Inhibition by Isoniazid of Synthesis of Mycolic Acids in *Mycobacterium tuberculosis*

By F. G. WINDER AND P. B. COLLINS

Department of Biochemistry, University of Dublin, Trinity College,
Dublin 2, Ireland

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SUMMARY

Exposure of growing *Mycobacterium tuberculosis* BCG to 1 µg. isoniazid/ml. inhibited the incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]$ and $[\text{2-}^{14}\text{C}]$ glycerol and $[\text{1-}^{14}\text{C}]$ -glutamate into its walls by about 50 % over 12 h. because ^{14}C incorporation into the mycolic acids of the walls was prevented. Isoniazid, 0.5 µg./ml. with *M. tuberculosis* BCG or 0.1 µg./ml. with *M. tuberculosis* H37Ra, inhibited incorporation of ^{14}C from $[\text{U-}^{14}\text{C}]$ glycerol into total mycolic acids by about 90 % over 6 h., indicating that inhibition began within 1 h. of the addition of the drug. There was no effect on mycolic acid synthesis in an isoniazid-resistant strain of *M. tuberculosis* BCG. The primary inhibitory action of isoniazid in sensitive mycobacteria is probably on mycolic acid synthesis, and this leads to formation of defective boundary layers of the bacteria.

INTRODUCTION

A detailed examination of the effects of isonicotinic acid hydrazide (isoniazid) on the carbohydrates of *Mycobacterium tuberculosis* suggested that this drug interferes with the formation of the cell envelope of this organism (Winder & Rooney, 1970). Other lines of evidence, discussed by these authors, support this conclusion. This present paper gives an account of subsequent studies into the inhibition of mycolic acid synthesis by isoniazid. Some of these results have appeared in preliminary form (Winder, Collins & Rooney, 1970).

METHODS

Organisms and growth conditions. *Mycobacterium tuberculosis*, var. *bovis*, strain BCG was obtained from Glaxo Laboratories Ltd, Greenford, Middlesex. The isoniazid-resistant strain was obtained by transfer of a heavy inoculum onto Löwenstein-Jensen medium containing 100 µg. isoniazid/ml., followed by repeated subculture on the same medium. *Mycobacterium tuberculosis*, var. *hominis*, strain H37Ra was obtained from Trudeau Laboratories, Saranac Lake, New York, U.S.A. Both organisms were grown in shaken culture at 37° as previously described (Winder, Brennan & McDonnell, 1967). The medium was based on Sauton medium (Soltys, 1952), but had less glycerol (7.55 g./l.) and asparagine was replaced by 11.2 g. monosodium glutamate/l.

Exposure to ^{14}C glycerol and isoniazid. After about 9 days similar small amounts of $[\text{2-}^{14}\text{C}]$ glycerol or $[\text{U-}^{14}\text{C}]$ glycerol of high specific activity was added to each culture. Isoniazid was simultaneously added to half of the cultures, the remainder serving as controls, and both sets of cultures were harvested after 6 or 12 h. by filtration through

Whatman no. 1 paper in a Büchner funnel. The collected material was washed several times on the filter with 0.9 % (w/v) NaCl, the fluid removed by suction and the bacteria weighed (wet weight).

Preparation of bacterial walls. The bacteria were suspended in 67 mM-phosphate buffer, pH 7.8 (10 g. wet wt of bacteria to 50 ml. of buffer), cooled in ice and treated for 20 min. with a 100 W 20 kHz ultrasonic generator (Soniprobe type 1130A, Dawe Instruments Ltd, London W.3), turned to maximal output. The homogenate was then fractionated as described by Kotani, Kitaura, Hirano & Tanaka (1959) to give a 330g sediment (unbroken bacteria and clumps), a 4200g sediment (crude-wall fraction) and a 4200g supernatant. The crude-wall fraction was treated with trypsin, as described by Kotani *et al.* (1959), after which the residue was freeze-dried. To remove unbound lipids, the walls were then treated for 2 days with chloroform + methanol (2 + 1, v/v) at room temperature, centrifuged after adjustment of the chloroform:methanol ratio to unity, and the residue extracted with ethanol + water (1 + 1, v/v) at 40° for 1 h. The residue of purified cell walls was then dried *in vacuo*. The chloroform + methanol and ethanol + ether extracts were pooled to provide the unbound lipids.

Extraction of mycolic acids from bacterial walls. Purified walls were saponified by refluxing with 2.5 % (w/v) KOH in methanol + benzene (1 + 1, v/v) for 6 h. After removing the insoluble residue by centrifuging, the extract was adjusted to pH 4.0, and mycolic acids (along with other fatty acids and possibly other lipids) were extracted with ether. Mycolic acids were precipitated from the ethereal extract by adding two volumes of ethanol, separated by centrifugation and dried *in vacuo*.

Extraction of mycolic acids and other fatty acids from whole bacteria. The bacteria were extracted three times with ethanol + ether (3 + 1, v/v) at 60° for 10 min. to remove unbound lipids. The extracts were pooled, evaporated to dryness, saponified and fatty acids extracted as described above. The bacterial residue was then saponified, fatty acids extracted and mycolic acids precipitated as described above.

Thin-layer chromatography of mycolic acids. Mycolic acids were converted to their methyl esters by the use of diazomethane (Lipsky & Landowne, 1963). The esters were chromatographed on thin-layer plates of silica gel G (E. Merck, A.-G., Darmstadt, West Germany) using light petroleum (b.p. 40 to 60°) + diethyl ether (9 + 1, v/v) as solvent. Samples of authentic mycolic acid esters (gifts from Dr P. J. Brennan of this Department) were used as standards. Mycolic acid esters and other lipids on the plates were detected by exposing plates to I₂ vapour or by spraying with Rhodamine 6G. The chromatogram of each sample was divided into 2 cm. bands, the gel removed from each band and its radioactivity counted.

Measurement of ¹⁴C. All samples were counted in Bray's solution (Bray, 1960) containing 2 % Cab-O-Sil (Packard Instrument Company) in a Packard 3380 liquid scintillation counter. The results were converted to d.p.m. using the external standard channels ratio method in conjunction with a quench curve prepared with the same scintillation mixture.

Materials. Isotopically labelled compounds were obtained from The Radiochemical Centre, Amersham, Buckinghamshire.

RESULTS

Effects of isoniazid on bacterial wall synthesis

The incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{glycerol}$ into *Mycobacterium tuberculosis* BCG and fractions derived therefrom is shown in Table 1. Over 90% of the ^{14}C taken up was found in the 4200g supernatant and in the 330g sediment. Exposure to isoniazid $1\text{ }\mu\text{g./ml.}$ for the 12 h. uptake period had no appreciable effect on the total uptake of ^{14}C . It had a very slight effect on the relative amounts of radioactivity in the 330g sediment and the 4200g supernatant fluid; this was probably due to a greater fragility of isoniazid-treated bacteria, which was more apparent when shorter periods of disintegration were used (Winder *et al.* 1970). Isoniazid slightly decreased the radioactivity in the crude-wall fraction; when this material was further fractionated, the ^{14}C content of the purified walls was half that of the control while there was little effect on the trypsin extract or the free lipid.

Table 1. *Effect of isoniazid on the incorporation of ^{14}C from $[^{14}\text{C}]\text{glycerol}$ into *Mycobacterium tuberculosis* BCG and its subcellular fractions*

Fifteen 100 ml. cultures were treated with $1\text{ }\mu\text{g.}$ isoniazid/ml. for 12 h. and the same number used as controls. $[2\text{-}^{14}\text{C}]\text{Glycerol}$ ($0\cdot26\text{ }\mu\text{Ci/culture}$) was added to give a final specific activity of approximately 66 d.p.m./ μmole . Yields of bacteria (g. wet wt) were: control, 2.6; isoniazid-treated, 2.3.

Fraction	^{14}C content (d.p.m.)	
	Control	Isoniazid-treated
330g sediment	127,000	111,000
4200g supernatant	189,000	207,000
Wall fractions (4200g sediment)		
Trypsin extract	20,000	17,000
Unbound lipid	4,000	3,800
Purified walls	5,900	3,000
Total in bacteria	346,000	342,000
	Yield (mg. dry wt)	
Purified walls	12.6	14.0
	Specific activity (d.p.m./mg. dry wt)	
Purified walls	468	214

Further experiments confirmed this result. In three separate experiments, the incorporation of ^{14}C from $[2\text{-}^{14}\text{C}]\text{glycerol}$ into purified walls over 12 h. in the presence of $1\text{ }\mu\text{g.}$ isoniazid/ml. was $0\cdot51 \pm 0\cdot03$ (S.E.M.) of the control incorporation, and in two further experiments the incorporation in the presence of $10\text{ }\mu\text{g.}$ isoniazid/ml. was $0\cdot38 \pm 0\cdot08$ of the control. In neither set of experiments did the total incorporation of ^{14}C into the bacteria in the presence of isoniazid differ appreciably from the control value. Thus isoniazid had a specific inhibitory effect on the incorporation of carbon from glycerol into the cell walls of *Mycobacterium tuberculosis* BCG.

In a similar experiment in which $[1\text{-}^{14}\text{C}]\text{-DL-glutamate}$, the other carbon source in the medium, was used at $1\cdot25\text{ }\mu\text{Ci/l.}$ of medium, the carbon from glutamate was incorporated into walls to a smaller extent than carbon from glycerol. This incorporation was reduced by 20% during a 12 h. exposure to $1\text{ }\mu\text{g.}$ isoniazid/ml. Thus isoniazid inhibited the total incorporation of carbon into the bacterial wall.

To identify the wall component whose synthesis was affected by isoniazid, purified

walls from bacteria which had been exposed to [^{14}C]glycerol in the presence and absence of isoniazid were separately saponified and extracted with ether at pH 4.0. Three fractions were obtained: a mycolic acid fraction; an aqueous fraction which contained part of the cell-wall polysaccharide; and a residue mainly of peptidoglycan. Isoniazid strongly inhibited the incorporation of radioactivity into the mycolic acid fraction, had a much smaller effect on incorporation into the aqueous fraction and no appreciable effect on the residue (Table 2). These results suggested that isoniazid had a primary effect on the synthesis of mycolic acid or its incorporation into the wall and that the effect on the polysaccharide fraction was secondary to this.

Table 2. *Effect of isoniazid on the incorporation of ^{14}C from [^{14}C]glycerol into fractions obtained on saponification of purified walls of *Mycobacterium tuberculosis* BCG*

Seventeen 100 ml. cultures were treated with 1 μg . isoniazid/ml. for 12 h. and the same number used as controls. [$2\text{-}^{14}\text{C}$]Glycerol (0.63 $\mu\text{Ci/culture}$) was added to give a final specific activity of approximately 168 d.p.m./ μmole . Yields of bacteria (g. wet wt) were: control, 15.5; isoniazid-treated, 15.4.

Fraction	^{14}C content (d.p.m.)	
	Control	Isoniazid-treated
Purified walls	2,944	1,508
Mycolic acids	768	126
Aqueous fraction (polysaccharide)	1,112	552
Insoluble residue	800	694

Table 3. *Effect of isoniazid on the incorporation of ^{14}C from [^{14}C]glycerol into fatty acids from free and bound lipids of *Mycobacterium tuberculosis* BCG*

Six 100 ml. cultures were used for each treatment (6 h.). [$\text{U-}^{14}\text{C}$]Glycerol (1.25 $\mu\text{Ci/culture}$) was added to give a final specific activity of approximately 333 d.p.m. Yields of bacteria (g. wet wt) were: control, 1.0; with 0.1 μg . isoniazid/ml., 1.2; with 1 μg . isoniazid/ml., 0.9.

Fraction	Control	Isoniazid-treated	
		0.1 $\mu\text{g./ml.}$	1 $\mu\text{g./ml.}$
		^{14}C content (d.p.m.)	
Fatty acids from free lipids	53,470	50,440	63,120
Fatty acids from bound lipids			
Mycolic acids	11,840	9,040	1,112
Other fatty acids	19,902	25,050	13,640
		Yield (mg. dry wt)	
Mycolic acids	16.4	18.0	11.8
		Specific activity (d.p.m./mg.)	
Mycolic acids	722	502	94

Effects of isoniazid on mycolic acid synthesis

To determine whether this effect of isoniazid was on the synthesis of mycolic acid or on its incorporation into the wall, experiments were made in which the total mycolic acid was extracted from *Mycobacterium tuberculosis* BCG which had been exposed to [^{14}C]glycerol for 6 h. with and without isoniazid. The results of a typical experiment (Table 3) showed that isoniazid 0.1 $\mu\text{g./ml.}$ slightly inhibited the synthesis of mycolic acid and that it had a strong inhibitory effect at 1 $\mu\text{g./ml.}$ Other experiments showed

that results with 0.5 $\mu\text{g./ml.}$ were similar to those with 1 $\mu\text{g./ml.}$ Effects on the free lipids, and on the non-mycolic fatty acids and related compounds of the bound lipid fraction were relatively small.

Since incorporation of ^{14}C from $[^{14}\text{C}]\text{glycerol}$ in the medium into the mycolic acid of control bacteria is effectively linear under these experimental conditions for the period involved (F. G. Winder, P. J. Brennan & S. A. Rooney, unpublished observations), the degree of inhibition by 1 $\mu\text{g.}$ isoniazid/ml. of incorporation of ^{14}C into mycolic acid over a 6 h. period indicated that it produced an inhibition of synthesis of mycolic acid between 90 % immediately and 100 % after a delay of less than 1 h. In view of this degree of inhibition, we conclude that the synthesis of all the mycolic acids present in major amount in this organism was affected.

Fig. 1. Thin-layer chromatography of the methylated mycolic acids from the bound lipids of *Mycobacterium tuberculosis* BCG which had been exposed to $[2-^{14}\text{C}]\text{glycerol}$ with and without 1 $\mu\text{g.}$ isoniazid/ml. for 6 h. (a) Distribution of radioactivity in chromatograms of control (—) and isoniazid-treated (---) material. (b) Diagram of spots on plate after exposure to iodine vapour.

Thin-layer chromatography of the methyl mycolates showed that they were contaminated with small amounts of fatty acids, but that most of the radioactivity in the case of mycolic acids from the control cells was associated with the mycolic acid area of the plate (Fig. 1). The amount of radioactivity associated with the mycolic acid area from isoniazid-treated cells was much less than with that from control cells. Separation of different components of the mycolic acid fraction was only slight with the chromatographic system used, but the chromatograms confirmed that the major mycolic acid components were all affected by isoniazid, though there was some suggestion that not all were affected equally.

In contrast to the above results with an isoniazid-sensitive strain of BCG, isoniazid

did not inhibit the incorporation of ^{14}C into the mycolic acids of an isoniazid-resistant strain (Table 4).

When similar experiments were made with isoniazid-sensitive *Mycobacterium tuberculosis* H37Ra, it was found that isoniazid at $0.1\text{ }\mu\text{g./ml.}$ led to almost complete inhibition of mycolic acid synthesis over 6 h. (Table 5).

Table 4. *Effect of isoniazid on the incorporation of ^{14}C from [^{14}C]glycerol into mycolic acids of isoniazid-resistant *Mycobacterium tuberculosis* BCG*

Nine 100 ml. cultures were treated with $1\text{ }\mu\text{g.}$ isoniazid/ml. for 6 h. and the same number used as controls. [$\text{U-}^{14}\text{C}$]Glycerol ($1.25\text{ }\mu\text{Ci/culture}$) was added to give a final specific activity of approximately $333\text{ d.p.m./}\mu\text{mole}$. Yields of bacteria (g. wet wt) were: control, 1.2 ; isoniazid-treated, 1.4 .

	Control	Isoniazid-treated
		d.p.m.
Total ^{14}C mycolic acids	14,108	17,736
		mg. dry wt
Yield of mycolic acids	29.8	34.2
		d.p.m./mg.
Specific activity of mycolic acids	474	519

Table 5. *Effect of isoniazid on the incorporation of ^{14}C from [^{14}C]glycerol into mycolic acids of *Mycobacterium tuberculosis* H37Ra*

Five 100 ml. cultures were treated with $0.1\text{ }\mu\text{g.}$ isoniazid/ml. for 6 h. and the same number used as controls. [$\text{U-}^{14}\text{C}$]Glycerol ($2.5\text{ }\mu\text{Ci/culture}$) was added to give a final specific activity of approximately $666\text{ d.p.m./}\mu\text{mole}$. Yields of bacteria (g. wet wt) were: control, 0.68 ; isoniazid-treated 0.58 .

	Control	Isoniazid-treated
		d.p.m.
Total ^{14}C in mycolic acids	2,660	80
		mg. dry wt
Yield of mycolic acids	11.5	8.2
		d.p.m./mg.
Specific activity of mycolic acids	231	9.7

DISCUSSION

These results show that isoniazid at $0.5\text{ }\mu\text{g./ml.}$ and upwards produced complete, or almost complete, inhibition of synthesis of mycolic acids in 1 h. or less in growing *Mycobacterium tuberculosis* BCG, while total incorporation of carbon into the cells as a whole was substantially unaffected for much longer periods. Isoniazid at $0.1\text{ }\mu\text{g./ml.}$ gave even more rapid and complete inhibition of synthesis of mycolic acids in *M. tuberculosis* H37Ra.

Two questions arise. First, is this inhibition related to the bactericidal action of the drug or is it some completely unrelated phenomenon? The fact that this inhibition occurred to a marked extent in both sensitive strains at isoniazid concentrations close to the minimal inhibitory ones under the conditions of the experiment, but did not occur under similar conditions with an isoniazid-resistant strain, is strong evidence that this effect is related to the bactericidal action of the drug. This conclusion is supported further in that isoniazid also inhibits mycolic acid synthesis in *Mycobacterium*

bacterium smegmatis and *M. phlei* but requires higher concentrations, proportional to their minimal growth inhibitory ones (P. J. Brennan, P. B. Collins & F. G. Winder, unpublished observations).

The second question which arises is, accepting that inhibition of mycolic acid synthesis is related to the bactericidal action of isoniazid, where does it fit into the chain of events involved? We suggest that inhibition of mycolic acid synthesis leads to the formation of envelope material low in mycolic acid and hence defective, that this results in loss of further materials from the boundary layers of the bacteria, and that it leads ultimately to loss of acid-fastness and death. We base this hypothesis partly on evidence in the present paper but mainly on evidence discussed elsewhere (Winder & Rooney, 1970). The inhibition of mycolic acid synthesis is established quickly as compared with the time required for most other observed effects of isoniazid (see Winder 1964; Youatt, 1969), while most such effects can be interpreted in terms of it (Winder & Collins, 1969; Winder & Rooney, 1970). The only effects of isoniazid action, other than inhibition of mycolic acid synthesis, which have been shown to occur within 1 h. of exposure are the uptake of the drug, its metabolism and the formation of pigments (Youatt, 1969); these effects may be involved in its action on mycolic acid synthesis. Hence we suggest that the primary site of the inhibitory action of isoniazid lies somewhere in the biosynthetic pathway to the mycolic acids. Since the biosynthetic pathway to mycolic acids is almost unknown (Étémadi, 1967), we do not suggest which enzyme or enzymes may be sensitive to isoniazid nor a mechanism by which isoniazid acts.

The relatively slight inhibitory effect on the incorporation of radioactivity into the polysaccharide fraction derived from the bacterial walls (Table 2) is presumably an indirect effect of inhibition of mycolic acid synthesis. Some of the polysaccharides in this fraction occur, prior to saponification, as mycolic acid esters (Imaeda, Kanetsuna & Galindo, 1968) and failure of mycolic acid synthesis may result either in an indirect inhibition of synthesis of the polysaccharides or in their elution from the bacterial envelope, a process for which there is evidence (Winder & Rooney, 1970).

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