Short Communications

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The path of hydrogen in the formation of cyclopropane fatty acids

Although transmethylation reactions from the sulfur atom of methionine to other electron-rich atoms have been investigated extensively, those which involve transalkylation from sulfur to carbon were reported only recently and they have not yet been thoroughly studied. The report of BIRCH *et al.*[1] that the methionine methyl group can serve as a precursor of a carbon–methyl group in mycophenolic acid first indicated the possibility of transalkylation reactions from sulfur resulting in carbon–carbon bonds. The investigations of ALEXANDER *et al.*[2-4] and PARKS[5] demonstrated that yeast enzymes are capable of catalyzing a transmethylation from sulfur to carbon resulting in the branched side chain of ergosterol.

Experiments reported earlier with growing bacterial cultures[6] and with bacterial extracts[7] have led us to believe that cyclopropane acid formation involves a novel type of transmethylation reaction resulting ultimately in the formation of two carbon–carbon covalent bonds. Whether this overall process involves methylated intermediates with only one bond to the added carbon atom cannot be judged at present. Experiments were devised to determine how many of the three hydrogens of the methyl group were retained in the transalkylation reaction, using methionine doubly labeled in the methyl group with ^{14}C and with ^{3}H or using [*Me*-$^{2}H_3$]methionine.

Escherichia coli, methionine auxotroph 113-3, which was used throughout these experiments, was the gift of Dr. B. D. DAVIS. The organism was cultivated in a synthetic medium[6] to which 20 mg of L-methionine per liter had been added. The isolation of fatty acids from whole bacterial cells has been described previously[6].

TABLE I

ISOTOPE INCORPORATION INTO CYCLOPROPANE FATTY ACIDS FROM MIXED ^{14}C- AND ^{3}H-METHYL-L-METHIONINE

100 ml of minimal medium containing 1.0 ml of mixed isotopic L-methionine (^{14}C, $9.43 \cdot 10^5$ counts/min/ml; ^{3}H, $4.03 \cdot 10^5$ counts/min/ml) and 1 mg non-isotopic methionine were inoculated with *E. coli* 113-3. The culture was incubated in a shaker flask at 37° for 36 h. The organisms were harvested and saponified with 10% KOH in 50% methanol at 60° for 15 min. After removal of neutral materials with light petroleum, the aqueous solution was acidified with HCl and extracted with ether. The crude fatty acid fraction thus obtained was converted to esters by treatment with diazomethane. An aliquot of the mixture was subjected to gas–liquid chromatography, using 20% diethylene glycol succinate on Chromosorb W packed in a ¼ × 8 ft stainless-steel column in the F and M Scientific Co. Instrument, Model 500. The fractions were collected at the outlet in glass U-tubes cooled in acetone–dry ice. The recovery of radioactive compounds from the column was approx. 65%. All samples were dissolved in a polyether scintillation fluid[6] and counted in a Packard Tri Carb scintillation counter. The values obtained were corrected for the overlap of ^{14}C during ^{3}H counting by the use of appropriate standards.

	^{3}H (counts/min)	^{14}C (counts/min)	$^{3}H/^{14}C$
0.1 ml methionine solution	$4.03 \cdot 10^4$	$9.43 \cdot 10^4$	0.427
Methylenehexadecanoic acid	$0.66 \cdot 10^3$	$1.69 \cdot 10^4$	0.39

Radioactive L-methionine samples were purchased from New England Nuclear Corp. For a ^{14}C–^{3}H doubly labeled mixture, the corresponding samples were mixed and chromatographed on sheets of Whatman No. 3 filter paper, using ethanol–acetic acid–water (64:1:35)[8]. The methionine area was cut from the strip and eluted with distilled water in a nitrogen-filled chamber. This operation served to remove oxidized impurities invariably present in commercial methionine samples. Deuterium-labeled methionine was prepared by the method of MELVILLE *et al.*[9], using *S*-benzyl-L-homocysteine (Cyclo Chemical Corp., Los Angeles, Calif.) and $CI^{2}H_3$ (Iso-Serve, Inc., Cambridge, Mass.). The product used for these experiments was twice recrystallized (atom per cent excess ^{2}H, 24.3 %, $[\alpha]_D^{25} = +20.7°$). A third recrystallization raised the deuterium content to 25.3 atom per cent excess, 93 % of theory for $C_5H_3[^{2}H_3]NO_2S$. Deuterium determinations were performed by Mr. J. NEMETH, Urbana, Ill., by the falling-drop method.

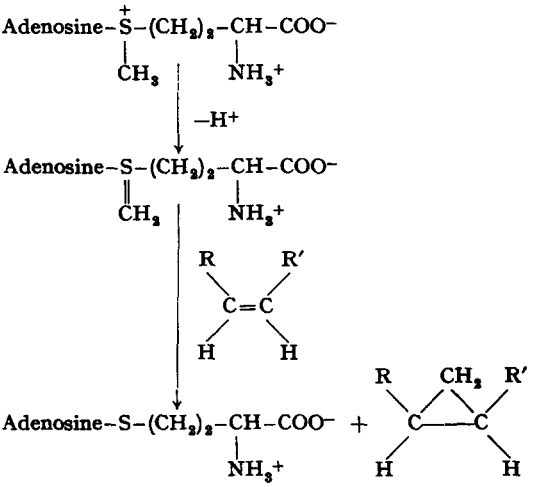
The 9,10-methylenehexadecanoic acid was prepared synthetically by the method of SIMMONS AND SMITH[10], using methyl palmitoleate. A sample of pure methyl palmitoleate prepared by partition chromatography of a commercial sample (Mann Research Laboratories, New York, N.Y.) was generously supplied by Miss A. T. NORRIS. The cyclopropane ester product was freed of starting material by chromatography on silicic acid impregnated with silver nitrate[11]. Silicic acid (Mallinckrodt No. 2847, screened to 100–200 mesh), 20 g, was slurried with 3 g $AgNO_3$ in water, and this suspension was frozen and lyophilized to dryness. The resulting powder was packed in a column under 3% ethyl ether in hexane. The sample was applied and eluted with the same solvent. The cyclopropane ester emerged before the olefinic ester and was cleanly separated from it. The product was characterized by gas–liquid chromatography and infrared spectrum.

The results of an experiment employing ^{14}C–^{3}H doubly labeled methionine are given in Table I. It can be seen that the results are ambiguous. One can make an interpretation that actually three hydrogens were transferred with only 10% loss of hydrogen. This is the conclusion drawn by ALEXANDER AND SCHWENK[4] in the case of ergosterol synthesis, where the $^{3}H/^{14}C$ ratio in the product was also 10% lower than that of the methionine used. An alternate interpretation would be that a large isotope effect in the breaking of C–^{3}H bonds has obscured the loss of one or more hydrogens during the transfer reaction.

In order to clarify this point another experiment using deuterium-labeled methionine was carried out. In this case *E. coli* 113-3 was cultured in 2 l of minimal medium containing 20 mg/l of deuterium-labeled methionine. The fatty acids were isolated as previously described, and the methyl esters were separated by gas–liquid chromatography. In this manner about 5 mg of deuterium-labeled methyl methylenehexadecanoate was collected.

Samples of isotopic and non-isotopic methyl 9,10-methylenehexadecanoate were subjected to mass spectrometry. The spectra indicated clearly that the deuterium-labeled sample contained two atoms of heavy hydrogen which increased the mass of the parent ion and of some of the larger fragments by two units. Unfortunately it was not possible to assign the exact position of the deuterium atoms within the molecule by examination of the cracking pattern, but it is likely that they are located in the methylene bridge. LIU AND HOFMANN[12] have shown that the carbon of the methionine methyl group is incorporated specifically into the methylene bridge.

The process of cyclopropane fatty acid synthesis therefore involves the transfer of the methionine methyl group along with two of its hydrogens to an unsaturated fatty acid chain. Since it has been demonstrated that the active methyl donor is actually *S*-adenosylmethionine[7,13,14], the overall process may involve the loss of a proton by such a reaction as the speculative one shown below:



A somewhat analogous chemical reaction in which a sulfoxonium methylide serves as the donor of a methylene bridge in a cyclopropane ring has been reported by COREY AND CHAYKOVSKY[15].

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1 A. J. BIRCH, R. J. ENGLISH, R. A. MASSY-WESTROPP, M. SLAYTOR AND H. SMITH, *J. Chem. Soc.*, (1958) 365.
2 G. J. ALEXANDER, *Science*, 126 (1957) 1065.
3 G. J. ALEXANDER, A. M. GOLD AND E. SCHWENK, *J. Biol. Chem.*, 232 (1958) 599.
4 G. J. ALEXANDER AND E. SCHWENK, *J. Biol. Chem.*, 232 (1958) 611.
5 L. W. PARKS, *J. Am. Chem. Soc.*, 80 (1958) 2023.
6 J. H. LAW, H. ZALKIN AND T. KANESHIRO, *Bicchim. Biophys. Acta*, 70 (1963) 143.
7 H. ZALKIN, J. H. LAW AND H. GOLDFINE, *J. Biol. Chem.*, 238 (1963) 1242.
8 F. SCHLENK AND R. E. DEPALMA, *J. Biol. Chem.*, 229 (1957) 1037.
9 D. B. MELVILLE, J. R. RACHELE AND E. B. KELLER, *J. Biol. Chem.*, 169 (1947) 419.
10 H. E. SIMMONS AND R. D. SMITH, *J. Am. Chem. Soc.*, 81 (1959) 4256.
11 B. DE VRIES, *Chem. Ind. London*, (1962) 1049.
12 T. Y. LIU AND K. HOFMANN, *Biochemistry*, 1 (1962) 189.
13 H. ZALKIN AND J. H. LAW, *Federation Proc.*, 21 (1962) 287.
14 W. M. O'LEARY, *J. Bacteriol.*, 84 (1962) 967.
15 E. J. COREY AND M. CHAYKOVSKY, *J. Am. Chem. Soc.*, 84 (1962) 867.

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