the eluting phosphate buffer was changed to the concentrations shown.

Analysis of Fatty Acids Produced by the Enzyme Reaction.—The radioactive phospholipid product of the enzyme reaction was hydrolyzed with 50% methanol (v/v) and 8% KOH (w/v). The fatty acids were extracted with diethyl ether after acidification of the saponified mixture. The ether extract was concentrated under a stream of nitrogen and the methyl esters of the fatty acids were made with freshly distilled diazomethane. Carrier methyl esters of known fatty acids were added and the mixture was subjected to gas-liquid chromatography. The methyl esters of the fatty acids emerging from the chromatographic column were separately collected in U-tubes cooled in a dry ice–acetone bath. The contents of each U-tube was transferred into scintillation vials with 15 ml of scintillation fluid and the radioactivity was determined with a Packard Tri-Carb scintillation counter (Packard Instruments Co., Inc., La Grange, Ill.). The scintillation fluid consisted of 15.18 g 2,5-diphenyloxazole and 189 mg 2,2-*p*-phenylene-bis-(5-phenyloxazole) dissolved in 1 gallon of toluene.

RESULTS

Purification.—The elution patterns of protein and enzyme activity from a typical Hypatite C fractionation are shown in Figure 1. The cyclopropane–fatty acid–synthetase system was adsorbed firmly to the Hypatite C which allowed the elution of considerable amounts of inactive protein at moderately high concentrations of phosphate buffer. Table I summarizes the results of a purification experiment. An increase in specific activity of approximately 50-fold was obtained for the most active enzyme preparation. The final recovery of enzyme after the two purification

TABLE I
PURIFICATION OF ENZYME

Fractions	Volume (ml)	Total Protein (mg)	Total Enzyme (enzyme units)	Specific Activity (enzyme units/mg protein)	Recovery (%)	Purification
Original enzyme	50	620	10,000	16	100	
DEAE-cellulose eluate	210	58	5,500	96	54	6
Hypatite C eluate						
Fraction 8	50	1.75	780	445	8	28
9	50	1.60	1,040	661	10	41
10	50	1.20	1,000	833	10	52
11	50	2.20	1,345	612	13	38

steps was about 40%. Fraction 11 of the Hypatite C step in Table I was concentrated about 5-fold as described under Methods. The recovery of enzyme activity was approximately 20% and the specific activity of the enzyme had decreased to 180 enzyme units per mg of protein. This preparation was used in some of the subsequent experiments. Further purification of the enzyme was difficult because concentration of the dilute enzyme solution resulted in considerable loss of activity. Furthermore, fractionation with ammonium sulfate, acetone, ethanol, and carboxymethyl-cellulose resulted in irreversible loss of activity and no additional purification of the enzyme system.

Lipid Substrate Specificity.—The results of experiments to determine the phospholipid-substrate specificity of the cyclopropane synthetase are summarized in Table II. As shown in the table, crude phosphatidylethanolamine was the best substrate for the enzyme. Purified phosphatidylethanolamine was a less effective substrate. The low levels of incorporation of the methyl group of *S*-adenosylmethionine into phosphatidylserine and phosphatidylinositol can probably be ascribed to the presence of phosphatidylethanolamine which contaminated these commercial samples. Purified phosphatidylcholine was not only a very poor substrate, but also inhibited the incorporation of the methyl group of *S*-adenosylmethionine into phosphatidylethanolamine (*vide infra*).

Nature of the ^{14}C-labeled Fatty Acids Synthesized by the Enzyme Reaction.—The distribution of radioactivity in the fatty acids of the phospholipid product of the enzyme reaction is given in Table III; more than 98% of the recovered radioactivity was located in the 17- and 19-carbon cyclopropane fatty acids. In this experiment, purified homogeneous phosphatidylethanolamine was the lipid substrate and the crude extract of *Clostridium butyricum* cells was the source of enzyme. Subsequent experiments with the purified synthetase system gave similar results. The results demonstrated conclusively that phosphatidylethanolamine was the lipid substrate of the enzyme and that the methyl group of *S*-adenosylmethionine was incorporated exclusively into cyclopropane fatty acids.

Effects of Surfactants.—The effect of sodium lauryl sulfate on the rate of incorporation of the methyl group of *S*-adenosylmethionine into cyclopropane fatty acids at three different concentrations of phosphatidylethanolamine is shown in Figure 2. The enzyme preparation used in this series of experiments was partially purified and concentrated by the methods already described. There was a marked effect of sodium lauryl sulfate on the rate of the enzyme reaction. Increasing concentrations of the surfactant resulted in an increased rate of reaction up to a maximal rate. Beyond this point the rate decreased with further increase in the surfactant concentration. The optimal rate of reaction occurred in a narrow concentration range of sodium lauryl sulfate. However, this concentration range was

TABLE II
LIPID SUBSTRATE REQUIREMENTS FOR CYCLOPROPANE–FATTY ACID SYNTHESIS[a]

Additions	*S*-Adenosylmethionine Incorporated into Cyclopropane Fatty Acids (mμmoles/hr/mg protein)
Azotobacter agilis phospholipid, 1.2 μmoles	12.8
Azotobacter agilis phosphatidylethanolamine, 2.6 μmoles	4.7
Phosphatidylserine, 2.5 μmoles	1.5
Phosphatidylinositol, 2.5 μmoles	1.8
Phosphatidylcholine, 4.0 μmoles	0.2

[a] Each reaction tube contained [*methyl*-^{14}C]-*S*-adenosylmethionine; 560 mμmoles (50,000 dpm) *Clostridium butyricum* extract; 8–12 mg protein; Tris-acetate, *p*H 7.0, 11–56 μmoles or potassium phosphate, *p*H 7.0, 35 μmoles; and lipid as indicated. Final volume was 1 ml.

TABLE III
DISTRIBUTION OF RADIOACTIVITY IN THE FATTY ACIDS SYNTHESIZED BY *Clostridium butyricum* EXTRACTS FROM [*methyl*-^{14}C]-*S*-ADENOSYLMETHIONINE AND *A. agilis* PHOSPHATIDYLETHANOLAMINE[a]

Fatty Acids[b]	Net dpm	Recovered Radioactivity (%)
14:0–16:1	95	0.7
17:Cy	9283	70.3
18:0–18:1	81	0.6
19:Cy	3728	28.2
>19:Cy	0	0

[a] The reaction mixture consisted of 560 mμmoles of [*methyl*-^{14}C]-*S*-adenosylmethionine, 10^6 dpm; 55 μmoles potassium phosphate buffer, *p*H 7.0; 2.6 μmoles purified *A. agilis* phosphatidylethanolamine; 20 mg *Clostridium butyricum* extract; the total volume was 1.9 ml and the mixture was incubated for 1 hour at 30°. An aliquot of the methyl esters obtained from the fatty acids of the saponified phospholipid product was subjected to gas-liquid chromatography. The aliquot contained 17,100 dpm. The net dpm was obtained by subtracting from the radioactivity of the fractions the small radioactive background of the column. Recovery from the column was 70%. [b] The abbreviations used for fatty acids are as follows: 14:0, tetradecanoic acid; 16:0, hexadecanoic acid; 16:1, hexadecenoic acid; 17:Cy, methylenehexadecanoic acid (cyclopropane); 19:Cy, methylenehexadecanoic acid (cyclopropane).

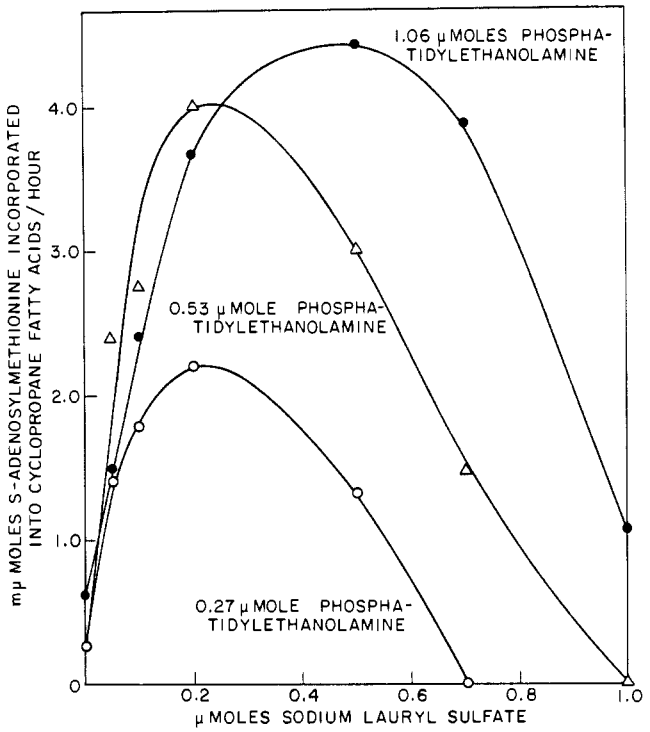

FIG. 2.—The effect of concentration of sodium lauryl sulfate on the rate of incorporation of *S*-adenosylmethionine into cyclopropane fatty acids at different lipid concentrations. Each incubation mixture contained: Tris-acetate buffer, *p*H 7.0, 100 μmoles; [*methyl*-^{14}C]-*S*-adenosylmethionine, 83 mμmoles, 100,000 dpm; partially purified enzyme-0.014 mg; sodium lauryl sulfate as indicated; phosphatidylethanolamine as indicated; water to make a total volume of 1 ml. Labeled fatty acids were extracted and radioactivity was counted as indicated.

somewhat broader at the higher lipid concentrations. This phenomenon may be the result of protection of the enzyme, by the lipid, from the denaturing effect of high concentrations of the surfactant.

The effects of dicetylphosphate, DL-α-tocopherylphosphate, sodium oleate, hexadecyltrimethylammonium bromide, Tween 80, and octadecenylphosphate on the rate of the enzyme reaction are shown in Figures 3 and 4. In this series of experiments a dialyzed preparation of the crude *Clostridium* extract was used as the enzyme. The results demonstrate that those compounds which produced negatively charged amphipathic ions had stimulatory effects on the enzyme reaction. Hexadecyltrimethylammonium bromide, which produced a positively charged amphipathic ion, was inhibitory. The neutral surfactant, Tween 80, was also inhibitory. In a separate experiment it was demonstrated that Cutscum, a neutral surfactant, also inhibited the enzyme reaction at comparable concentrations. The surfactant molecules which were examined were not substrates for the enzyme reaction when tested at the concentrations which produced maximal stimulatory effects in the presence of phosphatidylethanolamine.

Effect of Phosphatidylcholine on the Enzyme System.—Phosphatidylcholine was not a substrate for the cyclopropane–fatty acid–synthetase system, and addition of positively or negatively charged ions did not enhance its ability to act as a substrate. At equimolar concentrations of phosphatidylcholine and *A. agilis* phospholipid there was greater than 80% inhibition of the enzyme reaction. This is presumed to be the result of the tendency of phosphatidylcholine to serve as a positively charged amphipathic agent.

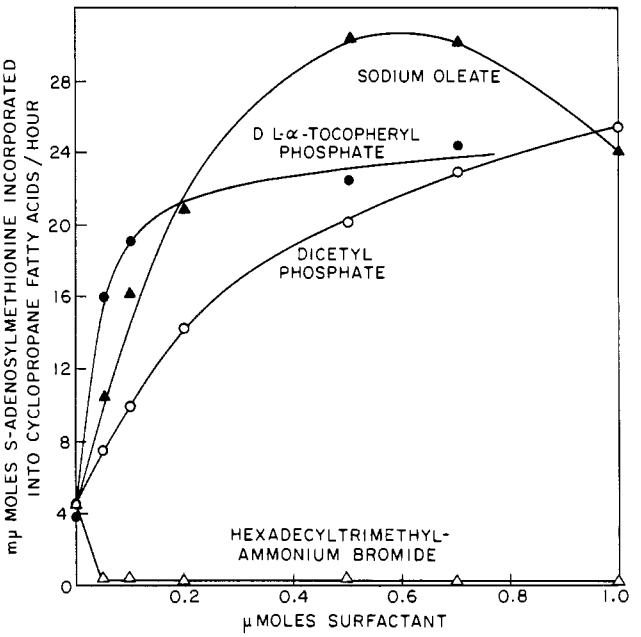

FIG. 3.—Effects of surfactants on the rate of incorporation of [*methyl*-^{14}C]-*S*-adenosylmethionine, 82 mμmoles, 50,000 dpm; Tris-acetate buffer, *p*H 7.0, 100 μmoles; phosphatidylethanolamine, 0.53 μmole; *Clostridium butyricum* extract, 2.7 mg; surfactant as indicated; water to a total volume of 1 ml. Incubation was for 15 minutes at 30°. Fatty acids were extracted and radioactivity was counted as described.

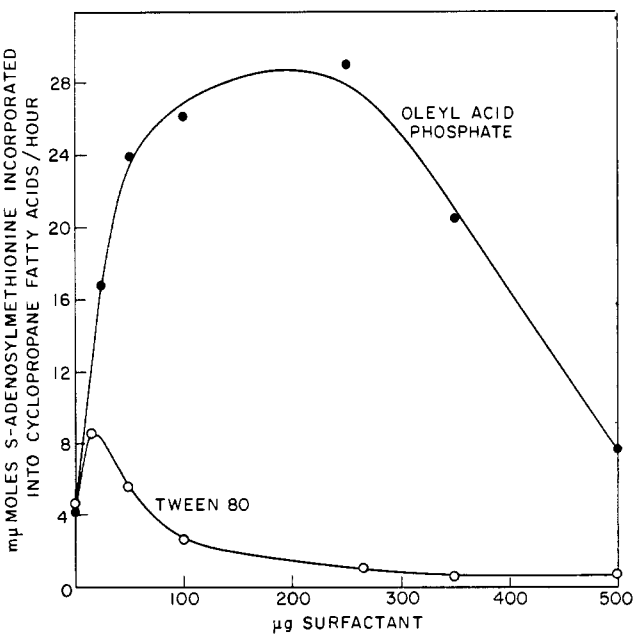

FIG. 4.—Effect of surfactants on the rate of incorporation of [*methyl*-^{14}C]-*S*-adenosylmethionine into cyclopropane fatty acids. The experimental conditions were as described for Fig. 3.

Substrate Affinities.—The K_m values for phosphatidylethanolamine and *S*-adenosylmethionine were determined at 30° by the method of Lineweaver and Burk (1934). The plots for phosphatidylethanolamine and *S*-adenosylmethionine are presented in Figures 5 and 6, respectively. The K_m values obtained from these plots were 5.7×10^{-5} M for *S*-adenosylmethionine and 5.3×10^{-4} M for phosphatidylethanolamine. The K_m value for *S*-adenosylmethionine was determined in the

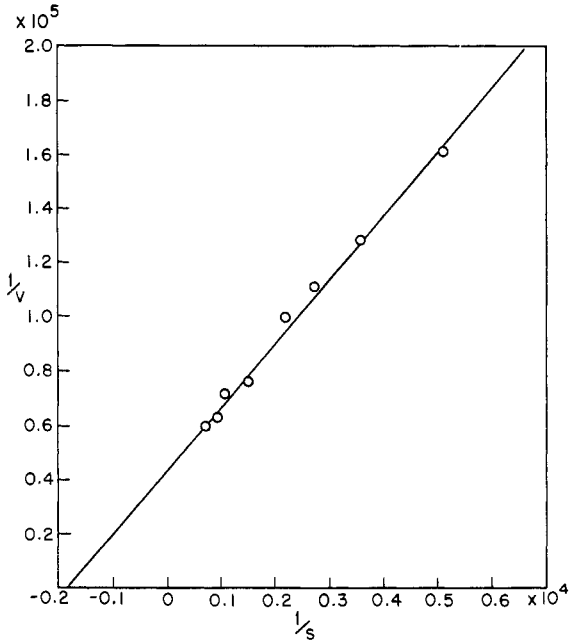

FIG. 5.—The reciprocal of the velocity of the formation of cyclopropane fatty acids as a function of the reciprocal of phosphatidylethanolamine concentration. The reaction mixture contained: Tris-acetate buffer, *p*H 7.0, 38–66 μmoles; potassium phosphate buffer, *p*H 7.0, 60 μmoles; partially purified enzyme, 0.05 mg; [*methyl*-^{14}C]-*S*-adenosylmethionine, 83 mμmoles, 150,000 dpm; phosphatidylethanolamine as indicated; water to a total volume of 1 ml.

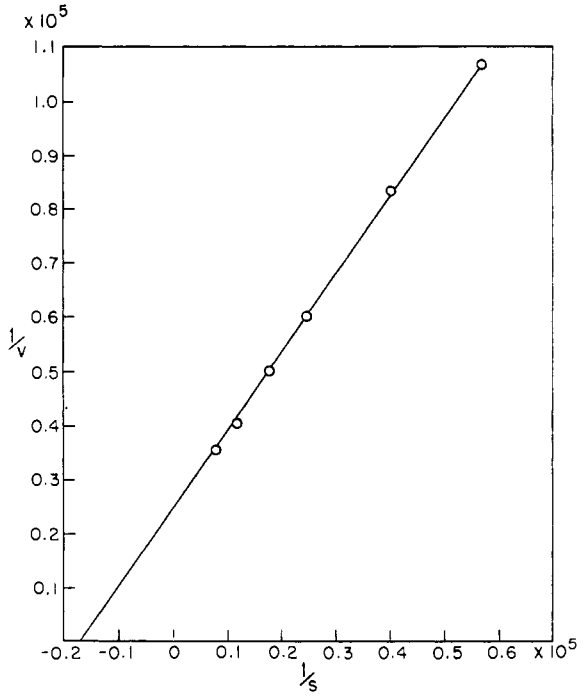

FIG. 6.—The reciprocal of the velocity of the formation of cyclopropane fatty acids as a function of the reciprocal of *S*-adenosylmethionine concentration. The reaction mixture contained: Tris-acetate buffer, *p*H 7.0, 100 μmoles; sodium lauryl sulfate, 0.2 μmole; phosphatidylethanolamine, 0.53 μmole; partially purified enzyme, 0.014 mg; *S*-adenosylmethionine as indicated, specific activity 1800 dpm/mμmole; water to a total volume of 1 ml.

presence of 2×10^{-4} M sodium lauryl sulfate. The addition of the surfactant was necessary, since for the particular preparation of phosphatidylethanolamine used the higher concentrations of *S*-adenosylmethionine produced turbidity and low incorporations in the assays in the absence of the surfactant. Attempts to determine a satisfactory K_m value for the phosphatidylethanolamine in the presence of sodium lauryl sulfate were unsuccessful. The kinetic plots did not conform to the Michaelis-Menten analysis. The effect of increasing concentrations of phosphatidylethanolamine on the rate of incorporation of the methyl group of [*methyl*-^{14}C]-*S*-adenosylmethionine into cyclopropane fatty acids in the presence or absence of 2×10^{-4} M sodium lauryl sulfate is shown in Figure 7.

Heat of Activation for the Enzyme Reaction.—The heat of activation for the enzyme reaction was determined under different experimental conditions over the temperature range 0–40°. The Arrhenius plots of log initial velocity of the reaction against the reciprocal of the absolute temperature are presented in Figure 8. From the slopes of the curves the heats of activation were determined in the conventional manner. In curves A and B the phospholipid substrates were purified phosphatidylethanolamine and crude phosphatidylethanolamine, respectively. For curve C purified phosphatidylethanolamine was the lipid substrate, but in addition each assay tube contained sodium lauryl sulfate at a final concentration of 2×10^{-4} M.

In each of the three experimental conditions a sharp break was observed in the corresponding Arrhenius plot. This discontinuity occurred between 17 and 23°.

The heats of activation for the reaction with purified phosphatidylethanolamine as the substrate were 32 kcal over the temperature range 0–21° and 18.4 kcal over the temperature range 21–40°. In contrast to these values the heats of activation in the presence of sodium lauryl sulfate were 13.6 kcal over the tempera-

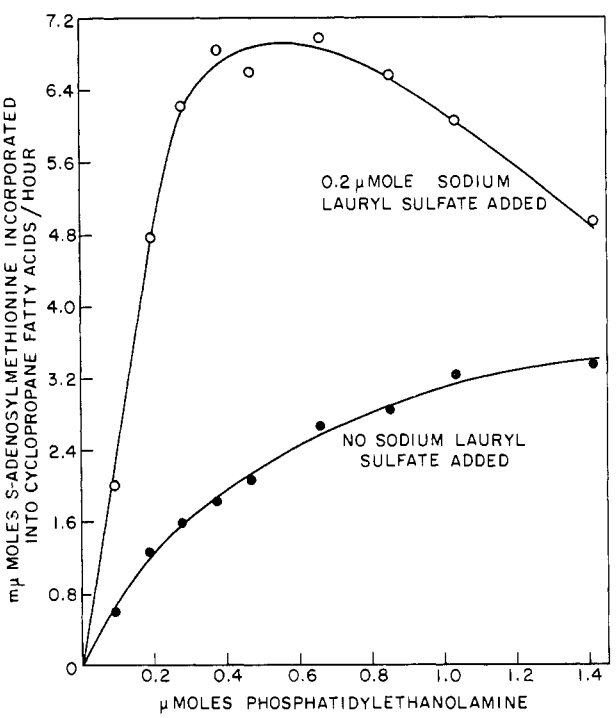

FIG. 7.—The rate of incorporation of [*methyl*-^{14}C]-*S*-adenosylmethionine into cyclopropane fatty acids as a function of phosphatidylethanolamine concentration in the presence or absence of sodium lauryl sulfate. The experimental conditions were similar to those described for Fig. 5 except that the mixture contained 0.2 μmole sodium lauryl sulfate when added, and the concentration of phosphatidylethanolamine was varied as indicated.

ture range 0–17° and 7.4 kcal over the temperature range 17–40°. The values obtained when crude phosphatidylethanolamine was used as substrate were surprisingly similar to the latter values; 13.8 and 7.1 kcal were obtained over the temperature ranges 0–23° and 23–40°, respectively.

Discussion

The results of both *in vivo* and *in vitro* experiments have indicated that the process of cyclopropane–fatty acid formation involves the addition of the methyl group of methionine, in the activated form, *S*-adenosylmethionine, to the double bond of an unsaturated fatty acid derivative (Liu and Hofmann, 1962; O'Leary, 1962; Zalkin and Law, 1962; Zalkin *et al.*, 1963). The experiments of Liu and Hofmann (1962) and Pohl *et al.* (1963) demonstrate that the methionine methyl group is incorporated into the methylene bridge of the cyclopropane ring and that two hydrogens accompany the methyl carbon. The overall reaction therefore constitutes a novel type of transmethylation in which two carbon-carbon covalent bonds are formed.

A consideration of the mechanism of this unusual reaction involves several questions: (1) How many enzymes are involved? (2) What are the reactants and the products? (3) What intermediates and what cofactors are involved? (4) What is the exact sequence of chemical events? (5) What is the nature of the active site of the enzyme or enzymes which catalyze the reaction?

Unfortunately the first question cannot yet be answered with certainty. The possibility that more than one enzyme is involved in the reaction cannot be ruled out, since the purification procedures so far have resulted in only about 50-fold increase in specific activity.

The second question can now be answered in some detail: The process involves a reaction between an olefinic linkage in the fatty acid chain of phosphatidylethanolamine and the *S*-adenosylmethionine methyl group, which results in a phosphatidylethanolamine molecule which contains a cyclopropane fatty acid. The other products of the reaction have not been characterized.

It is highly unlikely that any loosely bound cofactor is involved in the reaction, since the purification procedure and the extensive dialysis of the enzyme solution would surely remove it, and consequently greatly diminish the catalytic activity of the enzyme. The presence of some bound cofactor which might serve to transfer the methyl carbon from *S*-adenosylmethionine to the olefinic linkage (e.g., a reduced folate or a cobamide derivative), however, cannot be ruled out.[1]

There is no evidence for the accumulation of intermediates in this reaction. O'Leary (1962) has suggested that cyclopropene acids may be formed as precursors of the saturated compounds. This postulated addition of the methylene group to the olefinic linkage *with retention of the double bond* is very difficult to envision from the point of view of a reasonable mechanism. Our studies offer no evidence in support of this hypothesis (see Table III).

[1] We have determined that free N^5 methyltetrahydrofolic acid will not serve as a source of the methylene carbon in this reaction. On the basis of earlier work (Pohl *et al.*, 1963) the involvement of cobamide derivatives in the formation of cyclopropane fatty acids in *E. coli* may be ruled out. In these experiments *E. coli* 113-3, a B_{12} auxotroph, was shown to form cyclopropane fatty acids when it was grown on methionine, which spares the B_{12} requirement of the organism.

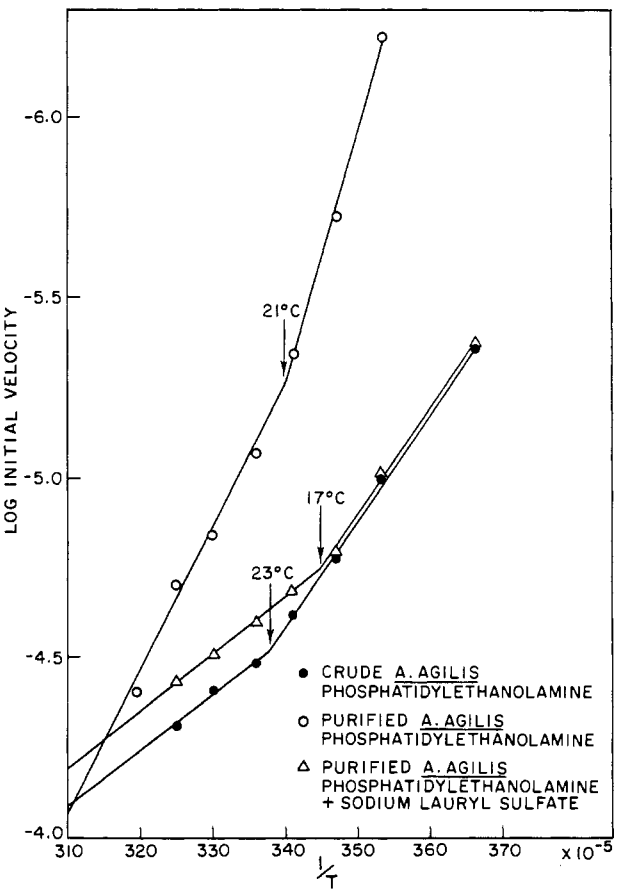

FIG. 8.—The effect of temperature on the rate of incorporation of [*methyl*-^{14}C]-*S*-adenosylmethionine into cyclopropane fatty acids. The reaction mixture contained: Tris-acetate buffer, *p*H 7.0, 60 μmoles; potassium phosphate buffer, 50 μmoles; partially purified enzyme, 0.05 mg; sodium lauryl sulfate when added, 0.2 μmole; [*methyl*-^{14}C]-*S*-adenosylmethionine, 82 mμmoles, 150,000 dpm; phospholipid, phosphatidylethanolamine, 0.94 μmole or crude phosphatidylethanolamine, 0.97 μmole; water to a total volume of 1 ml. Incubation was carried out for 30 minutes at temperatures indicated.

The effect of surfactants in the enzymatic reaction is a particularly interesting feature. The resemblance of this system to that studied by Bangham and Dawson (1959) was first suspected when it was found that saturation of the enzyme with a micellar preparation of pure phosphatidylethanolamine gave a maximum velocity only 30% of that observed when the system was saturated with the crude phospholipid preparation. Bangham and Dawson (1959) in their studies on the phospholipase of *Penicillium notatum* discovered that this enzyme will attack only phospholipid micelles which bear a net negative surface charge. The surface charge, which was reflected in the electrophoretic mobilities of the micelles, could be altered by the addition to the reaction medium of ionic surfactants, metal cations, and anions, or by a change in *p*H. We have found that the addition of a surfactant to the cyclopropane-synthetase system was necessary to produce maximal catalytic activity. Only anionic surfactants stimulated the reaction; the cationic surfactant hexadecyltrimethylammonium bromide and the neutral surfactants, Tween 80 and Cutscum, were inhibitory. Although the evidence indicates strongly that the cyclopropane-synthetase system is analogous to that of Bangham and Dawson, we have yet to correlate the zeta potential of the micelles with the catalytic activity of the enzyme.

The function of the charges on the micelle is not known, but as Bangham and Dawson (1959) have suggested there may be complementary charges on the enzyme molecule; the interaction of the charges facilitates the catalytic reaction either by the proper orientation of the substrate and active site of the enzyme, or by aiding the penetration of the enzyme into the micelle. This may be of particular importance in the case of the cyclopropane-synthetase system, because the enzymatic reaction involves a double bond located in the middle of an aliphatic chain, which may lie deep within the micelle.

We have observed that the order in which the reagents were added to the assay medium was critical. The addition of surfactant to the enzyme solution before the addition of phospholipid and *S*-adenosylmethionine resulted in either no stimulation or an inhibition of the reaction. It thus appears that sodium lauryl sulfate has also an inhibitory effect, perhaps because of denaturation of the enzyme; the phospholipid in some way protects the enzyme from this second effect.

The Arrhenius plots of the logarithm of the initial velocity of the enzyme reaction against the reciprocal of the temperature of reaction were discontinuous. The heats of activation calculated from the slopes of the curves were higher at the lower temperature ranges than at the higher temperature ranges. The heats of activation which were obtained when phosphatidylethanolamine alone was used as substrate were considerably higher than the corresponding values obtained in the presence of sodium lauryl sulfate. The latter values were almost identical to those obtained with a crude phosphatidylethanolamine substrate. The effect of sodium lauryl sulfate appears to be similar to that of a natural activator which is present in the crude phosphatidylethanolamine. An analogous observation was made by Dawson (1957, 1958), who reported that the natural activators monophosphoinositide and a polyglycerol phospholipid were necessary for the hydrolysis of phosphatidylcholine by the phospholipase of *Penicillium notatum*.

The sharp breaks observed in the Arrhenius plots are not unique to this system. Similar discontinuities have been observed for the reactions catalyzed by pancreatic lipase, trypsin, urease (Sizer, 1943), and fumarase (Massey, 1953). The reasons for the discontinuity are not known. However, in a system which involves the interaction of lipid micelles and a water-soluble-enzyme system the state of the micellar aggregation must be of considerable importance. The critical micelle concentration of amphipathic molecules, in general, increases with temperature (Brady and Huff, 1948; Klevens, 1947; Wright, *et al.*, 1939), and therefore it is possible that at the temperature where the discontinuities are observed the micelles may have reverted to monomolecular species.

The enzyme system which has been discussed here offers an opportunity to study the mechanism of an unusual and possibly unique enzymatic reaction as well as the complex problem of the interaction of lipid micelles with a water-soluble enzyme. What meaning this has in terms of *in vivo* reactions occurring at cell membranes cannot be judged at present.

Acknowledgment

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