explained by a readier access of choline to the enzyme *in vitro*.

It has always been difficult to accept that the toxicity of HC3 is due to effects on the central nervous system (Schueler, 1955; Kasé & Borison, 1958) because it is unlikely that a bisquaternary ammonium compound of this type would penetrate sufficiently. Recently, evidence has appeared suggesting that HC3 interrupts neuromuscular transmission in the respiratory musculature (Longo, 1959; Holmes & Wilson, 1960). This effect appears only when the rate of stimulation is high and sustained; this would deplete the acetylcholine in the nerve endings and would aggravate any limitation on its resynthesis. Moreover, the effect was rapidly and readily reversed by choline, which is in keeping with the nature of the inhibition found in the present work.

SUMMARY

1. The inhibition by HC3 of acetylcholine synthesis in minced mouse brain has been confirmed. The inhibition was observed also in minced guinea-pig brain.

2. The synthesis of acetylcholine by homogenates of guinea-pig brain or by particles obtained from them was inhibited by HC3 only if the concentration of choline was low.

3. When these preparations were treated with ether the increased synthesis of acetylcholine that occurred was activated by HC3. This effect was more marked with the cruder preparations.

4. It was concluded that HC3 does not inhibit choline acetylase directly but that it competes with

choline for access to the enzyme through structural barriers in the cell.

5. The relation of these findings *in vitro* to the high toxicity of HC3 is discussed.

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Incorporation of [2-¹⁴C]Mevalonic Acid and [2-¹⁴C]Acetic Acid into Lipids of Mycobacteria

By T. RAMASARMA*

Department of Biochemistry

AND T. RAMAKRISHNAN

Pharmacology Laboratory, Indian Institute of Science, Bangalore 12, India

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The present investigation was started with a view to studying the biosynthesis of vitamin K₂, which is known to occur in mycobacteria (Snow, 1952; Brodie, Davies & Fieser, 1958). Vitamin K₂ isolated from *Mycobacterium tuberculosis* (Brev-

annes) has nine isoprene units in its side chain (Noll, 1958; Noll, Ruegg, Gloor, Ryser & Isler, 1960). Evidence has been presented by Brodie & Ballantine (1960) that vitamin K has an essential role in electron transport and oxidative phosphorylation in preparations from mycobacteria. It is known that animal tissues do not synthesize

* Scientists' Pool Officer of Council of Scientific and Industrial Research, India.

vitamin K. Hence, an understanding of the biosynthetic pathway of vitamin K₂ in *M. tuberculosis* might offer a possibility of finding a suitable inhibitor of one of the steps and thereby act as a therapeutic agent.

[2-¹⁴C]Mevalonic acid, the recognized precursor of isoprenoid compounds in Nature, was found to be incorporated into the lipids of *M. tuberculosis*. Since this organism lacks the other generally occurring isoprenoids, sterols (Anderson, 1932), co-enzyme Q (Lester & Crane, 1959) and possibly also α -tocopherol (Green, Price & Gare, 1959), it was expected that [2-¹⁴C]mevalonate would be exclusively incorporated into vitamin K₂. However, fractionation of these lipids by silicic acid column and paper chromatography revealed that the ¹⁴C-activity appeared in the neutral and esterified lipids, but not significantly in vitamin K₂. A comparative study on the incorporation of [2-¹⁴C]mevalonate and [2-¹⁴C]acetic acid into the lipids of *M. tuberculosis* and *Mycobacterium phlei* will be described in this paper.

MATERIALS AND METHODS

Proportions of solvents in mixtures are given by volume throughout.

Labelled compounds. DL-[2-¹⁴C]Mevalonic acid lactone (15 μ C/mg.) was a gift from Dr O. Isler to Professor P. S. Sarma. Samples were delactonized before use by incubating in 0.05N-NaOH for 15 min. at 37°. Sodium [2-¹⁴C]-acetate (60 μ C/mg.) was purchased from The Radiochemical Centre.

Cultures. Two species of mycobacteria were used: *M. tuberculosis* H₃₇R_v, the human virulent strain, and *M. phlei*, the non-pathogenic strain. These were grown in the synthetic medium of Youmans & Karlson (1947) containing glycerol as the carbon source. The ¹⁴C-labelled compounds were added directly to the medium before sterilization and the bacteria were grown for the desired period (*M. tuberculosis*, 21 days and *M. phlei*, 4 days). No attempt was made to trap the respired CO₂. At the end of the incubation

period, the media containing the bacteria were sterilized and filtered. The bacteria were washed thoroughly with distilled water and dried in a desiccator.

Extraction and fractionation of lipids. The dried bacteria were extracted successively with two 5 ml. portions of acetone, diethyl ether and light petroleum (boiling range 40–60°) and the pooled extract was evaporated. Vitamin K₂ was completely extracted by the acetone treatment. Subsequent extractions with ether and light petroleum removed the bulk of the lipids, but no more vitamin K₂. Further extraction of the cell residue with ethanol-ether (1:1), as used by Jacobsen & Dam (1960) for extracting vitamin K from bacteria, did not remove any more vitamin K₂ or lipids. However, no attempt was made to extract all the conjugated lipids. The lipids were fractionated on silicic acid-Celite columns as described by Jayaraman & Ramasarma (1961), with light petroleum, ether-light petroleum (1:9), ether-light petroleum (1:4) and ethanol successively as eluents. The ether-light petroleum (1:9) fraction, which contains vitamin K₂ and also some glycerides, was rechromatographed on a reverse-phase paper system (Lester & Ramasarma, 1959) and vitamin K₂ was detected by the modified leucomethylene blue spray (Jayaraman & Ramasarma, 1961).

Utilization of [2-¹⁴C]mevalonic acid in vitro. The reaction mixture consisted of mevalonic acid (2.0 μ moles; 30 000 counts/min.), ATP (5 μ moles), MgSO₄ (5 μ moles), ascorbic acid (10 μ moles), phosphate buffer (40 μ moles; pH 7.2), menadione (1.0 μ mole) and *M. tuberculosis* extract (1.6 mg. of protein) in a total volume of 0.8 ml. After incubation at 37° for 3 hr., the reaction was stopped by heating at 100° for 2 min. and the lipids were extracted by shaking twice with a mixture of 4 ml. of light petroleum and 1 ml. of ethanol. The aqueous layer was evaporated and the phosphorylated intermediates were separated by paper chromatography as described by Bloch, Chaykin, Phillips & de Waard (1959), the corresponding spots were cut out and the counts determined.

The cell-free extract was prepared by grinding the harvested cells with double the weight of glass powder, extracting with 0.25M-tris buffer (pH 8.0) and centrifuging down the debris at 10 000g.

Measurement of radioactivity. All the samples were plated on aluminium planchets and counted in a windowless gas-flow counter and the counts were corrected for self-absorption.

Table 1. Incorporation of [2-¹⁴C]mevalonate and [2-¹⁴C]acetate into *Mycobacterium tuberculosis* and *M. phlei*

The cells from 75 ml. and 50 ml. of media for *M. tuberculosis* and *M. phlei* respectively were extracted. The media contained mevalonate (6.0 μ moles for *M. tuberculosis* and 3.0 μ moles for *M. phlei*) or acetate (1.0 μ mole for *M. tuberculosis* and 0.5 μ mole for *M. phlei*).

	<i>M. tuberculosis</i>				<i>M. phlei</i>			
	Mevalonate		Acetate		Mevalonate		Acetate	
	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)
Added to medium	4 390 000	100	2 864 000	100	2 508 500	100	1 000 000	100
Lost from medium	410 000	9.3	1 549 000	54	305 000	12	845 000	84.5
Cell residue	290 000	6.6	704 300	24.5	5 620	0.22	42 580	4.3
Lipids	6 920	0.16	75 950	2.6	670	0.03	6 060	0.6
Weight of dried cells extracted (mg.)	258.0		107.0		7.5		2.0	
Weight of cell residue (mg.)	245.0		100.0		5.2		1.4	
Weight of lipids (mg.)	12.4		6.0		2.0		0.5	

RESULTS

Mevalonate was utilized by *M. tuberculosis* as shown by Table 1. The ^{14}C -activity lost from the medium as well as that incorporated in the cell with mevalonate as substrate was lower than that with acetate. Whether this effect is because of poor utilization of mevalonate or because only one of the isomers of DL-mevalonate serves as the substrate is not clear. A greater part of the ^{14}C -activity was left in the cell residue. This activity may represent some unconverted mevalonate or some of the phosphorylated intermediates of mevalonate metabolism (which are shown to be formed in the experiment *in vitro*, Table 6) or even some water-soluble degradation products of mevalonate within the cells. Further extraction of the cell residue with ethanol-ether (1:1) and chloroform, successively, did not remove any of this activity.

The lipids were fractionated on a silicic acid-Celite column and the distribution of the ^{14}C -activity in the various fractions is shown in Table 2. The recoveries were poor, indicating that some highly polar components not eluted with ethanol also possessed ^{14}C -activity. With *M. tuberculosis*, a majority of mevalonate appears to have been incorporated in the fraction eluted with ether-light petroleum (1:9) consisting of vitamin K_2 , triglycerides and, if any, sterols and mycolic acid. On the other hand, ^{14}C -activity from acetate was incorporated both in the ether-light petroleum (1:9) and the ethanol fractions. The acetate incorporation in the latter fraction can be expected since it consists of phospholipids among other polar compounds. The difference in the pattern of distribution of ^{14}C -activity in ether-light petroleum (1:9) and ethanol fractions indicate that mevalonate was not incorporated via acetate. With *M. phlei* the incorporation of acetate was predominantly in the ether-light petroleum (1:9) and (1:4) fractions but with mevalonate the ^{14}C -activity appeared in the non-polar hydrocarbon fraction representing carotenes known to be present in this organism (Goodwin & Jamikorn, 1956).

The ether-light petroleum (1:9) fraction of *M. tuberculosis* lipids was further fractionated on a second silicic acid column according to the procedure of Noll & Bloch (1955), in which mycolic acid was eluted by benzene-light petroleum (1:1) (Table 3). This fraction was evaporated, taken up in ether and extracted with 10% alkali to remove the free fatty acids. No ^{14}C -activity was found in the free fatty acid fraction.

The fractions from chromatogram I were further separated by the reverse-phase paper chromatography in which the triglycerides remained at the origin and vitamin K_2 moved with R_f about

Table 2. Fractionation of the lipids on silicic acid column (chromatogram I)

Fraction	<i>M. tuberculosis</i>						<i>M. phlei</i>					
	Mevalonate			Acetate			Mevalonate			Acetate		
	(counts/ min.)	(%)	Lipid (mg.)	(%)	(counts/ min.)	(%)	(counts/ min.)	(%)	(counts/ min.)	(%)	(counts/ min.)	(%)
Added	6220	100	10.8	100	68	100	600	100	6060	100	6060	100
Light petroleum	41	0.7	0.4	4	44	0.07	146	24	75	0.1	75	0.1
Ether-light petroleum (1:9)	4022	65	4.6	43	16	250	47	8	1158	19	1158	19
Ether-light petroleum (1:4)	157	2.5	0.6	6	1	550	54	9	599	10	599	10
Ethanol	603	9.7	1.8	17	12	175	*	—	151	2.5	151	2.5

* Less than background (about 20 counts/min.).

Portions of lipids applied on the columns were: samples from *M. tuberculosis*, nine-tenths for both mevalonate and acetate; from *M. phlei*, nine-tenths for mevalonate and entire amount for acetate.

0.15–0.20. All ether–light petroleum (1:9) fractions showed positive spots with leucomethylene blue spray, which occasionally streaked up to R_F 0.40. Table 4 shows that in all the cases the majority of the counts were found in the origin spot and only few in the region of R_F 0.10–0.40 or above. From the activity in the 'vitamin K_2 fraction' in Table 4, taking the amount of sample into con-

sideration, it was calculated that a total of 183 and 2260 counts/min. representing 2.6 and 3.0 % of the counts in the whole lipids were incorporated from mevalonate and acetate respectively. It was thought desirable to determine whether these counts were not because of any contamination since a larger area of paper chromatogram was eluted to include the streaked quinone spots. These

Table 3. *Rechromatography of ether–light petroleum (1:9) fraction of Mycobacterium tuberculosis lipids (chromatogram II)*

The amounts used were one-half and one-fifth of the fractions from chromatogram I for mevalonate and acetate respectively.

Fraction	Mevalonate		Acetate	
	(counts/min.)	(%)	(counts/min.)	(%)
Added	2011	100	3250	100
Light petroleum	*	—	*	—
Benzene–light petroleum (1:1)	800	40	882	27
Benzene–ether (1:1)	485	24	2457	76
Methanol–ether (1:9)	*	—	215	6
Methanol	*	—	*	—

* Less than background (about 20 counts/min.).

Table 4. *Reverse-phase paper chromatography of fractions from chromatogram I*

Portions of fractions from chromatogram I (see Table 2) were applied to the papers. In those from *M. tuberculosis* the amounts of each fraction used were: mevalonate, ether–light petroleum (1:9), one-half; acetate, ether–light petroleum (1:9) and ethanol, one-fifth and one-tenth respectively. The entire fractions from *M. phlei* were applied.

Fraction	<i>M. tuberculosis</i>						<i>M. phlei</i>			
	Mevalonate, ether–light petroleum (1:9)		Acetate, ether–light petroleum (1:9)		Acetate, ethanol		Acetate, ether–light petroleum (1:9)		Acetate, ether–light petroleum (1:4)	
	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)
	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)	(counts/min.)	(%)
Added	2011	100	3250	100	1218	100	1159	100	599	100
Origin spot (R_F 0–0.1)	1311	65	1873	58	297	24	506	44	461	77
Middle spot (R_F 0.1–0.4)	82	4	406	12.5	50	4	86	7	34	6
Top spot (R_F 0.4–1.0)	33	1.3	36	1	183	15	*	—	*	—

* Less than background (about 20 counts/min.).

Table 5. *Saponification of the glyceride fractions from paper chromatogram*

The origin spots (R_F 0–0.1, see Table 4) were eluted and saponified.

Fraction	Counts/min.		Percentage of recovered counts/min.	
	Saponifiable	Unsaponifiable	Saponifiable	Unsaponifiable
<i>M. tuberculosis</i>				
Mevalonate, ether–light petroleum (1:9)	180	408	30	70
Acetate, ether–light petroleum (1:9)	481	493	49	51
Acetate, ethanol	133	91	59	41
<i>M. phlei</i>				
Acetate, ether–light petroleum (1:9)	149	36	81	19
Acetate, ether–light petroleum (1:4)	93	90	51	49

Table 6. Utilization of [2-¹⁴C]mevalonate by *Mycobacterium tuberculosis* in vitro

Details of the reaction mixture, incubation and fractionation procedures are given in the Methods section.

Fraction	Counts/min.
Origin spot	172
<i>R_F</i> 0.25–0.35 spot (pyrophosphomevalonate)	129
<i>R_F</i> 0.50–0.60 spot (5-phosphomevalonate and isopentenyl pyrophosphate)	191
<i>R_F</i> 0.80–0.85 spot (mevalonate)	26 800
Lipids	842

fractions were oxidized with potassium permanganate, the laevulinic acid formed was converted into its dinitrophenylhydrazone, and this was separated along with an authentic sample (Lester, Hatefi, Widmer & Crane, 1959). The hydrazones in either case showed no significant counts, showing that vitamin K₂ had no ¹⁴C-activity.

The glyceride fractions from the paper chromatograms (origin spots, Table 4) were eluted and were saponified with 0.2 ml. of 40 % sodium hydroxide in 4 ml. of ethanol in the presence of pyrogallol for 20 min. at 100°. The ¹⁴C-activity was distributed between the saponifiable and unsaponifiable matter (Table 5). Only for the ether-light petroleum (1:9) fraction of mevalonate-incorporated lipids of *M. tuberculosis* was a greater proportion of the counts recovered in the unsaponifiable matter. The identification of the active component could not be pursued because of the small quantity of material available.

Recently Schiefer & Martius (1960) have demonstrated the synthesis of homologues of vitamin K₂ by isolated ox-heart mitochondria when incubated with 2-methyl-1:4-naphthaquinone and isoprene alcohol pyrophosphates. The formation of 5-phosphomevalonate, 5-pyrophosphomevalonate and isopentenyl pyrophosphate from mevalonate has been demonstrated by Bloch *et al.* (1959). Since mevalonate was utilized by *M. tuberculosis*, the possibility of the formation of the phosphate intermediates and also vitamin K₂ was investigated with a cell-free extract. The ¹⁴C-activity was present in the phosphorylated intermediates although their identification is tentative, based only on the *R_F* values (Table 6). The lipid extract also contained significant ¹⁴C-activity; but none of this appeared in the vitamin K₂ after reverse-phase paper chromatography.

DISCUSSION

Although vitamin K₂ is the only well established isoprenoid compound in *M. tuberculosis*, mevalonate incorporation into it was insignificant. This is

obviously not due to the inability of the cell to utilize mevalonate. The experiment with cell-free extract also showed that mevalonate can be metabolized to form the phosphorylated intermediates. It is possible that mevalonate was rapidly degraded *in vivo* to some intermediate unavailable for vitamin K₂ synthesis. Since acetate was also not incorporated into vitamin K₂, it is difficult to visualize the probable intermediate at the moment. It may be pointed out that Anderson, Norgard & Porter (1960) reported that [2-¹⁴C]-mevalonate served as a precursor for β-carotene in *Blakeslea trispora* but not in *Chlorella pyrenoidosa*, and suggested the possibility of different mechanisms operating for isoprene synthesis in different species.

Most of the ¹⁴C-activity incorporated from mevalonate appeared in the unsaponifiable matter. The active component also appears to be more polar than hydrocarbons in elution properties. A similar incorporation of [2-¹⁴C]mevalonate into some component in the unsaponifiable matter that is not a carotene, is not precipitated by digitonin and does not have the properties of intermediates in the squalene synthesis, has been reported in *Chlorella* by Anderson *et al.* (1960). They suggested that it could be a polyene; the presence of such a substance in *M. tuberculosis* has yet to be established. A carotene, leprestone, similar to β-carotene, has been indicated to be present in a *M. tuberculosis* strain by Takeda & Ohto (1944). Its existence in the organism used by us and also its properties are not known. If the mevalonate was not extensively degraded, other branched methyl compounds present in *M. tuberculosis*, tuberculo-stearic acid, mycocerosic acid and phthiocerol, could also incorporate ¹⁴C-activity from mevalonate since these can be visualized as having isoprene units with the double bond being saturated. Their biosynthesis would be of interest to study in view of their contribution to the characteristic toxicity of this bacillus.

SUMMARY

1. [2-¹⁴C]Mevalonic acid and acetic acid were incorporated into the lipids of *Mycobacterium tuberculosis* and *Mycobacterium phlei*. The ¹⁴C-activity was distributed in saponifiable and unsaponifiable matter.

2. It appears that mevalonic acid did not serve as a precursor of the isoprenoid side chain of vitamin K₂.

3. The majority of ¹⁴C-activity from mevalonic acid was found in an unidentified component of *Mycobacterium tuberculosis* lipids which appears to be more polar than are hydrocarbons in its elution properties.

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Observations on the Effects of Methionine and Homocysteine on Growth of a Cholineless Mutant of *Neurospora crassa*

By F. R. DALAL, D. V. REGE AND A. SREENIVASAN*
Department of Chemical Technology, University of Bombay, India

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Besides choline, *Neurospora crassa* strain no. 34486 (a cholineless mutant) can also utilize for growth monomethylethanolamine and dimethylethanolamine, the immediate precursors of choline (Horowitz, Bonner & Houlahan, 1945); this indicates that the block in synthesis of choline from ethanolamine in this mutant precedes monomethylethanolamine (Horowitz, 1946). Methionine is also active (Horowitz & Beadle, 1943) though at much higher concentrations. It was therefore of interest to determine whether homocysteine could, either partially or totally, replace methionine for growth of the mutant, and whether the addition of C₁ donors or pteroylglutamate, whose derivatives are recognized cofactors in transformylation reactions (Huennekens & Osborn, 1959), or both, facilitated the methylation either of monomethylethanolamine to choline or of homocysteine to methionine.

EXPERIMENTAL

Organism and media. Stock cultures of the cholineless mutant no. 34486 were maintained on agar slants of the following composition (J. F. Nyc, personal communication) in g./l.: potassium tartrate, 5; NaNO₃, 4; KH₂PO₄, 1; MgSO₄·7H₂O, 0.5; NaCl, 0.1; CaCl₂, 0.1; glycerol, 20; hydrolysed casein, 0.25; yeast extract, 5; malt extract, 5; agar, 15. The pH was 5.6–5.8. The basal medium used in

these experiments was the same as that used for choline assay by Horowitz & Beadle (1943).

Growth studies. Growth studies were carried out in 50 ml. Erlenmeyer flasks containing 10 ml. of medium. After autoclaving at 15 lb. for 15 min. the flasks were cooled and inoculated with 0.2 ml. of a spore suspension in sterile distilled water. They were incubated at 30° for 72 hr. except where stated otherwise. The mycelial pads were then removed, pressed out on filter paper, dried at 80° and weighed to the nearest 0.1 mg. Flasks were kept in duplicate and each experiment was repeated at least twice.

[Me-¹⁴C]Methionine uptake during synthesis. In experiments with ¹⁴C additions, the washed mycelial pads were disintegrated in a Potter–Elvehjem glass homogenizer and made up to 6 ml. with distilled water. Portions (2 ml.) were plated on stainless-steel planchets and dried under an infrared lamp, and the radioactivity was determined directly, with the Tracerlab-SC-16 windowless gas-flow counter in conjunction with a Tracerlab-SC-51 autoscaler. Weight of dried mycelial matter on each planchet was determined to the nearest 0.1 mg.

Determination of free sulphhydryl groups. This was by the method of Grunert & Phillips (1951).

RESULTS

Effects of homocysteine on growth. Homocysteine, by itself, failed to replace choline for growth at any concentration from 5 to 400 µg./ml. Addition of DL-serine (50 µg./ml.) or formate (50 µg./ml.) as C₁ donors did not increase growth response over the blank, indicating a lack of methylating ability under the experimental conditions. Addition of

* Present address: Central Food Technological Research Institute, Mysore, India.