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Mevalonic Acid Is Partially Synthesized from Amino Acids in *Halobacterium cutirubrum*: a ^{13}C Nuclear Magnetic Resonance Study†

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^{13}C nuclear magnetic resonance revealed an unusual pathway for the biosynthesis of lipids in *Halobacterium cutirubrum* and *H. halobium*. Mevalonic acid was not synthesized from three acetyl-coenzyme A molecules, as has been suggested previously, and the branch-methyl and methine carbons in phytanyl chains were derived from neither acetate nor glycerol. Instead, they were supplied by the degradation of amino acids, in particular of lysine. Presumably, two different types of two-carbon fragments were used simultaneously by halobacteria for the biosynthesis of mevalonate. The labeling pattern of squalene supported the above conclusions. Based on these data, a general scheme is proposed to account for the contribution of lysine-to-lipid biosynthesis.

Our previous papers on the application of the ^{13}C-nuclear magnetic resonance (NMR) technique to study the metabolic pathways of methanogenic bacteria (7, 8, 10) showed several significant differences in biosynthesis when compared with the biosynthesis of other amino acids by bacteria and eucaryotes. Similar studies for comparison with halobacteria are lacking. Halobacteria are archaebacteria also, but with an aerobic rather than anaerobic mode of metabolism.

Halobacteria require quite complicated media for growth, and, in contrast to the aceticlastic methanogens, acetate can be used for growth only when combined with several additional carbon sources. An amino acid mixture is necessary (18), and other organic compounds such as glycerol can stimulate growth (18). Much research has been done on amino acid transport in halobacteria (19), and it was concluded that all amino acids except cysteine were transported through the membrane. On the other hand, the metabolism of halobacteria has been relatively little studied (19). In *Halobacterium salinarium*, all key enzymes of the tricarboxylic acid cycle and of the glyoxylic acid pathway have been detected (1). Lipid biosynthesis in halobacteria was studied also, with the conclusion that it is similar to the pathway leading to synthesis of isoprenoid chains in higher organisms (14, 15). Our preliminary results of labeling of lipids with [^{13}C]acetate (9) show that in fact not all positions in the phytanyl chains originate from acetate. The present study addresses this problem in more detail. Because of the presence of many carbon sources in the medium, the biosynthesis of amino acids was also monitored to obtain information about carbon flow through the major pathways of these organisms.

MATERIALS AND METHODS

Growth conditions. *H. cutirubrum* (NRC 34001) and *H. halobium* (NRC 34003) were grown aerobically in the dark at 37°C in 100-ml volumes or in 50-ml volumes in experiments with ^{13}C-labeled lysine. The synthetic growth medium described by Grey and Fitt (12) was used, except in amino acid biosynthesis experiments, where the amino acid being studied was omitted from the medium. Sodium citrate, originally present in the medium (12), was replaced by sodium acetate (12 mM solution) for all experiments. ^{13}C labeling was performed as follows. To observe the labeling patterns from acetate, sodium acetate in the medium was replaced with an equivalent amount of [1-^{13}C]-, [2-^{13}C]-, or [1,2-$^{13}C_2$]acetate. For labeling from [1,3-$^{13}C_2$]glycerol, 50-ml cultures were used, and the concentration of glycerol in the medium was decreased to 0.5%. For experiments with [U-^{13}C]lysine, the labeled compound was diluted with unlabeled lysine in a ratio of 1:2.

Fractionation of cells for NMR experiments. Bacteria were harvested by centrifugation, and lipids were extracted by the Bligh and Dyer procedure (3). Polar lipids were precipitated from chloroform solution by adding 5 volumes of cold acetone and centrifuging the precipitate (13). The acetone-soluble fraction was evaporated under a nitrogen stream, and the squalenes were obtained from it by a solvent partition method which used hexane–95% methanol (vol/vol) (13). It was found that under our conditions of growth, the hexane fraction contained only trace amounts of dihydro- and tetrahydrosqualene, so that no further purification of squalene was necessary. Amino acids were obtained from delipidated cells by hydrolysis in 6M HCl at 110°C for 30 h.

For ^{14}C labeling, 5 μCi of each substrate was added to 100 ml of medium containing the full complement of amino acids, glycerol (100 mg/100 ml), and $CH_3COONa \cdot 3H_2O$ (100 mg/100 ml). The substrates and amounts were as follows: 1 μM [2-^{14}C]mevalonic acid (dibenzoylethylenediamine salt), 1.0 μCi/μmol; 13.2 mM [U-^{14}C]glycerol, 3.8 μCi/mmol; 7.3 mM [1,2-^{14}C]acetic acid, 6.85 μCi/mmol; 3.3 μM [2-^{14}C]pyruvate, 15.3 μCi/μmol; 2.1 mM L-[U-^{14}C]threonine, 23.8 μCi/mmol; 0.22 mM L-[U-^{14}C]proline, 0.23 μCi/μmol; 1.2 mM L-[U-^{14}C]arginine, 43.5 μCi/mmol; 2.9 mM L-[U-^{14}C]serine, 17.2 μCi/mmol; 2.9 mM L-[U-^{14}C]lysine, 17.2 μCi/μmol; 0.79 mM L-[U-^{14}C]phenylalanine, 63.3 μCi/mmol; 3.1 mM L-[U-^{14}C]leucine, 16.4 μCi/mmol. Cultures of 100-ml volume were harvested by centrifugation, washed in 10 ml of salt solution, and suspended in 10 ml of salt solution. Each 100-ml culture was divided into equal portions for the study of lipids and proteins. Cells were broken by passing through a French pressure cell operated at 100 MPa. Samples were then centrifuged at 12,000 × *g* for 30 min, and the proteins were precipitated with ethanol (60% [vol/vol]) at 4°C.

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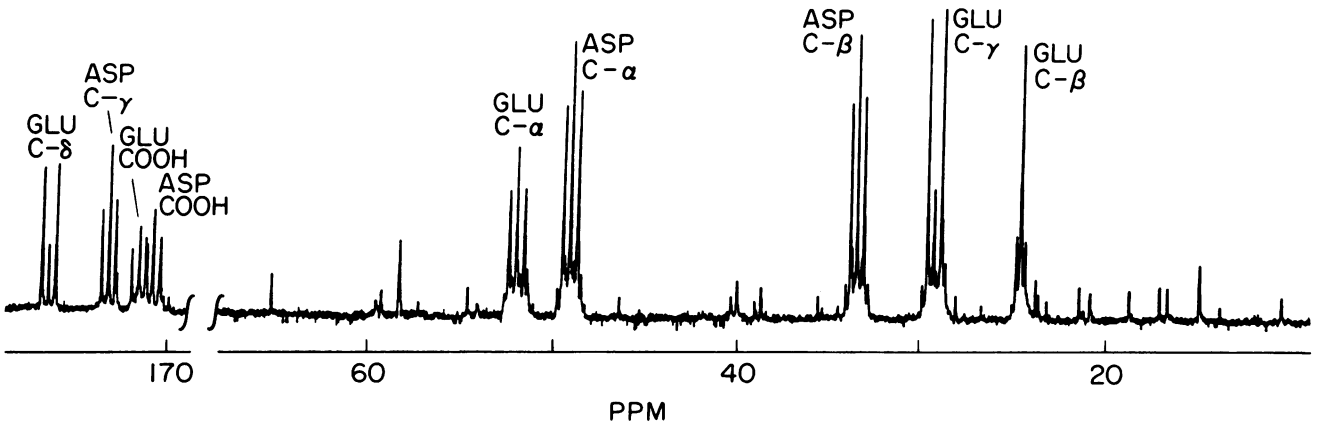

FIG. 1. ^{13}C NMR spectrum (75.5 MHz) of the mixture of amino acids obtained from *H. cutirubrum* grown in the presence of [1,2-$^{13}C_2$]acetate.

^{13}C NMR spectra. For NMR analysis, amino acids were dissolved in 0.1 M HCl containing 90% ^2H_2O. Lipids were analyzed in deuterated chloroform-methanol (7:1 [vol/vol]). ^{13}C NMR spectra were recorded at 75.5 MHz on a Bruker CXP 300 spectrometer. Chemical shifts were referenced to tetramethylsilane in a concentric capillary. Spectral parameters were typically 18-kHz sweep width, 60° pulses, 16,384 data points, and 2-s recycle time. All spectra were proton decoupled. For quantitation of intensities, the nuclear Overhauser effect was suppressed by gated decoupling, and the recycle time was 4 s. Integration was performed by using the NMRCAP program from Nicolet software.

Reagents. ^{13}C-labeled compounds were from Merck, Sharp & Dohme, Canada Ltd., Montreal, with enrichment levels of 90%. ^{14}C-labeled compounds were purchased from New England Nuclear of Canada, Lachine, Quebec.

RESULTS

Amino acids. Basic experiments were done by using the defined growth medium of Grey and Fitt (12), where citrate was replaced by labeled acetate. A ^{13}C NMR spectrum of the amino acid mixture, labeled from [1,2-$^{13}C_2$]acetate, is shown in Fig. 1. Only nine amino acid signals were significantly enhanced; this occurred at 24.92, 29.43, 52.44, 176.30, 171.84, 49.65, 33.89, 173.28, and 171.27 ppm. The first five could be assigned to glutamic acid, and the last four were assigned to aspartic acid. From the ^1H NMR spectra of labeled glutamic and aspartic acids (purified from a mixture of amino acids by using a column of Dowex 1-X8 in the acetate form), and from the relative intensities of multiplet components in the ^{13}C NMR spectrum of a sample labeled from [1-^{13}C]acetate, it was concluded that the labeled acetate was diluted about fivefold upon entering the tricarboxylic acid cycle (probably by acetyl-coenzyme A (CoA) derived from glycerol and some of the amino acids). [1,3-$^{13}C_2$]glycerol labeled glutamate and aspartate in the same way as did [2-^{13}C]acetate (spectra not shown). Omitting some of the amino acids, such as phenylalanine or alanine, stimulated their biosynthesis from glycerol. Glycerol also led to labeled histidine and serine. However, no significant labeling of phenylalanine, histidine, or serine was observed with acetate as the source.

Lipids. Figure 2 shows the ^{13}C NMR spectra of the polar lipids which were labeled from acetate and, for comparison, a spectrum of unlabeled (nonenriched) polar lipids. Identification of signals was based on earlier data (5, 7). If the mevalonate pathway were operating as expected (21), [1-^{13}C]acetate should have labeled carbons A and C in the repetitive subunit of the phytanyl chains (i.e., carbons 1, 3, 5, 7, 9, 11, 13, and 15 of the entire chain [Fig. 3]). Furthermore, [2-^{13}C]acetate should have labeled positions B, D, and E in the subunit (i.e., carbons 2, 4, 6, 8, 10, 12, 14, 16, 17, 18, 19, and 20 of the chain). It can be clearly seen from Fig. 2 that [1-^{13}C]acetate almost exclusively labeled A positions and only very weakly labeled C positions. [2-^{13}C]acetate predominantly labeled B and D positions but did not label E positions. Thus, acetate did not contribute carbon atoms to either the branch-methyl groups or the methine groups. To test whether the mevalonate biosynthesis pathway could be altered by the presence of acetate during growth, *H. cutirubrum* was grown in a medium where acetate was replaced by either [1,3-^{13}C]glycerol or [3-^{13}C]alanine. The spectra obtained were virtually identical to that shown for [2-^{13}C]acetate (Fig. 2b), indicating that both glycerol and alanine were converted through acetyl-CoA. These studies further confirmed an absence of labeling in the branch-methyl and methine carbon positions.

Since two carbons in each phytanyl chain subunit were not significantly labeled, either from acetate or from glycerol, and amino acids were the only other carbon sources added to the growth medium, these carbons must have been supplied by certain amino acids. To explore this possibility, ^{14}C labeling experiments were performed (Table 1). As expected, mevalonic acid was converted nearly exclusively to lipid. Acetate, pyruvate, and glycerol all contributed significantly to both lipids and amino acids. The highest level of labeling of lipids from those amino acids studied was found for lysine, but contributions from others, such as leucine, were also significant.

Some compounds may have been incorporated into the cellular material with significant scrambling of the label. This was observed in the case of *H. cutirubrum* for pyruvate; adding 1 mM or 0.5 mM [2-^{13}C]pyruvate to the medium led to incorporation of the label in an unspecific way (with nearly complete scrambling). Thus, it was necessary to test whether lysine was indeed incorporated into those positions of the phytanyl chains which were not labeled from acetate, or whether it was first degraded to acetate and then incorporated into lipids. For this purpose, *H. cutirubrum* was grown in the presence of uniformly labeled lysine. As seen in Fig. 4, this led to labeling of all the phytanyl positions. The ^{13}C NMR spectrum contained two important features. First,

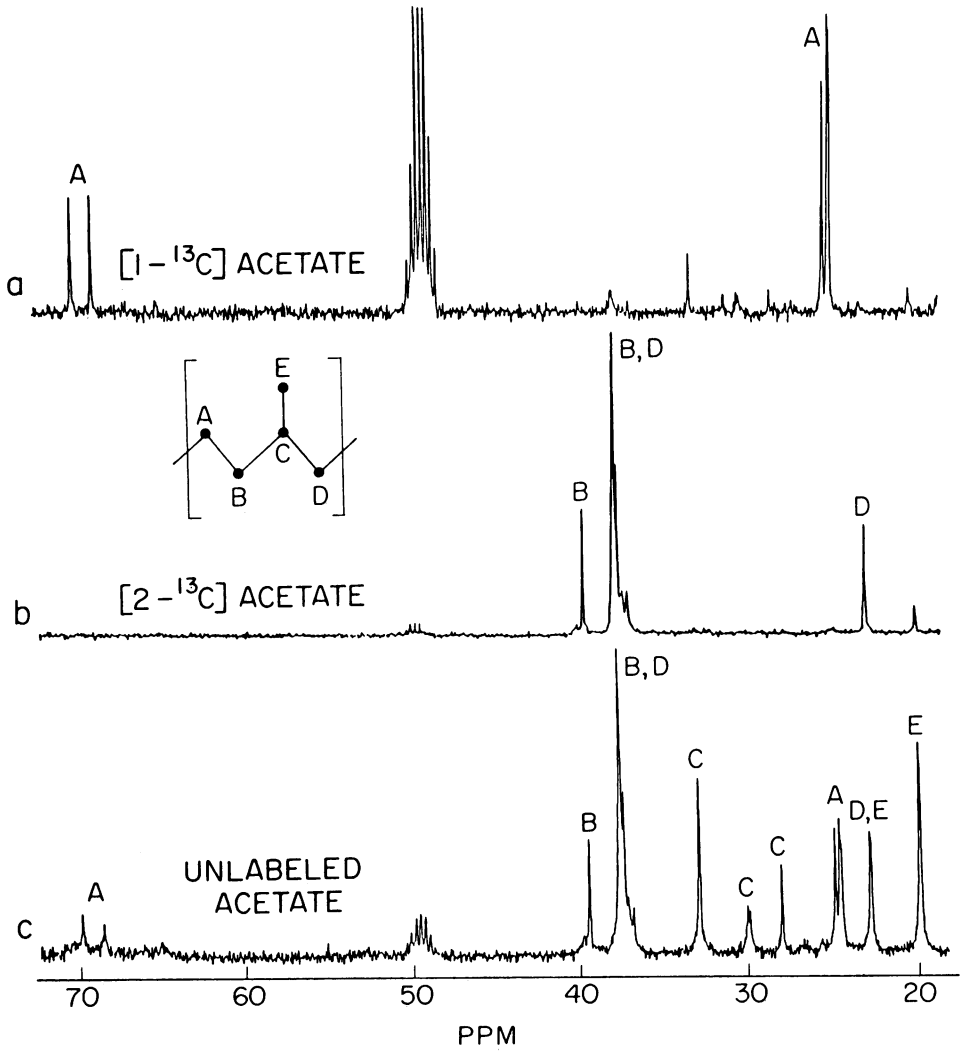

FIG. 2. ^{13}C NMR spectra (75.5 MHz) of the polar lipids extracted from *H. cutirubrum*. Peaks are labeled with letters showing assignment in the five-carbon repetitive subunit of phytanyl chains (drawn schematically between a and b). A multiplet at 49 to 50 ppm originated from deuterated methanol. Amounts of lipids used were as follows: [1-^{13}C]acetate (a), 3 mg; [2-^{13}C]acetate (b), 3 mg; unlabeled acetate (c), 27 mg.

the intensities of signals which were not labeled from acetate (Fig. 2, C and E positions) were greater than the others, showing that lysine had some specificity of incorporation into phytanyl chains. The incorporation of ^{13}C into other positions would have been diluted by biosynthetic acetate. Secondly, the signals of all carbons consisted of multiplets. In particular, nearly 80% of the intensity of the signal of the branch-methyl groups at 19.9 ppm was in the form of a doublet, with a separation characteristic of a one-bond carbon scalar coupling (35 Hz). This showed that lysine contributed two carbon fragments for synthesizing the methyl and methine groups of phytanol. More elaborate studies are necessary to determine whether an excess of labeling of these two positions relative to the others is sufficient to account for the total synthesis of methyl and methine groups and to test the significance of the contributions from other amino acids.

Experiments with [1-^{13}C]lysine were performed to explore the degradation of this amino acid. However, contrary to the case of [U-^{13}C]lysine, no incorporation of label into lipids was detected. This suggests that lysine was decarboxylated during degradation.

In parallel to the labeling of the phytanyl chains, incorporation of [2-^{13}C]acetate was examined for squalene. In this compound, only four signals were observed: at 124.38 and 124.26 ppm (carbons 3, 7, and 11 [Fig. 3]), at 39.73 ppm (carbons 5 and 9), and at 25.69 ppm (carbon 1). This was consistent with the labeling patterns in phytanyl chains, since signals caused by branch-methyl groups could not be detected.

Experiments on incorporation of acetate into phytanyl chains were also performed for *H. halobium*, with results identical to those for *H. cutirubrum*. Thus, the unusual metabolism to mevalonate was not restricted to only one species.

DISCUSSION

Since defined media for the growth of halobacteria contain many carbon sources, our initial studies were performed to determine the flow of carbon from labeled precursors. Our efforts were concentrated on the labeling patterns of amino acids, as this can provide an overall picture relatively easily. The only two amino acids strongly labeled from acetate were aspartic and glutamic acids. This occurred despite the pres-

TABLE 1. Labeling of lipids in *H. cutirubrum* from various carbon sources

Labeled precursor	% of cpm in lipids[a]	Lipid cpm/ protein cpm
Thr	1.2	0.02
Pro	0.6	0.01
Arg	0.1	0.00
Ser	5.3	0.08
Lys	23.5	0.49
Phe	ND[b]	0.04
Leu	ND	0.18
Acetate	ND	1.97
Mevalonate	ND	114.0
Glycerol	ND	0.75
Pyruvate	ND	1.50

[a] Labeling of the lipid fraction was expressed as a percentage of the total cpm taken up by the cells.
[b] ND, Not done.

ence of large quantities of glutamic acid in the growth medium, which is similar to results observed in another archaebacterium, *Methanococcus voltae* (6). Threonine can also be labeled if it is omitted from the growth medium. Other amino acids, which depend on glycolytic intermediates, such as phenylalanine, histidine and serine, although easily labeled from glycerol, were not labeled from acetate. Thus, acetate is primarily used in the biosynthesis of lipids and enters the tricarboxylic acid cycle. This is what could be expected from the scant information available on the metabolism of halobacteria (14, 15).

Previous studies on the biosynthesis of phytanyl chains in halobacteria concluded that synthesis began from acetate (acetyl-CoA) and proceeded according to the following general scheme (14): acetate → acetyl-CoA → mevalonate → phytanyl-pyrophosphate → lipids. Such a scheme implies that mevalonic acid is synthesized by halobacteria in the same way as by higher organisms. The previous study (14) showed that radiotracer-labeled acetate was incorporated into the phytanyl chains of *H. cutirubrum* but did not describe which of the carbon atoms were labeled. Our study, by using the ^{13}C NMR method, confirmed the incorporation of acetate into phytanyl chains and revealed an unexpected lack of label transfer from acetate, glycerol, or alanine to the branch-methyl and methine carbon atoms. Thus, the present results are in contradiction to this scheme and suggest that of three two-carbon units normally used as precursors of mevalonic acid, only two originate from acetate, presumably via acetyl-CoA. The third two-carbon unit, which supplies carbon atoms to the branch-methyl and methine groups of the phytanyl chain, is of a different origin; it is provided by amino acids, particularly lysine.

Other amino acids, especially leucine, also labeled lipids significantly (Table 1), and it is possible that they contributed two specific carbon fragments to phytanol. However, the labeling of lipids was most significant in the case of lysine, which justifies the concentration of our efforts on this amino acid.

The observed conversion of lysine into the polyisoprenoid chain is quite surprising. Although some amino acids, such as leucine, can be converted to mevalonic acid (11), we are not aware of any route for converting lysine to the phytanyl chains of lipids.

An impressive variety of catabolic pathways for lysine has developed in different classes of organisms (2). Further studies are necessary to determine whether any of them, or a completely new pathway, is used by halobacteria. The pathway used by *Clostridium* strain SB4 is of particular interest, since in this case lysine contributes directly to acetoacetate (23). However, in *Clostridium* strain SB4, carbons 1 and 2 of acetoacetate are derived from lysine, which could not account for labeling such as that occurring in the phytanyl chains of *H. cutirubrum*. More information about the degradation of lysine by *H. cutirubrum* could be obtained from studies on the metabolism of selectively labeled lysine.

The results described above lead to a general scheme for

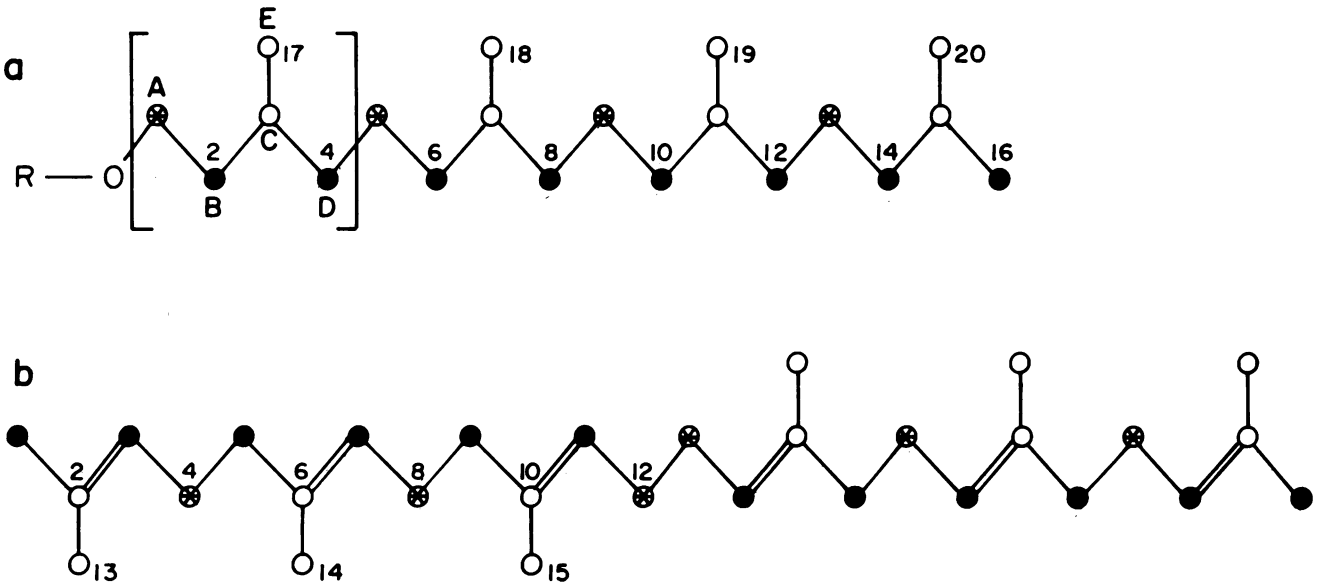

FIG. 3. Structures of a phytanyl chain (a) and squalene (b). Symbols for origin of carbon atoms: ●, methyl group of acetate; ⊛, carboxyl group of acetate; ○, amino acids. The carbon atoms of one unit (in brackets) derived from mevalonic acid are labeled A to E for comparison to Fig. 2.

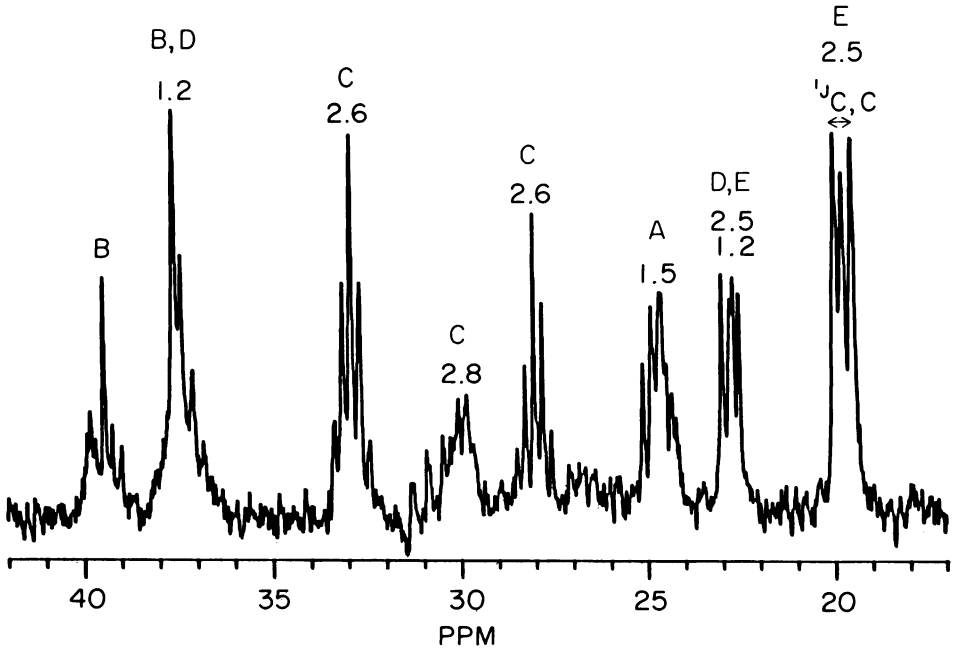

FIG. 4. High-field fragment of the ^{13}C NMR spectrum (75.5 MHz) of the total lipids from *H. cutirubrum* grown in medium supplemented with [U-^{13}C]lysine. Values shown above each multiplet represent total intensities measured by integration of multiplets. The assignment of signals was as illustrated in Fig. 2, using the letters A through E. $^1J_{C,C}$, Carbon-carbon coupling constant, calculated as 35 Hz for the signals of CH_3 groups and marked by ↔ above the multiplet.

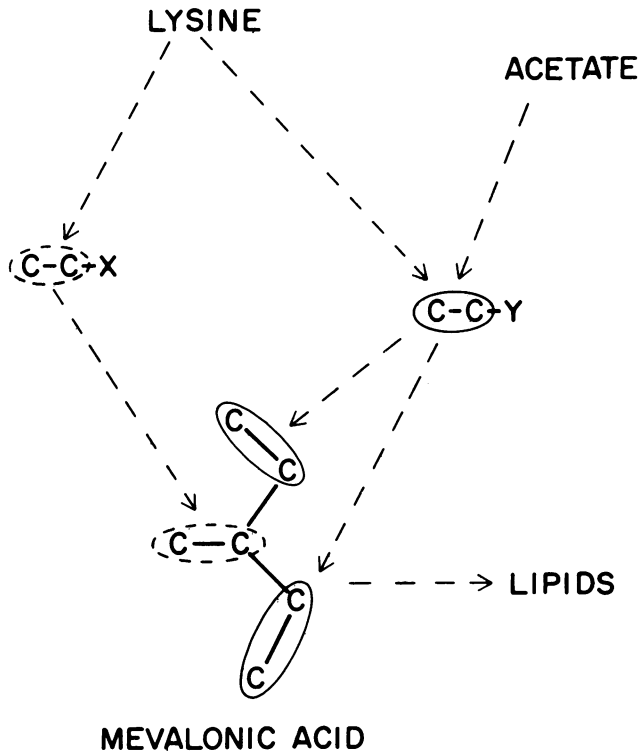

FIG. 5. Scheme of incorporation of acetate and lysine into mevalonic acid. The scheme accounts for the experimental evidence showing that acetate does not supply the branch-methyl and methine carbons and that label from [U-^{13}C]lysine can be incorporated into all positions in the phytanyl chain. The carriers of two-carbon units are shown as X and Y, where Y is likely to be a CoA.

the utilization of lysine for the synthesis of phytanol and squalene (Fig. 5). During degradation, lysine provides two types of carbon fragments. One type is used for making branch-methyl groups and positions adjacent to them; the second type is used in a way similar to that of exogenous acetate (or glycerol) and is incorporated into all other positions. The similar labeling patterns of phytanol and squalene support our hypothesis of an unusual biosynthetic step occurring at the level of mevalonic acid. Furthermore, the absence of carbon transfer from acetate to the branch-methyl and methine carbon atoms of the phytanyl chain was reproduced by growing *H. cutirubrum* in media containing [1,3-^{13}C]glycerol or [3-^{13}C]alanine in the absence of acetate. Thus, this newly described biosynthetic pathway for mevalonate synthesis was operative regardless of the presence of added acetate during growth.

All above results point to a difference in the biosynthetic pathway at an early stage in the synthesis of mevalonic acid. Since this compound originates from three two-carbon units, it is quite possible that the general scheme of the biosynthesis is the same as in the higher organisms; i.e., it proceeds via acetoacetyl-CoA, and the basic difference is in the enzymes used by halobacteria, especially in the acetoacetyl-CoA thiolase. In some systems, this enzyme was reported to use various thiol compounds (16, 20). Stern and Drummond (20) have described an enzyme fraction from ox liver which catalyzes a mixed thiolysis reaction according to the general scheme acetoacetyl-SR_1 + R_2SH ⇌ acetyl-SR_1 + acetyl-SR_2, where R_1 and R_2 are different thiol donors. Thiolases active with thiol donors other than CoA were also reported for yeasts (16). Operation of a mixed thiolase, which would use acetyl-CoA derived from acetate and acetyl-*X* from degraded amino acids, would explain the labeling pattern of the phytanyl chains observed in halobacteria. In contrast to ox liver, such an enzyme in *H. cutirubrum* would be used for synthesis rather than degradation of acetoacetyl-CoA.

In addition to the possibility of a mixed thiolase activity, mevalonic acid could be formed partially from malonyl-CoA. Such a mechanism has been postulated in animal systems (4) and examined extensively (17), with rather ambiguous conclusions. The major difficulties in testing the proposal are the presence of enzymes interconverting acetyl-CoA and malonyl-CoA and possible simultaneous involvement of enzymes used for catabolism or anabolism of fatty acids and for biosynthesis of sterols. Halobacteria offer a very unique opportunity to expand these studies. Not only do they contain only trace amounts of fatty acids (15), but also (as shown in this work) halobacteria form mevalonate from two-carbon units of two different origins.

The results presented here show a significant difference in the biosynthesis of mevalonic acid relative to that in other systems studied previously, such as mammals and yeasts (17). It is of interest to determine whether this unusual variation in the pathway to lipids is unique to halobacteria or whether it is also found in other archaebacteria. ^{13}C NMR studies of labeling of lipids in *Methanospirillum hungatei* (7) showed lipid labeling patterns as would be expected for the synthesis of mevalonic acid by the standard pathway found in mammalian and yeast cells. However, this may be a consequence of the fact that *M. hungatei* requires only acetate and CO_2 as carbon sources, resulting in a labeling of all positions in the phytanyl chains by acetate. Additional studies on the incorporation of deuterium into lipids in both methanogens and halobacteria are currently under way to gain more insight into the details of lipid biosynthesis in archaebacteria.

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