Synthesis of Fatty Acids by Extracts of Mycobacteria and the Absence of Inhibition by Isoniazid

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The synthesis of fatty acids by extracts of *Mycobacterium tuberculosis* BCG and *Mycobacterium smegmatis* has been studied and the results are presented in this paper. The findings supplement those obtained with other mycobacteria (Kusunose, Kusunose, Kowa & Yamamura, 1959, 1960; Ebina, Munakata & Motomiya, 1961*b*; Piérard & Goldman, 1963).

The synthesis of some types of lipid in *M. tuberculosis* has been reported to be inhibited by isonicotinic acid hydrazide (isoniazid) (Russe & Barclay, 1955; Ebina, Motomiya, Munakata & Kobuya, 1961*a*; Winder, 1964). It has been suggested that this effect might be due to inhibition by isoniazid of fatty acid synthesis (Ebina *et al.* 1961*b*), but the results in the present paper indicate that this is not the case.

MATERIALS AND METHODS

Growth of bacteria. M. smegmatis was grown on Proskauer & Beck medium under stationary conditions at 37° (Winder & O'Hara, 1962). 'Acetate-grown' bacteria were obtained by replacing half of the glycerol in the medium with an equal weight of sodium acetate. Cells were harvested after 4–5 days' surface growth unless the bacteria were to be exposed to isoniazid, in which case the flasks were transferred after 45 hr. to a rotary shaker (model G25; New Brunswick Scientific Co. Inc., New Brunswick, N.J., U.S.A.), and shaken at 37° at 200 rev./min. (amplitude 1 in.) for a further 3 hr. Isoniazid (10 μg./ml. of medium) was then added to half the cultures. Treated and control bacteria were harvested after suitable incubation periods.

M. tuberculosis var. *bovis*, strain BCG (Glaxo Laboratories Ltd., Greenford, Middlesex), was grown statically for 14 days at 37° on Sauton medium (Soltys, 1952), modified by the addition of casein hydrolysate (Youatt, 1958) and with the pH adjusted to 6·8 with aq. NH_3. However, when the bacteria were to be exposed to isoniazid, after 6–7 days' growth they were placed in the rotary shaker for a further 3–4 days. Isoniazid (10 μg./ml.) was then added to half the cultures. Treated and control bacteria were harvested after suitable periods.

Preparation of cell-free extracts. Bacteria were harvested by filtration, washed with deionized water and sucked dry. Extracts were prepared immediately by either of the two methods given below except in experiments involving the pre-exposure of cells to isoniazid: here each of these samples was further dried *in vacuo* over P_2O_5 and stored at $-20°$ for no longer than 48 hr. Extracts of these cells were prepared by grinding only.

Cells (about 3 g. dry wt.) were suspended in 15 ml. of the appropriate buffer, chilled to 1° and disrupted in a MSE–Mullard 60w ultrasonic disintegrator fitted with a $\frac{3}{8}$ in. diam. probe for 4–5 min.; the temperature was maintained at about 1°. Alternatively, 0·3 g. of Ballotini beads (no. 12) and 0·5 ml. of chilled buffer were added to each 0·05 g. dry wt. of cells, which were then ground vigorously in a chilled pestle and mortar for 4 min. In both cases the disintegrated material was centrifuged at 20 000*g* for 60 min. at 4°. The supernatant was used for all the assays, immediately after preparation. The ultrasonically prepared extract contained 3–10 mg. of protein/ml. and ground extract 1–3 mg./ml.

Assay of acetate kinase. Acetate kinase (ATP–acetate phosphotransferase, EC 2.7.2.1) was determined as described by Rose (1955). The assay mixture contained in a final volume of 3 ml.: 300 μmoles of tris buffer (pH 7·5), 100 μmoles of sodium acetate, 10 μmoles of $MgCl_2$, 800 μmoles of hydroxylamine hydrochloride neutralized with KOH, 10 μmoles of ATP and 1 ml. of ultrasonically prepared extract. Incubation was at 30° for 60 min. One unit of enzyme is defined as that amount which catalyses the formation of 1 μmole of acetylhydroxamate/min.

Assay of phosphate acetyltransferase. Phosphate acetyltransferase (acetyl-CoA–orthophosphate acetyltransferase, EC 2.3.1.8) was assayed by the method of Stadtman (1955), except that the reaction was initiated by the addition of acetyl phosphate and CoA (Dilworth & Kennedy, 1963). The reaction mixture contained in a final volume of 1·6 ml.: 50 μmoles of tris buffer (pH 7·9), 25 μmoles of KCl, 1 μmole of cysteine, 50 μmoles of potassium arsenate, 2 μmoles of acetyl phosphate (dilithium salt), 0·18 μmole of CoA and 1 ml. of ultrasonically prepared extract. Incubation was carried out at 30° for 60 min. One unit of enzyme is defined as that amount which catalyses the disappearance of 1 μmole of acetyl phosphate/min.

Assay of acetyl-coenzyme A carboxylase. Acetyl-CoA carboxylase [acetyl-CoA–CO_2 ligase (ADP), EC 6.4.1.2] was assayed by the method of Waite & Wakil (1962). The reaction mixture contained in a final volume of 1 ml.: 39 μmoles of acetyl-CoA, 1 μmole of ATP, 5 μmoles of $NaH^{14}CO_3$ (1 μc), 31 μmoles of potassium phosphate buffer (pH 6·5), 0·3 μmole of $MnCl_2$, 21 μmoles of isocitrate and 0·2 ml. of extract, prepared by either method. Incubation was at 37° for 15 min. After deproteinization by the addition of 0·1 ml. of 2 N-$HClO_4$ the solution was taken to dryness and counted. Counts were corrected for self-absorption by means of a standard curve. One unit of enzyme is defined as that amount which catalyses the formation of 1 μmole of malonyl-CoA/min.

Assay of incorporation of acetate into lipid. This activity was assayed with the following in a final volume of 1 ml.: 100 μmoles of potassium phosphate buffer (pH 7·1), 0·25 μmole of CoA, 25 μmoles of $KHCO_3$, 3 μmoles of ATP, 0·8 μmole of NADP, 2·5 μmoles of reduced glutathione, 1 μmole of sodium [1-^{14}C]acetate (0·5 μc) and 0·5 ml. of extract. Incubation was carried out in a glass-stoppered tube at 37° for 2 hr. In earlier work, the reaction was stopped by adding 1 ml. of 10% (w/v) KOH in ethanol, then saponified for 30 min. at 80–85° and, after acidification with HCl to pH 2, was extracted with 3 × 5 ml. of light petroleum (b.p. 40–60°) (Piérard & Goldman, 1963). In later work, 2 ml. of 10% KOH in ethanol was used and saponification was carried out by refluxing for 4·5 hr. at 107–110°. The 'non-saponifiable' lipids were extracted with 3 × 5 ml. of diethyl ether, the aqueous residue was adjusted with HCl to pH 2·0 and 'saponifiable' lipids were extracted with 3 × 5 ml. of diethyl ether. Ethereal extracts were evaporated to a small volume under a stream of air and transferred quantitatively to planchets, and the radioactivity was determined. Infinite thinness was assumed. The results were calculated as counts/min./mg. of protein originally present in the reaction mixture. The saponified lipids also were subjected to reversed-phase chromatography with 97% (v/v) acetic acid as solvent (Ballance & Crombie, 1958), the radioactive areas being located by radioautography and fatty acids by the copper acetate–potassium ferrocyanide procedure (Kaufmann & Nitsch, 1954).

Determination of protein. Protein was determined by a modified biuret method (Gornall, Bardawill & David, 1949), with bovine serum albumin (fraction V) as standard.

Chemicals. Acetyl phosphate (dilithium salt), ATP, DL-isocitric acid lactone, reduced glutathione and NADP were from Sigma Chemical Co., St Louis, Mo., U.S.A.; CoA was from Pabst Laboratories, Milwaukee, Wis., U.S.A.; acetyl-CoA was from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; sodium [1-^{14}C]acetate and $NaH^{14}CO_3$ were from The Radiochemical Centre, Amersham, Bucks.; tris and hydroxylamine hydrochloride were from Merck A.-G., Darmstadt, Germany; and L-cysteine was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Other chemicals were of A.R. grade.

Instruments. Extinction values were determined with a Unicam SP. 600 spectrophotometer, and pH determinations were made with a Radiometer pH-meter model 22 (Radiometer, Copenhagen, Denmark). Radioactivity was measured in a Nuclear–Chicago end-window counter standardized by the use of a ^{14}C reference source (The Radiochemical Centre). All samples were counted for a minimum of 5000 counts.

Statistical treatment of results. Where statistical treatment was possible, means ±S.E.M. are given with the numbers of determinations in parentheses.

RESULTS

The specific activity of acetate kinase in crude extracts of *M. smegmatis* is given in Table 1. The addition of CoA to the incubation mixture produced no stimulation of acetate activation, so that there was no evidence of acetyl-CoA synthetase [acetate–CoA ligase (AMP), EC 6.2.1.1] in the extracts. The activity of the enzyme was not affected even by high concentrations of isoniazid.

When an attempt was made to determine the specificity of the preparation the results given in Table 2 were obtained. Although propionate seemed to be activated to a similar extent to acetate, about half of the apparent activation was independent of ATP. Butyrate also seemed to be activated but, when a dialysed cell extract was used, the effect was entirely independent of ATP; the small effect of ATP with the undialysed extract was probably due to a small content of acetate. Thus the extracts

Table 1. *Activities of acetate kinase and phosphate acetyltransferase in ultrasonically prepared extracts of* Mycobacterium smegmatis *and the absence of inhibition by isoniazid*

Acetate-grown bacteria were obtained by replacing half the glycerol in the normal medium with an equal weight of sodium acetate. Assay mixtures were as described in the text. The final concn. of isoniazid, where added, was 250 μg./ml. of assay mixture. Values for phosphate acetyltransferase are the means of duplicate determinations.

Enzyme	Specific activity (milliunits/mg. of protein)	
	Isoniazid absent	Isoniazid present
Acetate kinase (normal bacteria)	3·98±0·01 (3)	4·08±0·20 (3)
Phosphate acetyltransferase (normal bacteria)	1·86	1·83
Phosphate acetyltransferase (acetate-grown bacteria)	4·5	4·5

Table 2. *Hydroxamic acid formation from acetate, propionate and* n*-butyrate in the presence and absence of adenosine triphosphate by ultrasonically prepared extracts of* Mycobacterium smegmatis

The assay mixture contained in a final volume of 1 ml.: 100 μmoles of potassium phosphate buffer (pH 7·1), 400 μmoles of sodium acetate, propionate or butyrate, 4 μmoles of $MnCl_2$, 10 μmoles of ATP, 700 μmoles of neutralized hydroxylamine and 0·5 ml. of fresh extract. Each substrate was tested in a separate experiment.

Substrate	$E_{540\,m\mu}$		
	Acetate	Propionate	*n*-Butyrate
Alterations to assay mixture			
None	0·466	0·796	0·651
ATP omitted	0·050	0·328	0·631
ATP and substrate omitted	0·020	0·017	0·022
Dialysed enzyme	—	—	0·606
Dialysed enzyme; ATP omitted	—	—	0·606
23 μmoles of KF added	—	—	0·022
100 μg. of isoniazid/ml. added	0·466	0·796	0·651

showed kinase activity towards propionate but none towards butyrate.

The apparent activation of propionate and butyrate in the absence of ATP could be accounted for by assuming the presence of an enzyme that catalysed the direct condensation of hydroxylamine with these acids. This enzyme was inhibited by fluoride and was unaffected by dialysis or by isoniazid.

The specific activity of phosphate acetyltransferase in ultrasonically prepared extracts of *M. smegmatis* is shown in Table 1. Activity was found to be greater in acetate-grown cells and was very low in extracts prepared by grinding. It was not inhibited by isoniazid. Sodium ions were inhibitory, since 50 % more activity occurred when potassium arsenate was used instead of sodium arsenate in the assay mixture.

Activity of acetyl-CoA carboxylase was variable in extracts prepared by both methods, but tended to be higher in the extracts prepared by grinding. There was a suggestion from the results that it might be slightly inhibited by a very large concentration of isoniazid (Table 3).

Overall incorporation of acetate by extracts of *M. smegmatis* into lipids extractable with light petroleum (b.p. 40–60°) after saponification by the method of Piérard & Goldman (1963) was not affected even by high concentrations of isoniazid (Table 4). Incorporation by extracts of *M. tuberculosis* appeared even to be slightly stimulated by 10 μg. of isoniazid/ml., though the statistical significance of the effect was marginal (Table 4).

Subsequent investigations showed that the method of Piérard & Goldman (1963) gave incomplete extraction of the radioactive lipids synthesized when cell-free preparations of *M. smegmatis* were incubated with [1-^{14}C]acetate (Table 5). Further, such fraction of the lipid as was rendered extractable by their saponification procedure could be extracted from the alkaline hydrolysate without acidification, indicating that the fatty acids in it still remained esterified. Much more vigorous saponification increased fourfold the total radioactivity extractable, and only about 1 % of this could then be extracted from the alkaline state (Table 5). Hence almost all the lipid synthesized from acetate by these extracts seemed to consist of fatty acids. Similar results were obtained when cell-free preparations of *M. tuberculosis* were used.

The extracted fatty acids produced by *M. smegmatis* were subjected to reversed-phase paper

Table 3. *Activity of acetyl-coenzyme A carboxylase in extracts of* Mycobacterium smegmatis *and the effect of isoniazid*

The assay mixture was as described in the text. The final concn. of isoniazid, where added, was 250 μg./ml. of assay mixture.

Method of preparation of extract	Alterations to assay mixture	Specific activity (microunits/mg. of protein)		Inhibition by isoniazid (%)
		Isoniazid absent	Isoniazid present	
Grinding	None	85	75	12
Ultrasonically	None	27	—	—
Ultrasonically	None	16	—	—
Ultrasonically	Dialysed enzyme	57	33	43
Ultrasonically	Dialysed enzyme; isocitrate omitted	23	22	5

Table 4. *Effect of isoniazid on the incorporation of radioactive acetate into total lipid extracted into light petroleum after saponification according to the method of Piérard & Goldman* (1963)

Extracts of the bacteria were incubated with [1-^{14}C]acetate as described in the text, and the mixtures were then saponified.

Concn. of isoniazid in assay mixture (μg./ml.) ...	Total radioactivity in light-petroleum fraction (counts/min./mg. of protein)					
	0	3·3	6·6	10·0	13·7	137
Source of extract						
M. smegmatis	208±9·8 (4)	—	210±14·7 (4)	—	—	—
	187	222	—	—	—	—
	269	—	—	—	236	—
	173	—	—	—	169	—
	318	—	—	—	—	318
M. tuberculosis BCG	525±13·6 (4)*	—	—	606±31·8 (4)*	—	—

* In this case, isoniazid showed an effect of marginal significance: $0{\cdot}10 > P > 0{\cdot}05$ (t test).

Table 5. *Incorporation of radioactive acetate into lipid by extracts of* Mycobacterium smegmatis: *comparison of two methods of saponification*

Extracts were incubated with [1-^{14}C]acetate as described in the text. The mixtures were then saponified as described below.

Method of saponification	Extracting solvent	Fraction	Radioactivity extracted (counts/min.)
1 ml. of 10% KOH in ethanol for 30 min. at 80–85°	3 × 5 ml. of diethyl ether*	'Non-saponifiable' (extracted before acidification)	1114
	3 × 5 ml. of diethyl ether*	'Saponifiable' (extracted after acidification)	28
2 ml. of 10% KOH in ethanol for 4·5 hr. at 107–110°	3 × 5 ml. of diethyl ether	Non-saponifiable	63
	3 × 5 ml. of diethyl ether	Saponifiable	4986

* Similar results were obtained when 3 × 5 ml. of light petroleum (b.p. 40–60°) was used.

Table 6. *Effect of isoniazid on the incorporation of radioactive acetate into total lipid extracted after vigorous saponification*

Extracts of *M. smegmatis* were incubated with [1-^{14}C]-acetate as described in the text. The mixtures were then saponified with 2 ml. of 10% KOH in ethanol for 4·5 hr. at 107–110°. Diethyl ether (3 × 5 ml.) was used as extracting solvent.

Fraction	Total radioactivity in lipid fraction (counts/min./mg. of protein): Isoniazid absent	Isoniazid present (10 μg./ml.)
Non-saponifiable lipids (extractable from alkaline solution)	21·4	20·2
Saponifiable lipids (extractable only after acidification)	1576	1608
Total lipids	1597	1628

chromatography followed by radioautography, after which the copper acetate–potassium ferrocyanide spray was applied. The radioactive areas were coincident with those spots which reacted with the reagent and corresponded, in R_F values, to a fatty acid of chain length less than C_6, *n*-octanoic acid, palmitic acid (C_{16}), stearic acid (C_{18}) and arachidic acid (C_{20}), together with at least two acids of chain length greater than C_{20} and a substantial amount of material that showed no sign of movement from the origin and could have been due to acids of even greater length.

When the more vigorous method of saponification was used, isoniazid again had no inhibitory effect on the incorporation of acetate into either the saponifiable or the small non-saponifiable lipid fraction (Table 6).

However, for reasons discussed below, it seemed possible that pre-exposure of the cells to isoniazid might result in extracts with a decreased lipid-synthesizing capacity. Hence whole cells were exposed to isoniazid for various periods of time, and the acetate-incorporating activity in cell-free extracts was compared with that in extracts from control cells. The results of typical experiments are shown in Table 7. There was no evidence that isoniazid adversely affected the ability of *M. smegmatis* extracts to incorporate acetate into either the saponifiable or non-saponifiable lipid fractions in less than 24 hr. In fact, there seemed to be a slight stimulation of activity when the period of exposure was between 8 and 20 hr. Similar results were obtained with extracts of cells of *M. tuberculosis* that had also been pre-exposed to isoniazid. Again some stimulation of the incorporation of acetate occurred when cells had been treated with isoniazid for moderately long periods of time.

DISCUSSION

A wide range of specific activities of acetate kinase had been recorded for different micro-organisms, ranging from 0·66 μm-mole/min./mg. of protein (*Clostridium kluyveri*) to 183 μm-moles/min./mg. of protein (*Proteus vulgaris*) (Rose, Grunberg-Manago, Korey & Ochoa, 1954). Our values for *M. smegmatis* fell towards the lower end of this range. Phosphate-acetyltransferase activity also varies considerably from organism to organism (Stadtman, 1955; Dilworth & Kennedy, 1963; Allen, Kellermeyer, Stjerholm & Wood, 1964), and the activity in *M. smegmatis* again is fairly low in this range. The specific activity of acetyl-CoA carboxylase in *M. smegmatis* extracts was close to that in crude extracts of chicken liver (Waite & Wakil, 1962).

The ability of *M. smegmatis* extracts to bring about the direct condensation of hydroxylamine with butyrate and propionate was presumably due to the presence of an esterase. Several types of esterase seem to be capable of bringing about such a

Table 7. *Effect of exposure of cells of* Mycobacterium smegmatis *and* Mycobacterium tuberculosis *to isoniazid on the activity of their extracts in incorporating acetate into lipid*

Extracts were incubated with [1-^{14}C]acetate as described in the text. Saponification was carried out with 2 ml. of 10% KOH in ethanol for 4·5 hr. at 107–110°; non-saponified lipids were extracted with 2 × 5 ml. of diethyl ether before acidification, and saponifiable lipids with 4 × 5 ml. of diethyl ether after acidification.

Fraction	Period of treatment ... Treatment of cells	Total radioactivity in extract (counts/min./mg. of protein)			
		8 hr.	12 hr.	20 hr.	24 hr.
M. smegmatis					
Non-saponifiable	Control	53	52	32	49
	Isoniazid (10 μg./ml.)	83	53	86	22
Saponifiable	Control	1454	1997	1739	2173
	Isoniazid (10 μg./ml.)	1652	3266	2130	1908
Total	Control	1507	2047	1771	2222
	Isoniazid (10 μg./ml.)	1735	3319	2216	1930
		Expt. 1	Expt. 2		
	Period of treatment ...	24 hr.	18 hr.	36 hr.	
M. tuberculosis BCG					
Non-saponifiable	Control	22	113	67	
	Isoniazid (10 μg./ml.)	117	116	110	
Saponifiable	Control	1466	666	961	
	Isoniazid (10 μg./ml.)	2275	577	1295	
Total	Control	1488	779	1028	
	Isoniazid (10 μg./ml.)	2392	693	1405	

condensation of hydroxylamine with fatty acids of various chain lengths (Lipmann & Tuttle, 1950; Hestrin, 1950; Ailhaud, Sarda & Desnuelle, 1962). The reaction catalysed by *M. smegmatis* extracts, like that catalysed by liver esterase (Lipmann & Tuttle, 1950), was inhibited by fluoride.

The range of fatty acids formed from acetate by extracts of *M. smegmatis* and of *M. tuberculosis* BCG was in agreement with the five even-chain acids from C_{16} to C_{24} reported by Kusunose *et al.* (1960) to be formed from acetate by extracts of *M. avium* Takeo. Ebina *et al.* (1961*b*) claimed, in addition, the formation of myristic acid (C_{14}) by the Takeo strain. Extracts of *M. tuberculosis* H37R$_a$ were also reported (Piérard & Goldman, 1963) to form from acetate the C_{16} to C_{24} acids and, in addition, the C_{26} acid was claimed in larger quantities together with moderate amounts of the even longer-chain acids up to C_{32}, though Lederer (1961) has stated that *M. tuberculosis* does not form straight-chain acids of greater length than C_{26}. Only Ebina *et al.* (1961*b*) mentioned the formation of acids of medium chain length such as the octanoic acid observed by us.

Evidence has already been obtained (Piérard & Goldman, 1963; Kusunose *et al.* 1959, 1960) that the 'malonyl-CoA pathway' (Wakil, 1961) is involved in the synthesis of the long-straight-chain acids in mycobacteria, and the findings in the present paper are in keeping with this. However, it is not clear whether this system alone is responsible for all the long-straight-chain acids produced or whether it forms mainly palmitic acid and the longer acids are then produced from this by an 'elongation system' utilizing acetyl-CoA (Wakil, 1961). From observations on other bacteria and on animal cells (Wakil, 1961) it appears likely that the octanoic acid found in our experiments arose through the action of such an 'elongation system'.

In the cases of both mycobacteria studied by us almost all the fatty acids formed were bound to material insoluble in lipid solvents. This is also true of a number of preparations from other organisms (Stumpf, 1961; Tietz, 1961).

In the experiments reported above there was no evidence of any important inhibitory effect of isoniazid either on the enzymes involved in the preliminary activation of acetate for fatty acid synthesis or on the overall incorporation of acetate either into esterified fatty acids or into the small amount of non-saponifiable material formed. This applied equally to extracts of *M. smegmatis* and of *M. tuberculosis*. In fact, there was even a suggestion that isoniazid can slightly stimulate acetate incorporation.

Further, the fact that incubation of cells with isoniazid did not decrease the acetate-incorporating activity of extracts subsequently prepared from them seems to eliminate the possibility that isoniazid, though not itself inhibitory, might be converted by the cells into an inhibitory derivative, or that isoniazid might slowly decrease the intra-

cellular concentration of fatty acid-synthesizing enzymes.

Hence the early inhibitory effect of isoniazid on the synthesis of phospholipids by *M. tuberculosis* (Winder, 1964) apparently must be explained other than by inhibition of the synthesis of straight-chain fatty acids.

SUMMARY

1. The activities of acetate kinase, phosphate acetyltransferase and acetyl-CoA carboxylase in cell-free extracts of *Mycobacterium smegmatis* were measured. Isoniazid had no important inhibitory effect on any of these enzymes.

2. Cell-free extracts of *M. smegmatis* and of *Mycobacterium tuberculosis* BCG incorporated acetate into the following acids: an acid of chain length less than C_6, *n*-octanoic acid, palmitic acid, stearic acid, arachidic acid and at least two longer-chain acids. These acids were esterified and bound to material insoluble in lipid solvents. Vigorous saponification was required to free them.

3. Isoniazid added to cell-free extracts had no inhibitory effect on the incorporation of acetate into lipids. Exposure of the organisms to isoniazid during growth resulted initially in a slight stimulation of the acetate-incorporating activity of extracts.

4. An enzyme present in *M. smegmatis* catalysed the direct condensation of hydroxylamine with butyrate and, to a less extent, with propionate.

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