Biosynthesis of Cyclopropane Compounds. VI. Product Inhibition of Cyclopropane Fatty Acid Synthetase by S-Adenosylhomocysteine and Reversal of Inhibition by a Hydrolytic Enzyme*

ALBERT E. CHUNG† AND JOHN H. LAW

From the James Bryant Conant Laboratory, Harvard University, Cambridge, Massachusetts

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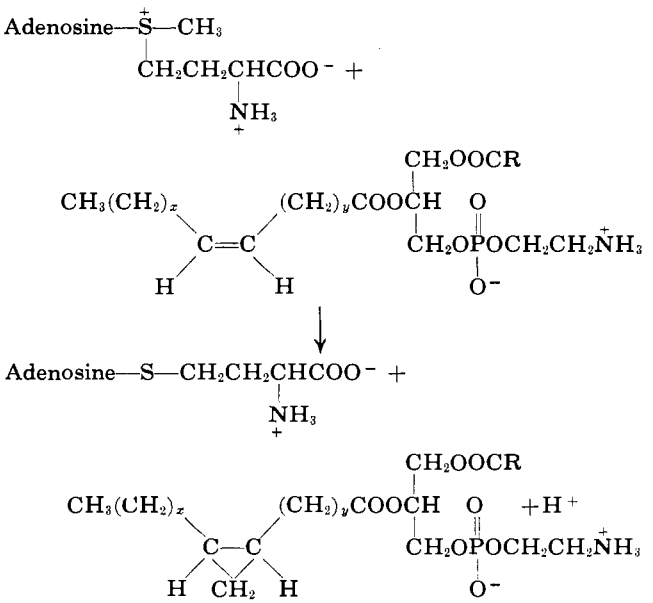
After purification by chromatography on Hypatite C, the cyclopropane fatty acid synthetase system of *Clostridium butyricum* showed a rapid decrease in rate after short periods of incubation. This rate decrease was prevented by addition of another protein fraction which had been separated during chromatography. The second fraction was shown to contain an enzyme which hydrolyzes *S*-adenosylhomocysteine to adenine and other products. Since *S*-adenosylhomocysteine seems to be a product of the synthetase reaction, the decrease in rate must be the result of product inhibition, which is reversed by the presence of the hydrolytic enzyme.

The enzymatic synthesis of cyclopropane fatty acids in extracts of *Clostridium butyricum* and *Serratia marcescens* was first reported by Zalkin *et al.* (1963). More recently Chung and Law (1964) have described a method for obtaining a partially purified preparation of the cyclopropane fatty acid synthetase from *C. butyricum* extracts. The enzyme system catalyzes the transfer of the methyl groups of *S*-adenosylmethionine to a monounsaturated fatty acid chain of phosphatidylethanolamine. The products of the reaction are phosphatidylethanolamine, which contains a cyclopropane fatty acid in ester linkage, and presumably *S*-adenosylhomocysteine, by analogy with other transmethylating reactions which utilize *S*-adenosylmethionine as the methyl donor (Mudd and Cantoni, 1964). Stoichiometry requires that a proton is lost during the reaction:



$x + y$ = 12 or 14, R = fatty acid chain

The reaction is shown for a fatty acid in the β-position of phosphatidylethanolamine, but fatty acids in the γ-position react as well (Hildebrand and Law, 1964).

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† Present address: Department of Biochemistry, University of Colorado School of Medicine, Denver.

The partially purified enzyme system showed no requirements for any cofactors, and the reaction appeared to be catalyzed by a single protein fraction.

Further experiments with purification of the enzyme system revealed that recombination of two protein fractions gave an enhancement of enzymatic activity as measured under the standard conditions described earlier (Chung and Law, 1964). This appeared at first to be the consequence of separating the enzyme system into two active fractions each of which catalyzed a partial reaction, but several observations were soon made which were not in accord with this hypothesis. The second component, which led to an apparent enhancement of cyclopropane fatty acid synthetase activity, was shown to be a hydrolytic enzyme that destroyed a product of the reaction, *S*-adenosylhomocysteine, and thereby relieved the powerful product inhibition exerted by this compound. This paper presents the experimental observations upon which these conclusions are based.

EXPERIMENTAL

Materials.—Hypatite C was the product of the Clarkson Chemical Co., Williamsport, Pa. *S*-[methyl-^{14}C]Adenosylmethionine was purchased from Tracerlab, Waltham, Mass. Unlabeled *S*-adenosylmethionine was prepared from activated baker's yeast according to Schlenk *et al.* (1959) and purified by the method of Mudd (1959). Adenine and DL-homocysteine were purchased from Nutritional Biochemicals, Inc., Cleveland, Ohio. *S*-Adenosyl-L-homocysteine was prepared by the method of Sakami (1961). *Azotobacter agilis* phospholipids were prepared as previously described (Chung and Law, 1964).

Methods.—PURIFICATION OF CYCLOPROPANE FATTY ACID SYNTHETASE.—The enzyme system from the *C. butyricum* extracts was purified by a slight modification of the methods described previously (Chung and Law, 1964). Chromatography of the enzyme on Hypatite C was carried out with a linear gradient of potassium phosphate buffer at *p*H 7.0 instead of the discontinuous gradient used previously. For a 2.5 × 10-cm column the mixing chamber initially contained 250 ml of 0.05 M potassium phosphate buffer and the reservoir chamber contained 250 ml of 0.5 M potassium phosphate buffer. Fractions of approximately 20 ml were collected with an automatic fraction collector (Research Specialties, Richmond, Calif.). The fractions were analyzed for protein content, enzyme activity, and phosphate buffer concentration. The phosphate buffer concentration was determined by measuring the re-

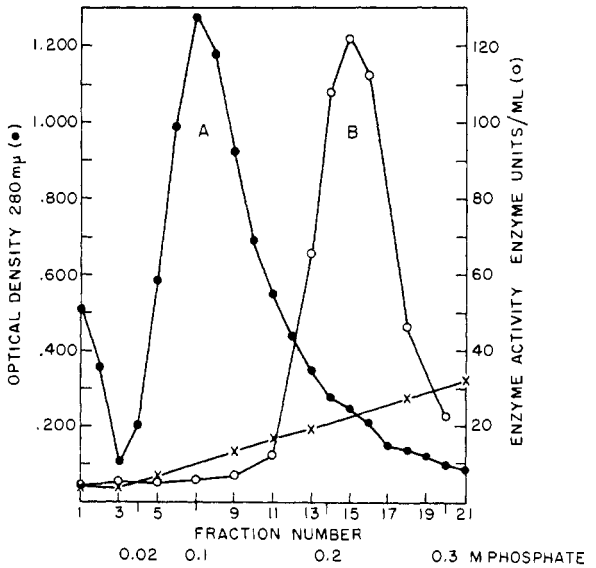

FIG. 1.—Fractionation of cyclopropane fatty acid synthetase on Hypatite C. The material applied to the column had been previously purified by chromatography on DEAE-cellulose by the method described earlier (Chung and Law, 1964). Simultaneous patterns of optical density at 280 mμ (●——●), and enzyme activity per ml (○——○) against fraction number, are presented. The concentration of potassium phosphate buffer (x.....x) in the various fractions is also presented. The enzyme activity in each fraction was determined in the presence of an equal volume of fraction 7.

sistance of the fractions with a conductivity bridge (Industrial Instruments, Cedar Grove, N.J.). The bridge was standardized with potassium phosphate solution of known molarity.

ASSAY OF ENZYME ACTIVITY.—The enzyme activity was determined as previously described (Chung and Law, 1964) unless otherwise noted. The cyclopropane fatty acids were extracted from the saponified reaction mixtures and the radioactivity was measured with a Packard Tri-Carb scintillation counter (Packard Instruments Co., Inc., La Grange, Ill.), using the scintillator solution described in a previous paper (Chung and Law, 1964).

PAPER CHROMATOGRAPHY.—Paper chromatography of the various samples was carried out on Whatman No. 3 MM chromatography paper in a descending manner with one of the following solvent systems: (*a*) 600 g $(NH_4)_2SO_4$, 1000 ml 0.1 M sodium phosphate buffer *p*H 6.8, and 20 ml 1-propanol (Sakami and Stevens, 1958), (*b*) ethanol–ammonia (28% NH_3)–water, 20:1:4 (Sakami and Stevens, 1958), and (*c*) butanol–water–formic acid (98%), 77:13:10 (Littlefield and Dunn, 1958).

RESULTS AND DISCUSSION

The elution patterns from the Hypatite C column are shown in Figure 1, in which ultraviolet absorption at 280 mμ, enzyme activity, and phosphate buffer concentration are plotted against fraction number. The tubes which contained cyclopropane fatty acid synthetase activity are referred to as fraction B, while the protein peak which preceded the synthetase fractions is referred to as fraction A. The various fractions were stored at −20°.

Earlier attempts to demonstrate a separation of the cyclopropane fatty acid synthetase system into two or more active fractions had not met with success (Chung and Law, 1964). It was therefore surprising when an apparent stimulation of activity was observed upon

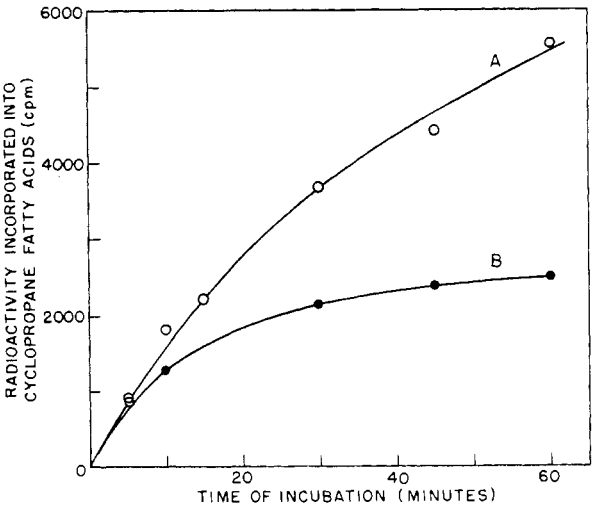

FIG. 2.—Kinetics of the synthetase reaction in the presence of fraction A. The reaction mixture contained 100 μmoles Tris-acetate buffer *p*H 7.0; 0.96 μmole *A. agilis* phospholipid; 80 mμmoles *S*-[methyl-^{14}C]adenosylmethionine, 100,000 dpm; 0.12 mg fraction A when added; 0.06 mg fraction B; total volume 1 ml. The incubation was at 30°. At the times indicated samples were withdrawn from the bath and the reaction stopped by the addition of 1 ml methanol and 0.2 ml 85% (w/v) KOH. Fatty acids were extracted after saponification and acidification for radioactivity measurements.

TABLE I
RECOMBINATION OF FRACTIONS A AND B[a]

Fraction A	Fraction B	Radioactive Methyl Groups Incorporated into Fatty Acids (cpm)
+	−	30
−	+	1782
+	+	4295
+ (boiled)	+	1852
+	+ (boiled)	81

[a] The reaction mixture contained: 0.12 mg fraction A (when added); 0.06 mg fraction B (when added); 81 mμmoles *S*-[methyl-^{14}C]adenosylmethionine, 50,000 dpm; 40–60 μmoles Tris-acetate buffer *p*H 7; 0.96 μmoles *A. agilis* phospholipid; total volume 1 ml. The fractions (where indicated) were boiled for 1 minute. The incubation was carried out at 30° for 30 minutes. The incorporation into the extracted cyclopropane fatty acids was determined. A blank incubation mixture, which contained no phospholipid, was subtracted from each of the above values for the incorporation.

combination of fractions A and B, described above. These results are shown in Table I. It can be seen that fraction B alone had enzymatic activity, while fraction A had no activity in the absence of fraction B. Addition of fraction A to fraction B stimulated the incorporation of radioactive methyl groups into fatty acids by a factor of 2. The stimulatory activity of fraction A was lost upon heating for 1 minute at 100°.

The effect of varying either fraction in the presence of a constant amount of the other was next tested. These results, shown in Table II, indicate that the stimulatory effect of fraction A reached a plateau while the addition of larger amounts of fraction B continued to raise the synthetase activity.

At this point several experiments were performed in an attempt to demonstrate some intermediate in the overall reaction which could be formed in the presence of fraction A and then converted to the final product

TABLE II
THE EFFECT OF VARYING THE CONCENTRATION OF FRACTIONS A AND B ON THE INCORPORATION OF LABEL INTO CYCLOPROPANE FATTY ACIDS

Experiment I[a]		
Fraction A (mg)	Fraction B (mg)	Incorporation (cpm)
0	0.06	453
0.06	0.06	1442
0.12	0.06	1869
0.18	0.06	2137
0.24	0.06	2207
0.30	0.06	2034
Experiment II[b]		
Fraction A (mg)	Fraction B (mg)	Incorporation (cpm)
0	0.10	1214
0.28	0.10	2329
0.28	0.19	3895
0.28	0.29	4993
0.28	0.38	5622

[a] The conditions of incubation were as described in Table I, except that the lipid used was a pure preparation of *A. agilis* phosphatidylethanolamine at a concentration of 0.95 μmoles per assay. Sodium lauryl sulfate was added at a concentration of 5×10^{-4} M. [b] The experimental conditions were as described for Experiment I. Fractions A and B, however, were first concentrated by precipitation with $(NH_4)_2SO_4$ and dialyzed against 0.04 M potassium phosphate buffer at *p*H 7.0.

in the presence of fraction B or *vice versa*. It was assumed at this point that fraction B contained a small amount of the fraction A stimulatory factor which accounted for the synthetase activity of fraction B by itself. All of these experiments failed, and attention was directed to other possibilities.

The time course of the synthetase reaction in the presence and absence of fraction A was next examined (Fig. 2). In the absence of fraction A the reaction rate decreased rapidly (curve B), while with fraction A added (curve A), the reaction continued at a rapid rate for the duration of the experiment. This shows clearly that the stimulatory effect of fraction A did not alter the initial rate of the reaction, but only affected the maintenance of the initial rate. The standard assay conditions, in which the reaction mixtures were incubated for 30 minutes, therefore gave the true initial velocity of the reaction with the purified synthetase only in the presence of fraction A, whereas in the crude synthetase mixtures no additions were necessary.

Two possible explanations for the rate maintenance effect of fraction A were considered and tested; either fraction A protected the synthetase from inactivation during the incubation or it destroyed an inhibitor formed during the course of the reaction. The first possibility was investigated in experiments shown graphically in Figure 3. Fraction B was preincubated for 30 minutes with buffer only, after which time substrates were added (arrow) and the subsequent course of the reaction recorded (curve C). A second experiment was run under the same conditions, except that after the preincubation period substrates and fraction A were added (curve A). In a third experiment the reaction was followed for the complete system, without fraction A, from zero time; at the point indicated by the arrow fraction A was added and the subsequent course of the reaction recorded (curve B). It can be seen that in the absence of fraction A the initial rate of reaction was unaffected by preincubation of fraction B (compare curve C with the first part of curve B).

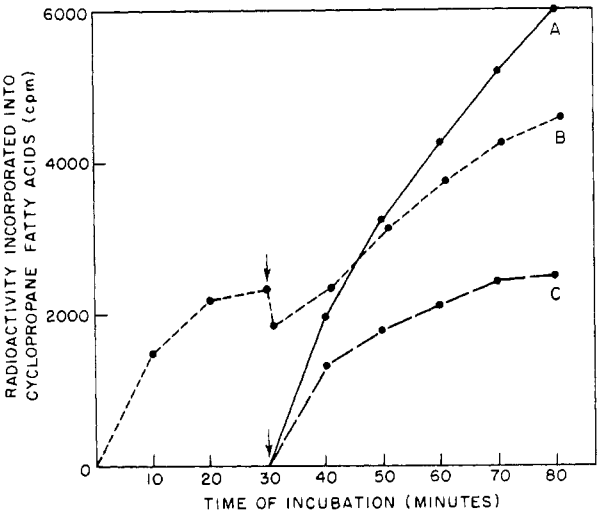

FIG. 3.—The stability of the cyclopropane fatty acid synthetase in the absence of fraction A. The reaction mixture for curve A contained 0.48 mg fraction B; 750 μmoles Tris-acetate buffer *p*H 7.0; 2.25 ml water. After incubation for 30 minutes at 30°, 7.2 μmoles *A. agilis* phospholipids, 600 mμmoles *S*-[methyl-^{14}C]adenosylmethionine, 0.375 μc, and 0.92 mg fraction A were added. An aliquot of 1 ml of the reaction mixture was immediately removed for extraction of fatty acids. The incubation was continued and samples were removed at 10-minute intervals thereafter for analysis. The reaction mixture and experimental procedures for curve C were similar to that for curve A but fraction A was omitted and an equivalent volume of water added to the reaction vessel instead. In curve B the initial reaction mixture contained 0.68 mg fraction B, 800 mμmoles *S*-[methyl-^{14}C]adenosylmethionine, 0.5 μc, 9.6 μmoles *A. agilis* phospholipids, 5 ml of water. The mixture was incubated at 30° and 1-ml samples were removed at the appropriate times for analysis. At the point indicated by the arrow 0.22 mg of fraction A was added and the experiment continued.

Furthermore, the effect of the addition of fraction A on the course of the reaction was the same whether it was added after fraction B had been preincubated with buffer only (curve A), whether it was added to fraction B which had not been preincubated (Fig. 2, curve A), or whether it was added after the enzyme reaction had proceeded for 30 minutes (second part of curve B). The following conclusions may be drawn from these experiments: (*a*) fraction B was stable under the experimental conditions, and (*b*) fraction A did not affect the stability of fraction B.

The results further indicated that the function of fraction A was to reverse an inhibition of the synthetase reaction which developed during the reaction, i.e., a product inhibition. It was assumed that *S*-adenosyl-L-homocysteine was a product of the synthetase reaction, in spite of the fact that this compound could not be detected by paper chromatography of the water-soluble reaction products obtained with partially purified synthetase preparations. This compound was therefore chosen for inhibition studies. The time course of the reaction in the absence of fraction A and the presence or absence of low concentrations of *S*-adenosyl-L-homocysteine was followed (Fig. 4, curves 2 and 4). This compound was indeed a potent inhibitor of the reaction. When fraction A as well as *S*-adenosylhomocysteine was added to the incubation mixture (curve 5), the rate was at first low, owing to the inhibition by *S*-adenosylhomocysteine, but soon increased until it matched the initial rate of the uninhibited reaction. Preincubation of *S*-adenosylhomocysteine and fraction A for 30 minutes before addition of fraction B and sub-

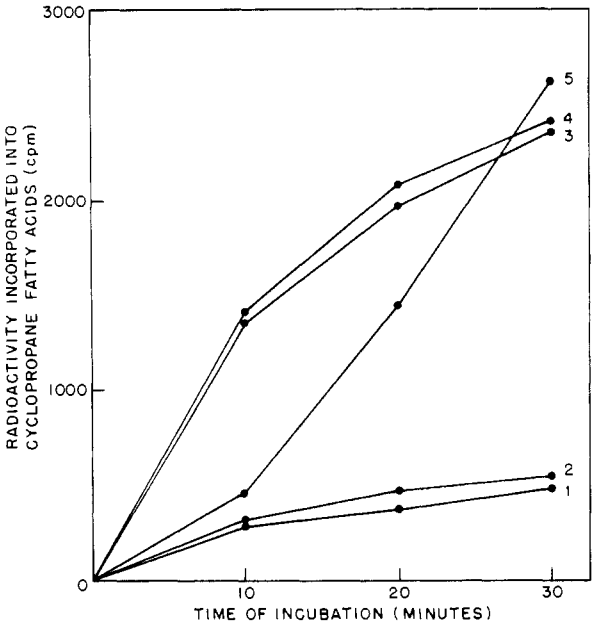

FIG. 4.—Inhibition of the cyclopropane fatty acid synthetase by *S*-adenosylhomocysteine and reversal of inhibition by fraction A. The reaction mixture for curve 4 contained 500 μmoles Tris-acetate buffer *p*H 7.0; 400 mμmoles *S*-[methyl-^{14}C]adenosylmethionine, 0.25 μc; 4.8 μmoles *A. agilis* phospholipids; 0.32 mg fraction B; total volume 5 ml. Incubation was at 30°. Aliquots of 1 ml were removed at predetermined times and the fatty acids were analyzed for radioactivity. The reaction mixture for curve 2 contained in addition 100 mμmoles of *S*-adenosylhomocysteine. For curve 5, 100 mμmoles of *S*-adenosylhomocysteine and 0.61 mg fraction A were added. The reaction mixtures for curves 1 and 3 were similar to that for curve 5 except that the *S*-adenosylhomocysteine and fraction A were treated as follows before incubation: For curve 1 fraction A was boiled for 1 minute and then incubated with *S*-adenosylhomocysteine for 30 minutes at 30°; at the end of this time the reagents and synthetase were added and the course of the reaction was followed. For curve 3, the *S*-adenosylhomocysteine was preincubated with 0.61 mg fraction A for 30 minutes at 30°. At the end of this time the mixture was boiled for 1 minute and, after cooling, the reagents and the synthetase were added. The reaction was followed as before.

strates produced no inhibition of the reaction (curve 3), but in an experiment that was identical except that boiled fraction A was used, the inhibitor was fully active (curve 1). The implication of these experiments is that fraction A contained an enzyme which destroyed *S*-adenosylhomocysteine.

In order to test this hypothesis, *S*-adenosylhomocysteine was incubated with fraction A and the reaction mixture was chromatographed on paper. Spots were located with a short-wave ultraviolet lamp (Mineral-light Model SL 2537, Ultra-violet Products, Inc., South Pasadena, Calif.). The results presented in Table III suggest that fraction A degraded *S*-adenosylhomocysteine to adenine and other products. In a separate experiment it was shown that 0.3 mg of fraction A completely degraded 80 mμmoles of *S*-adenosylhomocysteine in 60 minutes. This explains the earlier failure to detect *S*-adenosylhomocysteine as a product of the synthetase reaction with cruder enzyme preparations. The presence of the hydrolytic enzyme in crude extracts probably also accounts for the failure of Zalkin *et al.* (1963) to observe inhibition by *S*-adenosylhomocysteine on the crude cyclopropane fatty acid synthetase system of *Serratia marcescens*.

When *S*-adenosylhomocysteine was incubated with

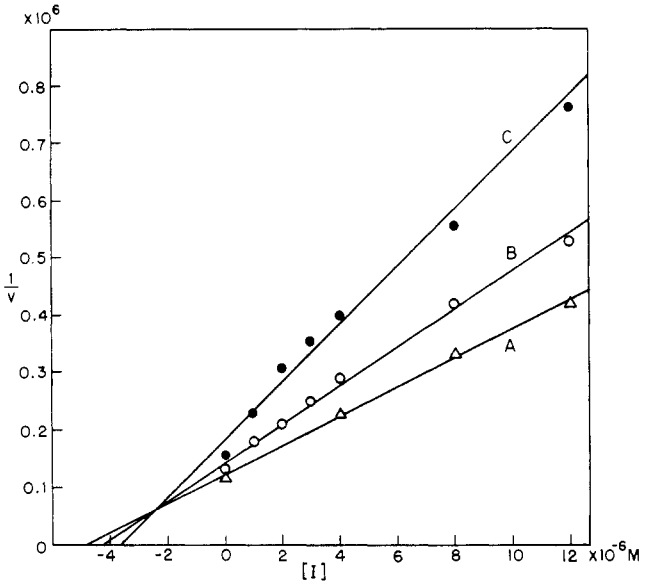

FIG. 5. Inhibition of cyclopropane fatty acid synthetase by increasing concentrations of *S*-adenosylhomocysteine at different concentrations of *S*-adenosylmethionine. Each reaction tube contained 0.95 μmole *A. agilis* phospholipids; 60 μmoles Tris-acetate buffer *p*H 7.0; 0.03 mg fraction B; *S*-adenosylhomocysteine as indicated; and 160, 80, and 40 mμmoles *S*-[methyl-^{14}C]adenosylmethionine for curves A, B, and C, respectively. Total volume of each reaction tube was 1 ml. Incubation was for 10 minutes at 30°. The rate of the reaction is expressed as moles *S*-adenosylmethionine incorporated into fatty acids/hour/liter.

TABLE III
HYDROLYSIS OF *S*-ADENOSYLHOMOCYSTEINE[a]

Samples	R_F Values: Solvent System A	Solvent System B
1. Boiled enzyme, *S*-adenosylhomocysteine	0.08	
2. Enzyme, *S*-adenosylhomocysteine	0.08; 0.36	0.04; 0.33
3. Adenine	0.36	0.30
4. Adenosine		0.17
5. *S*-Adenosylhomocysteine	0.08	0.04

[a] The reaction mixture contained: 400 mμmoles *S*-adenosylhomocysteine; 100 μmoles Tris-acetate buffer *p*H 7.0; 1.4 mg fraction A (precipitated with ammonium sulfate and dialyzed); water to a total volume of 1 ml. The mixture was incubated at 30° for 68 minutes; the reaction was stopped by heating for 1 minute at 100° and the precipitated protein removed by centrifugation. Samples were spotted on Whatman No. 3 MM paper and the chromatograms developed in solvent system A (ethanol–NH_3 (28%)–H_2O; 20:1:4) or solvent system B (butanol–H_2O–formic acid (98%); 44:13:10). The chromatograms were dried in a stream of warm air and the ultraviolet-absorbing spots detected by an ultraviolet lamp.

fraction B and the products were examined by paper chromatography, no hydrolytic degradation of the inhibitor was observed. Fraction B is, therefore, entirely free of the hydrolytic enzyme.

S-Adenosylhomocysteine appears to be in competition with *S*-adenosylmethionine, as shown in Figure 5. The reciprocal of the initial velocity is plotted against inhibitor concentration for three concentrations of *S*-adenosylmethionine, but at constant phospholipid concentration (Dixon, 1953). The K_I was estimated to be about 2.4×10^{-6} M. In another experiment *S*-

TABLE IV
INHIBITION OF CYCLOPROPANE FATTY ACID SYNTHETASE BY S-ADENOSYLHOMOCYSTEINE AT DIFFERENT CONCENTRATION OF PHOSPHOLIPID[a]

Inhibitor Concentration (M × 10^6)	Cyclopropane Fatty Acids Formed (mμmoles/hour)	
	0.19 μmoles Phospholipid/ml	0.57 μmoles Phospholipid/ml
0	7.0	8.9
1	5.5	6.5
2	4.8	4.6
3	4.3	4.4
4	3.3	3.9
8	2.3	3.0
12	2.0	2.3

[a] Each reaction vessel contained 80 mμmoles S-[methyl-^{14}C]adenosylmethionine, 110,000 dpm; 72 μmoles Tris-acetate buffer pH 7.0; *A. agilis* phospholipids as indicated; S-adenosylhomocysteine as indicated; 0.05 mg fraction B; total volume 1 ml. Incubation was for 10 minutes at 30°.

adenosylmethionine was held constant while S-adenosylhomocysteine and phospholipid were varied (Table IV). The inhibitor is not competitive with phospholipid.

Some other possible products of the synthetase reaction or of hydrolytic enzymes were tested as inhibitors of the synthetase. There was no inhibition when adenine, adenosine, or DL-homocysteine were tested at concentrations considerably higher than the K_I value for S-adenosylhomocysteine.

The product inhibition of the cyclopropane fatty acid synthetase system by S-adenosylhomocysteine is directly analogous to the tyramine methylpherase system reported by Mann and Mudd (1963). A hydrolytic enzyme in each case degrades the product, S-adenosylhomocysteine, to adenine and other compounds. Duerre (1962) reported the purification of an enzyme from *Escherichia coli* which cleaved S-adenosylhomocysteine to adenine and S-ribosylhomocysteine. This enzyme is probably identical to that found by Mann and Mudd (1963) in barley roots and the one in fraction A from *C. butyricum*. It seems likely that such enzymes will be found to occur generally in tissues where transmethylation reactions take place. A distinction should be noted between this type of enzyme, which can bring about complete hydrolysis of S-adenosylhomocysteine, and the yeast enzyme described by de la Haba and Cantoni (1958), which attacks at the sulfur atom and which catalyzes a reaction with an equilibrium greatly in favor of synthesis of S-adenosylhomocysteine.

Duerre (1962) noted that the S-adenosylhomocysteine hydrolase was unstable in solutions of low ionic strength and also that the purified enzyme lost activity on storage at −20°. Before the role of the hydrolytic enzyme in the cyclopropane fatty acid synthetase system was discovered it was observed that the partially purified synthetase lost considerable activity on concentration of dilute solutions and under various conditions used for enzyme purification (Chung and Law, 1964). It remains to be determined if this apparent loss of activity was merely the result of inactivation of the hydrolytic enzyme which would lead to false synthetase rate measurements, or if the synthetase itself was inactivated.

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