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N-METHYL GROUPS IN BACTERIAL LIPIDS

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Abstract

Goldfine, Howard (Harvard Medical School, Boston, Mass.), and Martha E. Ellis. *N*-methyl groups in bacterial lipids. J. Bacteriol. **87**:8–15. 1964.—The ability of bacteria to synthesize lecithin was examined by measuring the incorporation of the methyl group of methionine into the water-soluble moieties obtained on acid hydrolysis of bacterial lipids. Of 21 species examined, mostly of the order Eubacteriales, only 2, *Agrobacterium radiobacter* and *A. rhizogenes*, incorporated the methyl group of methionine into lipid-bound choline. Evidence was also obtained for the formation of lipid-bound *N*-methylethanolamine and *N,N'*-dimethylethanolamine in these two organisms. Two other species, *Clostridium butyricum* and *Proteus vulgaris*, incorporated the methyl group of methionine into lipid-bound *N*-methylethanolamine, but did not appear to be able to further methylate these lipids to form lecithin. The results of this study lend further strength to the generalization that bacteria, with the exception of the genus *Agrobacterium*, are unable to synthesize lecithin.

Lecithin has been found in the lipids of relatively few bacterial species (Asselineau and Lederer, 1960), and its synthesis was studied in only one of these organisms (Law, Zalkin, and Kaneshiro, 1963). Phosphatidyl ethanolamine, in contrast, appears to be a common, though not universal, constituent of bacterial lipids (Asselineau, 1962). The discovery of a phospholipid containing *N*-methylethanolamine (MEA) in *Clostridium butyricum* (Goldfine, 1962) led us to consider the possibility that bacterial species unable to synthesize lecithin may nevertheless possess the ability to synthesize phosphatidyl MEA and phosphatidyl *N,N'*-dimethylethanolamine (DMEA). Because MEA is not revealed as readily as ethanolamine on chromatograms stained with ninhydrin, and because DMEA is not revealed as readily as choline by Dragendorff's reagent, it is possible that MEA and DMEA have been overlooked in earlier studies of bacterial lipid hydrolysis products.

We have, therefore, undertaken a survey of a number of bacterial species for these *N*-methylated derivatives. Because methionine was shown to serve as a source of the methyl groups of *N*-methylethanolamine (Goldfine, 1962) and choline (Law et al., 1963) in bacterial lipids, we examined the ability of these bacteria to incorporate the methyl group of C^{14} (methyl) L-methionine into phospholipid bases.

Materials and Methods

Cultures and growth conditions. The strains studied, media used, and cell densities obtained are recorded in Table 1. All cultures were grown at or near optimal temperature (Breed, Murray, and Smith, 1957; Jacobs and Gerstein, 1960). Aerobes were grown in Erlenmeyer flasks (filled one-third to one-half) on rotary shakers. Lactobacilli were grown without shaking in 1-liter volumetric flasks filled to the neck. Clostridia were grown in volumetric flasks as described previously (Goldfine and Bloch, 1961).

Materials. C^{14} (methyl) L-methionine (33.3 or 67 μc/mg) was obtained from New England Nuclear Corp., Boston, Mass.; 10 μc were added to each culture with the exceptions of *Serratia marcescens* (12 μc) and *Proteus vulgaris* (40 μc).

Isolation of lipids. After being harvested by centrifugation in the cold and washed with cold distilled water, the cells were extracted three times with chloroform-methanol (2:1, v/v; Goldfine and Bloch, 1961). The duration of each extraction was 2 to 3 hr. The lipids, dissolved in chloroform-methanol, were washed with 0.05 N NaCl (Folch, Lees, and Stanley, 1957).

Hydrolysis of lipids. Lipids were hydrolyzed in 1 N HCl at 100 C, overnight. Usually 1 or 2 ml of 1 N HCl were used to hydrolyze half of the lipid extracted from each culture. Hydrolysis was carried out in tubes (16 by 125 mm) with Teflon-lined screw-caps. After being cooled, the hydrolysates were extracted three times with 10 ml of petroleum ether. The petroleum ether extracts, containing fatty acids and aldehydes

TABLE 1. *Growth of cells*

Organism	Source	Medium*	Volume	Final Klett reading (no. 66 filter)
			ml	
Aerobacter aerogenes	J. H. Law	A	1,000	197
Agrobacterium radiobacter	ATCC† 4718	B	1,000	210
A. rhizogenes	ATCC 11325	B	1,000	203
Alcaligenes faecalis	H. M. S.‡	C	800	344
Bacillus megaterium KM	M. Yudkin	L	1,000	128
B. subtilis	H. M. S.	D	1,000	152
Clostridium acetobutylicum	J. H. Law	M	1,000	260
C. butyricum	ATCC 8260	E	1,000	195
C. histolyticum	H. M. S.	F	1,000	
C. propionicum	E. R. Stadtman	G	1,000	200
C. tetanomorphum	ATCC 3606	H	1,000	151
Lactobacillus casei	K. Bloch	I	1,000	128
L. plantarum	ATCC 8014	I	1,000	62
Micrococcus lysodeikticus	E. Levin	J	800	190
Mycobacterium phlei	ATCC 356	K	800	
Proteus vulgaris	H. M. S.	D	4,000	
Pseudomonas aeruginosa	ATCC 7701	A	800	170
Sarcina lutea	H. M. S.	D	1,000	145
Serratia marcescens	ATCC 8195	A	800	185
Staphylococcus albus	H. M. S.	D	1,000	310
Streptococcus B	H. M. S.	D	1,000	100

* Key: A = medium C of Roberts et al. (1955); B = Starr (1946); C = C-broth of Roberts et al. (1955); D = tryptic digest broth, Fields (BBL); E = Casamino Acid medium of Broquist and Snell (1951); F = Thioglycollate broth (Difco); G = Goldfine and Stadtman (1960); H = Barker et al. (1959); I = Henderson and Snell (1948); J = 1% peptone (Difco), 0.5% NaCl, 1 N NaOH (approximately 3 ml per liter) at pH 7.5; K = Fulco and Bloch (1962); L = medium C of Roberts et al. (1955), plus 2.0 g of sodium glutamate and 0.003 mole of ferric citrate per liter; M = Hamilton (1959) and subsequent modifications by F. H. Westheimer (*unpublished data*). It contained (per liter of medium): Trypticase, 20 g; yeast extract, 1 g; KH_2PO_4, 1 g; asparagine, 0.6 g; $FeSO_4 \cdot 7H_2O$, 0.02 g; ascorbic acid, 0.02 g; L-arabinose, 4 g; $MgSO_4 \cdot 7H_2O$, 0.4 g; $MnSO_4 \cdot H_2O$, 0.0124 g; NaCl, 0.02 g.

† American Type Culture Collection.

‡ Collection of Department of Bacteriology and Immunology, Harvard Medical School.

if plasmalogens were present (Goldfine, 1963), were combined, and a sample was assayed for radioactivity. The aqueous phase was evaporated under a stream of nitrogen at 60 to 100 C, and the residue was placed under oil-pump vacuum for 15 to 30 min. The sample was dissolved in 70% ethanol for application to paper for chromatography. Samples, dissolved in water, were assayed for C^{14}.

Radioactivity assays. Total lipids and fatty acids were counted in toluene-plus scintillators (Goldfine and Bloch, 1961). Water-soluble compounds were counted in a dioxane-anisole-diethylene glycol dimethyl ether (750:125:125) solution which contained the same scintillators (Law et al., 1963). Liquid scintillation spectrometers (Nuclear-Chicago Corp., Des Plaines, Ill., and Packard Instrument Co., Lagrange, Ill.) were used in this study.

Paper chromatography of lipid hydrolysis products. The water-soluble lipid hydrolysis products were chromatographed on Whatman no. 1 paper in the solvent systems previously described (Goldfine, 1962). Paper dipped in 1 N KCl was used in solvent 1.

Thin-layer chromatography of lipids. Lipids were chromatographed on layers of silica gel G (Brinckman Instruments, Inc., Great Neck, L.I., N.Y.) approximately 0.5 mm thick. About 10 mg of lipid were spread out in a band 2 cm from the bottom of the plate. Plates were developed in chloroform-methanol-7 N NH_4OH (60:35:6). They were then dried in air and exposed to iodine vapors. The lipid zones were outlined, and

the silica gel containing each zone was stripped from the glass plates by means of a suction device (Hirsch and Goldrick, J. Lipid Res. *in press*). Each lipid fraction was eluted from the silica gel with chloroform-methanol (1:3), v/v) and assayed for phosphorus.

Phosphorus analysis. Phosphorus analyses were performed in duplicate by the method of Gomori (1942). Lipids were first digested in 10 N H_2SO_4 at 140 to 160 C for 70 to 100 min; the samples were cooled, 1 or 2 drops of 30% hydrogen peroxide were added, and digestion was continued for another 30 min.

RESULTS

Incorporation into lipid of C^{14} from methyl-labeled methionine. The incorporations of C^{14} into the total extractable lipids and into the acid hydrolysis products are given in Table 2. Recoveries of radioactivity in the hydrolysis products were satisfactory, except where total counts were low, and in the case of *Mycobacterium phlei*. The poor recovery of C^{14} from *M. phlei* lipids was probably due to an insoluble material produced on hydrolysis. The organisms studied can be divided into three groups on the basis of incorporation of C^{14} into the water-soluble products obtained on acid hydrolysis of the lipids.

Group 1. The first group of 13 organisms consists of those cultures which incorporated little or no C^{14} into the water-soluble lipid hydrolysis products. This group includes: *Aerobacter aerogenes, Bacillus megaterium, B. subtilis, C. acetobutylicum, C. histolyticum, C. propionicum, C. tetanomorphum, Lactobacillus casei, L. plantarum, Micrococcus lysodeikticus, Sarcina lutea, Staphylococcus albus*, and *Streptococcus* B.

Group 2. The second group of four organisms incorporated from 15,000 to 857,000 disintegrations per min of C^{14} into the water-soluble compounds obtained from lipid hydrolysates. This group includes: *Alcaligenes faecalis, M. phlei, Pseudomonas aeruginosa*, and *Serratia marcescens*. These labeled moieties were examined by paper chromatography in solvent 1 (Goldfine, 1962). The paper strips were scanned with a gas-flow counter (Actigraph, Nuclear-Chicago Corp.) and exposed to X-ray film for radioautography. No radioactive spots corresponding to carrier MEA or DMEA hydrochlorides or choline chloride were seen on the chromatograms of any of these lipid hydrolysis products. The major radioactive material extracted from the *M. phlei* lipid hydrolysate migrated (R_F = 0.86) ahead of choline chloride (R_F = 0.70). Only faint spots were seen on the radioautograms of the water-soluble products obtained from the lipid hydrolysates of *A. faecalis* and *P. aeruginosa*, and these compounds migrated more slowly than the salts of MEA (R_F = 0.36) and DMEA (R_F = 0.59).

Group 3. The third group of organisms, consisting of *Agrobacterium radiobacter, A. rhizogenes, C. butyricum*, and *P. vulgaris*, incorporated considerable radioactivity from methyl-labeled methionine into their lipids. In each case, radioactivity was found in both the petroleum ether-soluble and the water-soluble fractions of the lipid hydrolysates.

Identification of bases in lipids of organisms in group 3. The identification of MEA in *C. butyricum* (ATCC 6015) lipids has been described (Goldfine, 1962). The lipids synthesized by four other clostridia incorporated little or no C^{14} from methyl-labeled methionine into the water-soluble fraction (Table 2). *C. butyricum* (ATCC 8260), like the previously examined strain, incorporated radioactivity into MEA. This was revealed by chromatography on paper in solvent 1, followed by radioautography (Goldfine, 1962). No incorporation into DMEA or choline was observed in either strain of *C. butyricum.*

The labeled base in *P. vulgaris* lipids was also identified as MEA. The water-soluble lipid hydrolysis products were chromatographed on paper in solvents 1, 2, and 3 (Goldfine, 1962). In addition, they were chromatographed on paper in *n*-butanol–acetic acid–water (60:15:25). In all cases, the only radioactive spot migrated with the same R_F as did MEA hydrochloride.

Furthermore, the unknown base (20,000 counts per min) from *P. vulgaris* lipids was added to 100 μmoles of carrier MEA. The MEA was converted to choline by reaction with methyl iodide in basic solution (Bremer, Figard, and Greenberg, 1960). Choline was precipitated as the reineckate, and was recrystallized three times to constant specific activity (predicted, 557 counts per min per mg; found, 560 counts per min per mg).

The labeled bases in *A. radiobacter* and *A. rhizogenes* lipids were identified as choline, DMEA, and MEA. The radioactive compounds migrated with the same R_F as did standard salts of these compounds in solvents 1, 2, and 3 (Goldfine, 1962). DMEA was not well separated

TABLE 2. *Incorporation into lipids of C^{14} from methyl-labeled methionine*

Organism	Incorporation of C^{14} into total lipids (disintegrations per min)	Hydrolysis products		
		C^{14} in petroleum ether extract (disintegrations per min)	C^{14} in aqueous phase (disintegrations per min)	Recovery
				%
Aerobacter aerogenes	2,335,000	2,120,000	10,200	91
Agrobacterium radiobacter	4,750,000	3,530,000	1,230,000	100
A. rhizogenes	5,720,000	4,600,000	1,470,000	106
Alcaligenes faecalis	212,000	152,000	36,400	89
Bacillus megaterium KM	242,000	228,000	10,200	98
B. subtilis		13,100	4,000	—
Clostridium acetobutylicum	80,000	75,000	7,800	103
C. butyricum	107,000	76,000	43,000	111
C. histolyticum	2,000	1,310	320	82
C. propionicum	4,800	3,000	1,500	94
C. tetanomorphum	27,000	22,500	4,500	100
Lactobacillus casei	315,000	305,000	6,200	99
L. plantarum	13,200	11,300	800	92
Micrococcus lysodeikticus	21,500	9,000	9,000	84
Mycobacterium phlei	4,970,000	2,320,000	857,000	64
Proteus vulgaris	5,320,000	4,240,000	483,000	89
Pseudomonas aeruginosa	2,490,000	2,400,000	63,000	99
Sarcina lutea	3,600	1,900	3,300	144
Serratia marcescens	3,945,000	3,630,000	15,000	93
Staphylococcus albus	10,800	7,900	6,500	133
Streptococcus B	14,400	15,000	1,900	117

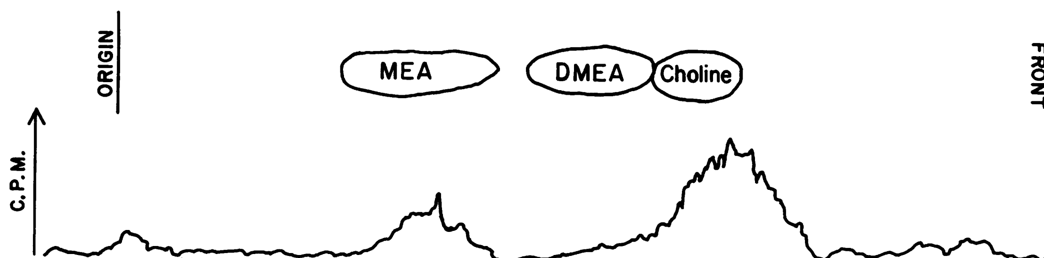

FIG. 1. *Radioactivity in bases derived from Agrobacterium radiobacter phospholipids. The location of the standards has been traced from the same chromatogram.*

in solvents 2 and 3, but could be distinguished in solvent 1.

A tracing of the radioactivity in the bases from *A. radiobacter* is shown in Fig. 1. Most of the C^{14} was in choline. The radioactivity in DMEA was not well resolved from that in choline in this tracing; however, it was shown to be radioactive after the lipids were separated on thin layers of silica gel and hydrolyzed as described below. These results are essentially identical with the findings of Law et al. (1963) on *A. tumefaciens* lipids.

Thin-layer chromatography of agrobacteria lipids. The phospholipids of both species of agrobacteria were resolved into three major bands which accounted for 80 to 90% of the total phosphorus. The major phospholipid band, which contained 65 and 49% of the total phosphorus in *A. rhizogenes* and *A. radiobacter*, respectively, migrated with the same R_F as did synthetic phosphatidyl ethanolamine (unknowns and standards, R_F, 0.43 ± 0.03). However, hydrolysis of the lipids, in this band after elution from silica gel, revealed that the major ninhydrin-

TABLE 3. *Incorporation of C^{14}-methyl methionine into trichloroacetic acid-insoluble fraction of cells*

Organism	C^{14} in lipids (disintegrations per min)	C^{14} in trichloroacetic acid-insoluble fraction (disintegrations per min)
Bacillus subtilis	17,000	710,000
Clostridium propionicum	4,800	2,260,000
Staphylococcus albus	10,800	895,000

positive materials in the water-soluble products were ethanolamine and MEA in both cases. (Work in this laboratory with phosphatidyl ethanolamine and phosphatidyl *N*-methylethanolamine, derived from *C. butyricum*, shows that these lipids cannot be resolved by thin-layer chromatography in this solvent system.) No radioactivity in choline or DMEA was detectable in the hydrolysates of the lipids in this band. Most of the radioactivity was localized in the MEA spots.

The band migrating slightly ahead of the major phospholipid band (R_F, 0.57) had 20% of the total phospholipid phosphorus. On chromatography of the water-soluble hydrolysis products on paper in solvent 1 (Goldfine, 1962), most of the radioactivity stayed near the origin, but some of it migrated with the same R_F as did DMEA hydrochloride.

The third band had the same R_F as did synthetic lecithin (R_F, 0.31 $\pm$ 0.02). It contained 8% of the phospholipid phosphorus from *A. rhizogenes* and 14% from *A. radiobacter*. On hydrolysis, all of the iodine-positive material migrated with choline chloride on paper (solvent 1), as did almost all of the radioactive material.

Incorporation of C^{14} from methyl-labeled methionine into the trichloroacetic acid-insoluble fraction. Although it is evident that methyl-labeled methionine was utilized for lipid synthesis in more than half of the organisms studied, the lack of incorporation into lipids in a number of organisms presented the possibility that these cells were unable to utilize exogenous methionine. Cells of three such species, which had been extracted with chloroform-methanol, were extracted twice at 0 to 2 C with 5 ml of 10% trichloroacetic acid. The residue was resuspended in 10% trichloroacetic acid, and a portion was filtered through a Millipore filter. The residues on the filters were counted with a thin-window Geiger tube (Table 3).

It is evident that these species incorporated C^{14} from methyl-labeled methionine into nonlipid, macromolecular fractions, presumably protein, nucleic acids, or both. Thus, lack of incorporation into lipids is not due to impermeability of these cells to methionine.

DISCUSSION

A number of reports on the presence of lecithin in bacteria do not provide satisfactory evidence for the synthesis of lecithin by these organisms. This is particularly the case when the organisms had been grown on media derived from animal tissues or yeast, which may contain choline and lecithin, and when the amount of choline found in the bacterial lipids represents only a small fraction of the total lipid nitrogen. Crowder and Anderson (1934) recognized this problem in their study of the lipids of *L. acidophilus*.

Choline was identified in hydrolysates of *A. tumefaciens* lipids by Geiger and Anderson (1939), and the presence of lecithin was confirmed by Kaneshiro and Marr (1962), who reported that this fraction constitutes 14% of the phospholipid of this organism. We have extended this finding to two other species of *Agrobacterium*: *A. radiobacter* and *A. rhizogenes*. The phospholipids of both of these species contain approximately 10% lecithin. None of the 19 other species of bacteria examined by us provided evidence of the *de novo* synthesis of lecithin, signaled by the incorporation of the methyl group of methionine into a choline moiety of phospholipids.

It is possible that a number of these organisms synthesize lecithin by pathways which do not utilize exogenous methionine as a methyl donor. To test this possibility, we have examined, in three organisms, the nonradioactive, water-soluble products of lipid hydrolysis. Paper chromatography, followed by staining with iodine vapor (Brante, 1949) and Dragendorff's reagent (Block, Durrum, and Zweig, 1958), revealed no trace of MEA, DMEA, or choline in the lipid hydrolysates of *Pseudomonas aeruginosa* and *C. acetobutylicum*. The lipids of *Alcaligenes faecalis* yielded barely detectable traces of DMEA and choline, which were not radioactive. It is worthwhile to note that this organism was grown in a rich medium.

Further evidence is provided by the published

analyses of the phospholipids of a number of organisms examined by us: *L. casei* and *L. plantarum* (Ikawa, 1963), *M. lysodeikticus*, (Macfarlane, 1961), *B. megaterium* (Yudkin, 1962), and *A. faecalis* (Saito and Akashi, 1957). All these analyses indicated the absence of lecithin in the phospholipids, in agreement with our finding that the methyl group of methionine is not incorporated into any *N*-methylated ethanolamine derivative in the lipids of these organisms.

The presence of phospholipids containing MEA in *C. butyricum* led us originally to consider the possibility that a number of bacteria may be found which cannot convert phospholipid ethanolamine to choline, but which can synthesize MEA- and DMEA-containing phospholipids. Our results indicate that this is generally not the case. [It should be noted that most of the organisms studied are in the order Eubacteriales. No generalizations can be made at this time about the order Pseudomonadales. The lipids of the mycobacteria have been studied in detail and they do not contain lecithin (Asselineau, 1962).] Only one other organism, *P. vulgaris*, was found which synthesizes phospholipids containing MEA, but not lecithin. In addition, as Law et al. (1963) and we have shown, MEA and DMEA, in addition to choline, can be found in hydrolysates of agrobacterial phospholipids. Phosphatidyl-MEA and phosphatidyl-DMEA have been implicated as precursors of lecithin by the in vitro studies of Bremer et al. (1960), and by Hall and Nyc (1961), who found that these lipids accumulate in *Neurospora* mutants which are unable to synthesize lecithin.

Our results lead to the conclusion that the incorporation of the methyl group of methionine into water-soluble moieties obtained on hydrolysis of bacterial lipids may be considered as necessary but not sufficient evidence for the presence of *N*-methylated ethanolamine derivatives. All five organisms studied by us and by Law et al. (1963), which have more than trace amounts of these bases in their phospholipids, incorporate the methyl group of methionine into these moieties. On the other hand, the present study shows that a number of organisms, e.g., *M. phlei*, *Pseudomonas aeruginosa*, and *A. faecalis*, which incorporate the labeled methyl group into water-soluble lipid moieties, have incorporated it entirely into compounds other than MEA, DMEA, or choline. The heavily labeled water-soluble product(s) obtained from *M. phlei* lipids may be derived from mycosides, which contain sugars with *O*-methyl groups, and which have been found in a number of mycobacteria (reviewed by Asselineau, 1962).

Although an examination of methyl groups in fatty acids was not the object of this study, a word should be said about the observed incorporation of C^{14} from the methyl group of methionine into the petroleum ether-soluble lipid hydrolysis products. The significant levels of incorporation seen in a number of organisms can be attributed to either the formation of cyclopropane rings in fatty acids (O'Leary, 1959; Liu and Hofmann, 1962), or the presence of branched-chain fatty acids in which the branched methyl group arises from methionine (Lennarz, Scheuerbrandt, and Bloch, 1962).

The presence of cyclopropane acids in lactobacilli, agrobacteria (Hofmann et al., 1955), *C. butyricum* (Goldfine and Bloch, 1961), *S. marcescens* (Law et al., 1963), streptococci (MacLeod and Brown, 1963) and *Aerobacter aerogenes* (O'Leary, 1962), and branched methyl groups in *M. phlei* (Lennarz et al., 1962) has been established. Uncharacterized branched-chain fatty acids have been found in *Pseudomonas aeruginosa* (James and Martin, 1956). The radioactivity observed in the petroleum ether-soluble fractions obtained from *A. faecalis*, *C. acetobutylicum*, and *P. vulgaris* should be investigated further, in view of the probable occurrence of branched-chain or cyclopropane fatty acids in these lipid extracts.

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