S-ADENOSYLMETHIONINE IN THE BIOSYNTHESIS OF BACTERIAL FATTY ACIDS

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ABSTRACT

O'LEARY, WILLIAM M. (Cornell University Medical College, New York, N.Y.). S-adenosylmethionine in the biosynthesis of bacterial fatty acids. J. Bacteriol. **84**:967-972. 1962.—The fatty-acid contents of a wild strain of *Aerobacter aerogenes* and of three auxotrophic mutants were determined by the use of gas-liquid chromatography. All strains studied exhibited essentially identical fatty-acid spectra. This organism was shown to contain large amounts of a C_{17} cyclopropane fatty acid, and it is suggested that it may be a convenient natural source of this compound. By use of the auxotrophic mutants which have varying requirements for exogenous sources of methionine and S-adenosylmethionine, it was shown by means of radioisotope tracer techniques that S-adenosylmethionine functions in this organism as a donor of a one-carbon unit that is used to form the ring structure in the biosynthesis of cyclopropane fatty acids.

In recent years, there has been considerable interest in the biosynthesis of bacterial fatty acids and particularly in those acids which contain cyclopropane rings. It has been shown that lactobacillic acid (*cis*-11,12-methylene-octadecenoic acid) is formed by the addition of a methylene group across the double bond of *cis*-vaccenic acid (*cis*-11,12-octadecenoic acid), and that the source of the added carbon atom is the methyl group of methionine (O'Leary, 1959*a*, *b*; Liu and Hofmann, 1962; Chalk and Kodicek, 1961). A similar mechanism has been suggested for the formation of C_{17} cyclopropane acid (O'Leary, 1959*b*; Law, 1961).

Although these studies provided the over-all information that the carbon chains of cyclopropane acids are supplied by analogous monoethenoid acids and that the ring carbons are derived from methionine, the finer details of the combination are as yet obscure. The chemical forms of the reacting substances, the intermediate compounds, and the specific reactions involved remain to be elucidated. Of particular interest in this respect was the question of whether methionine participates in such biosynthesis directly, or after conversion to S-adenosylmethionine, or whether some more circuitous route is involved. This adenosyl complex is known to participate in many biological transmethylation reactions (Shapiro and Schlenk, 1960), and it seemed possible that it might play a comparable role in the formation of a methylene bridge.

The experiments reported in this paper were designed to investigate the possible involvement of S-adenosylmethionine in the bacterial biosynthesis of cyclopropane fatty acids.

MATERIALS AND METHODS

Bacteria. To control experimental procedures as closely as possible, it was desirable to employ an organism that required an exogenous source of S-adenosylmethionine. No bacteria are known whose wild strains exhibit such a requirement, but fortunately some auxotrophic mutants of *Aerobacter aerogenes* suitable for use in this investigation were obtained through the generosity of S. Shapiro of the Argonne National Laboratory. These strains and the procedures used for their isolation have been described in detail (Shapiro, 1962). In the minimal medium of Davis and Mingioli (1950), strain AM-1 grew only if supplied with S-adenosylmethionine; strain 62 grew well if supplied with L-methionine, and to a limited extent if supplied with S-adenosylmethionine; and strain 68 grew well if supplied with either L-methionine or S-adenosylmethionine. A wild strain of *A. aerogenes* (ATCC 129) was also employed which required neither compound for growth in the minimal medium.

Media and culture methods. The chemically defined medium described by Davis and Mingioli (1950) was used for the mass culture of all organisms. When auxotrophic mutants requiring either L-methionine or S-adenosylmethionine were studied, the medium was supplemented with 0.2 μmole/ml of the appropriate substance. Solutions of these compounds were sterilized by filtration and then added to the otherwise complete, heat-sterilized medium. To produce the organisms for each fatty-acid analysis, 4 liters of medium were inoculated and incubated for 18 hr at 37 C. These cultures were aerated by vigorous agitation throughout the period of incubation. The cells were then collected, washed, and lyophilized as described elsewhere (O'Leary, 1959*a*). The average yield of dry cells was approximately 1 g/liter for all strains.

Chemicals. All chemicals employed were obtained from commercial sources with the exception of S-adenosylmethionine. This compound was prepared and purified by the methods of Schlenk, Dainko, and Stanford (1959), using *Saccharomyces cerevisiae* as the biosynthetic agent.

Extraction and analysis of fatty acids. Lyophilized cells (3 to 4 g) were refluxed under nitrogen with 30 ml of 20% KOH plus 30 ml of methanol for 4 hr. After removing the nonsaponifiable material with petroleum ether, the extract was acidified, and the free fatty acids were recovered by repeated extraction with ethyl ether. After the ether extract was dried over anhydrous sodium sulfate, the solvent was removed under reduced pressure, and the fatty-acid mixture stored in vacuo over phosphorus pentoxide.

During the preliminary studies of the incorporation of radioactive methyl-carbon into fatty acids, and for qualitative monitoring of these substances throughout the investigation, chromatography on silicone-impregnated paper was employed, following the methods of Schlenk et al. (1957). For more detailed analysis by gas-liquid chromatography, samples of mixed fatty acids were converted to their methyl esters by use of boron trifluoride in methanol, as described by Metcalfe and Schmitz (1961). The esters were analyzed with a Perkin-Elmer model 154D fractometer fitted with a 4-m silicone column. Individual fatty-acid fractions were collected in small-bore U tubes cooled in acetone-Dry Ice baths, and recovered by rinsing the tubes with acetone. To verify the presence of unsaturated compounds, samples were chromatographed both before and after catalytic hydrogenation, which was accomplished using procedures described by Kaneshiro and Marr (1961).

For the determinations of infrared absorption spectra, fatty acids were spread as thin films over KBr pellets or sandwiched between NaCl windows and examined with a Perkin-Elmer model 137 infrared spectrophotometer.

Measurements of radioactivity were made using a Nuclear-Chicago gas-flow proportional counter or a Packard Tri-Carb liquid scintillation counter.

RESULTS

Before proceeding with studies on the biosynthesis of fatty acids in *A. aerogenes*, it was important first to establish the fatty-acid composition of this organism, which had not been done previously, and to determine whether the mutants to be used in this investigation differed in any significant way with respect to their fatty-acid spectra.

Repeated analyses were made of samples of fatty acids extracted from cells of the wild strain both before and after catalytic hydrogenation. The fatty-acid composition determined from these studies is shown in Table 1. Infrared spectra of the mixed fatty acids exhibited an absorption peak at 9.8 μ which suggested the presence of some compound, or compounds,

TABLE 1. *Fatty-acid composition of Aerobacter aerogenes*

Gas chromatographic fraction	Fatty acid	Total acids
I	C_{10} saturated	1.1
II	C_{12} saturated	3.6
III	C_{13}*	0.9
IV	C_{14} saturated	10.5
V	C_{15}*	0.8
VI	C_{16} unsaturated	4.1
VII	C_{16} saturated	37.9
VIII	C_{17} cyclopropane	24.7
IX	C_{18} unsaturated	8.9
X	C_{18} saturated	1.4
XI	C_{19} cyclopropane	5.7
XII	C_{20}*	0.4

* Not further characterized.

containing a cyclopropane ring. Infrared examinations of each of the fractions isolated by gas-liquid chromatography showed that this same absorption characteristic was exhibited by fractions VIII and XI (Table 1). The chromatographic and infrared data thus indicated that fraction VIII is the methyl ester of a 17-carbon cyclopropane acid, and that fraction XI is a 19-carbon compound of the same type.

Fractions III and V (Table 1) exhibited chromatographic behavior that would be expected of C_{13} and C_{15} acids, respectively, but during this study it was not possible to collect enough of these fractions to permit identifying them further. Goldfine and Bloch (1961) reported observing similar acids in the lipids of *Clostridium butyricum*.

Exhaustive studies of the various mutant strains of *A. aerogenes* showed that, fortunately, none of their fatty-acid compositions differed significantly from that of the wild strain.

Some preliminary studies were performed to determine whether S-adenosylmethionine was involved in the biosynthesis of fatty acids by this organism. The AM-1 mutant, which required an exogenous source of this compound, was grown in medium containing 8×10^6 counts per min per liter of methyl-C^{14}-S-adenosylmethionine. The mixed fatty acids were isolated from these cells and were, indeed, found to be radioactive. Duplicate experiments yielded samples exhibiting 10×10^3 and 12×10^3 counts per min per mg, respectively. Once it was established that the methyl-carbon from S-adenosylmethionine could find its way into the fatty acids of this microorganism, more detailed studies of this phenomenon were justified.

TABLE 2. *Specific activities of fatty acids recovered from Aerobacter aerogenes strain AM-1 grown on methyl-C^{14}-S-adenosylmethionine*

Fatty acid	Specific activity*
C_{10} saturated	95
C_{12} saturated	150
C_{13}	870
C_{14} saturated	165
C_{15}	935
C_{16} unsaturated	2,950
C_{16} saturated	145
C_{17} cyclopropane	27,300
C_{18} unsaturated	2,810
C_{18} saturated	170
C_{19} cyclopropane	25,910
C_{20}	195

* Expressed as count per min per μmole.

Next to be investigated was the manner of distribution of methyl-carbon from this donor compound among the fatty acids of the AM-1 strain. This organism was grown in medium containing methyl-C^{14}-S-adenosylmethionine having a specific activity of 4×10^4 counts per min per μmole. The specific activities of the various fatty acids isolated from these cells are shown in Table 2. As was expected, the majority of the radioactivity appeared in the fractions containing the C_{17} and C_{19} cyclopropane acids. A more curious observation was that the levels of radioactivity found in the unsaturated acid fractions, while not as high as those of the cyclopropane compounds, were considerably above the activities found in the saturated-acid fractions. The possibility that the radioactivity found in the unsaturated fractions was due to contamination by small amounts of the highly radioactive cyclopropane compounds that were eluted later is unlikely, since the intervening saturated acids had much lower specific activities than either.

Fractions III and V (Table 1) also contained somewhat elevated levels of radioactivity. This may be an indication that these small fractions consist at least in part of cyclopropane compounds, but further careful study will be necessary to establish whether this is the case.

To simplify further studies, it was decided to minimize counting procedures by using one compound as an indicator of the degree of incorporation of methyl-carbon, rather than continue to determine the specific activity of every fatty acid in every experiment. As shown above, such incorporation is greatest in the two cyclopropane acids. Since, of these two, the C_{17} acid occurs in a much higher concentration, it was chosen as the indicator compound for the next series of experiments.

This series employed all four strains of *A. aerogenes* described above. Each strain was grown in four different media, each of which consisted of the basic medium of Davis and Mingioli plus one of the following: methyl-C^{14}-S-adenosylmethionine, methyl-C^{14}-L-methionine, methyl-C^{14}-S-adenosylmethionine plus unlabeled L-methionine, and unlabeled S-adenosylmethio-

TABLE 3. *Incorporation of methyl-carbon from labeled and unlabeled donor compounds and combinations thereof in C_{17} cyclopropane fatty acid by various strains of Aerobacter aerogenes*

Strain	Requirement for exogenous M or AM	Supplied in medium*			
		AM-C^{14}	M-C^{14}	AM-C^{14} + M	M-C^{14} + AM
129	Neither	3,030†	1,190	2,220	990
AM-1	AM	30,420	—‡	27,940	430
62	M	1,650	19,070	1,330	18,210
68	AM or M	27,760	20,100	21,640	13,540

*Both M (methionine) and AM (adenosylmethionine) supplied with specific activities of 40,000 counts per min per μmole.

† Counts per min per μmole of C_{17} cyclopropane acid.

‡ Insufficient growth for study.

nine plus methyl-C^{14}-L-methionine. The C_{17} acid was recovered from the cells of each of these 16 cultures by the methods already described, and specific activities were determined for all such fractions (Table 3). Both S-adenosylmethionine and methionine were incorporated to some extent by the wild strain which did not have an absolute requirement for either. In the AM-1 mutant which specifically required exogenous S-adenosylmethionine, there was little detectable uptake of L-methionine, but in the methionine-requiring strain 62, the reverse occurred. Most interestingly, strain 68, which had a nutritional requirement that was satisfied by either methionine or adenosylmethionine, can incorporate the methyl group of either compound into C_{17} acid. When both were present, this strain used both simultaneously. In both the wild strain and strain 68, S-adenosylmethionine appeared to be a more efficient methyl-carbon donor than L-methionine.

DISCUSSION

The determination of the fatty-acid composition of *A. aerogenes* is of particular interest in that relatively little information has been available on the lipids found in the members of this genus. In the only paper on this subject of which the author is aware, Sneed and Halvorson (1954) reported studies of the lipids of *A. cloacae*. By fractional distillation of the fatty acid methyl esters at low pressure, they were able to determine the percentage content in this organism of those fatty acids having chain lengths ranging from C_{10} through C_{20}. By this method, it was not possible for them to ascertain the proportions of unsaturated acids present.

Perhaps the most noteworthy aspect of the fatty-acid composition of *A. aerogenes* is the high content of C_{17} cyclopropane acid. This acid has been detected in other microorganisms, including *Escherichia coli* (O'Leary, 1959*b*; Dauchy and Asselineau, 1960; and others), *Bacillus subtilis* and *Pasteurella pestis* (Asselineau, 1961), and *Serratia marcescens* (Zalkin and Law, 1962). Kaneshiro and Marr (1961) showed that the C_{17} cyclopropane acid in *E. coli* is *cis*-9,10-methylene hexadecanoic acid, the homologue of 19-carbon lactobacillic acid. The high content of the C_{17} acid in *A. aerogenes* and the ease with which this organism can be cultured in simple, well-defined media suggest that it may be a convenient source of this compound.

After initial studies had shown that methyl-carbon from S-adenosylmethionine appeared in mixed fatty-acid fractions, it was expected, on the basis of experience with methionine (O'Leary, 1959*b*), that most of this methyl-carbon would be found in the cyclopropane acids. Studies of the individual acids showed that this was the case. Presumably, the added carbon was used to constitute a methylene bridge across the double bond in analogous unsaturated acids resulting in the formation of a three-membered ring. Such a reaction was conclusively demonstrated in other microorganisms by Liu and Hofmann (1962). Zalkin and Law (1962) reported a similar incorporation into the phospholipid fatty acids recovered from *S. marcescens*.

The presence of a significant amount of methyl-carbon from S-adenosylmethionine in chromatographic fractions usually considered to contain unsaturated acids was puzzling. Similar, and equally puzzling, effects were observed in earlier studies employing other organisms (O'Leary, 1959*b*; Liu and Hofmann, 1962). Why should methyl-carbon, one-carbon fragments, accumulate in unsaturated acid fractions? A possible explanation, one for which there is not yet any experimental evidence, is that the straight-chain unsaturated acids which usually comprise these fractions do not themselves contain the methyl-carbon, but that, instead,

there are present in these fractions other compounds which do contain the methyl-carbon and which cannot be separated from the usual unsaturated acids by the chromatographic methods usually employed. Such compounds might be cyclopropene acids, which have been suggested as possible intermediates in the conversion of straight-chain unsaturated acids to cyclopropane compounds. Indications of such compounds have been observed in recent studies of the fatty acids of pleuropneumonia-like organisms (O'Leary, 1962). Unfortunately, it has not been possible to obtain authentic samples of any cyclopropene acids to study their behavior in chromatographic systems such as those used in this investigation. This would appear to be a fruitful field for further studies, which might aid considerably in elucidating the finer details of cyclopropane-ring biosynthesis.

The data on the incorporation of C^{14}-labeled methyl-carbon from various donor sources strongly indicate that, in this microorganism, methionine participates in fatty-acid biosynthesis in the form of S-adenosylmethionine. Not only is incorporation of methyl-carbon from this compound very heavy, but also, in those strains which are able to utilize either donor compound. S-adenosylmethionine functions in this respect more efficiently than free methionine, and is used preferentially when both are present.

Recent years have witnessed considerable progress in the attempt to unravel the perplexing problem of how such molecular structures as cyclopropane fatty acids could be formed in a biochemical milieu. We know that the immediate precursor of such an acid is the analogous monounsaturated acid and that the third ring carbon can be derived from the methyl-carbon of methionine in the form of S-adenosylmethionine. While this is somewhat reminiscent of the involvement of S-adenosylmethionine in transmethylation reactions, clearly the details of the combination differ in important and as yet unknown respects from those of the usual methylations. The formation of a cyclopropane acid from the compounds known to participate would appear to involve three basic events: the transfer of a one-carbon fragment to the unsaturated acid, the formation of a methylene bridge at the site of the ethylenic bond, and the reduction of the ethylenic bond. The questions of whether these are discrete or concurrent events, the order in which they occur, and the reaction mechanisms involved all remain to be answered. As was mentioned above, it has been suggested that one intermediate in the conversion from a monoethylenic acid to a cyclopropane acid may be a cyclopropene compound, i.e., a methylene bridge across a still-existing double bond which would then be reduced to give the cyclopropane ring.

Beyond the matter of the final details of cyclopropane-ring biosynthesis, there is the even more important question of the role that these complex fatty acids play in the biochemistry of the bacterial cell. The relatively high concentrations in which they commonly occur and their high degree of structural specificity suggest that this role may be of considerable importance.

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