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Mycolic Acid Synthesis: a Target for Ethionamide in Mycobacteria?

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Striking structural analogies exist between the two specific antimycobacterial drugs ethionamide (ETH) and isoniazid (INH), and they share several inhibitory properties in susceptible species of mycobacteria. The effect of ETH on mycolic acid synthesis was studied in whole cells and in cell extracts of various species, since this synthesis is one direct target for INH, as we recently demonstrated in cell extracts of *Mycobacterium aurum*. It was shown in the present study that there is not a direct relationship between ETH susceptibility and mycolic acid inhibition. This observation could explain the lack of cross-resistance between the two drugs. The presence of ETH disturbed mycolic acid synthesis in both resistant and susceptible mycobacteria. Synthesis of oxygenated species of mycolic acid was inhibited, while that of diunsaturated acids was either slightly altered or even increased. In contrast, INH inhibited the synthesis of all kinds of mycolic acids in the same way in all susceptible strains and had no effect on mycolic acid synthesis in resistant strains. In the presence of ETH, the unsaturated mycolic acid molecules presented a methyl end different from the usual one. These data strongly suggest that the normal unsaturated mycolic acid species are not the precursors of the oxygenated types. Moreover, they show that ETH probably acts early in the pathway leading to oxygenated mycolic acid.

Isoniazid (INH) and ethionamide (ETH) are specific antituberculosis drugs. The mode of action of the former has been reviewed several times (5, 6, 17, 19), while for the latter there are only a few speculative data (19). Mycolic acid synthesis has been proposed as a target of INH action (18), and by using cell extracts (CE) able to synthesize mycolic acids, we have recently demonstrated their direct susceptibility to the drug (14). As mycolic acids [R_1-CH(OH)-CH(R_2)-COOH] represent specific and quantitatively major compounds (ca. 40%) of mycobacterial cell walls, inhibition of their formation may explain, on the one hand, the narrow specificity of INH for mycobacteria and, on the other hand, the death of cells as a consequence of the wall structure damage provoked by the drug.

ETH, α-ethylthioisonicotinamide, presents strong bacteriostatic properties against some mycobacteria and is rather more active against INH-resistant mutants (15). Structurally, it presents striking analogies with INH (Fig. 1), and similar modes of action have been proposed for the two drugs (19). The same effects on acid fastness and respiration were registered (3, 16), and identical decreases in the "alkali-soluble carbohydrates" of the cell envelope were noted (21). In both cases, the bacteria double in number before growth stops (15). Moreover, as observed after INH treatment (20), it was shown on whole ETH-treated cells of *Mycobacterium bovis* (BCG strain) that mycolic acid synthesis is strongly inhibited. However, no cross-resistance between the two drugs could be established (15, 19).

In this report, MICs of ETH were determined for different mycobacterial species containing various types of mycolic acids (unsaturated and oxygenated kinds [2]) and susceptible strains were chosen to analyze the effect of the drug on mycolic acid synthesis, both in whole cells and in CE prepared from nonpathogenic species. A clear-cut relationship was not established between ETH susceptibility and mycolic acid biosynthesis inhibition, as great variability in the behaviors of the different species was observed. Disturbances in oxygenated acid synthesis led us to propose a hypothesis for the mode of action of ETH which may cast some light on the metabolic relationship between different mycolic acid types.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. tuberculosis* CIPT 14 001 0060 (Canetti strain) and *Mycobacterium* sp. strain CR21, a new species from the environment (13), were grown on Sauton medium as a surface pellicle (30 ml per flask) at 37 and 33°C, respectively.

To obtain comparative inocula, the following technique was used (11). Cells from a 2- or 3-week culture were harvested by decantation, and the medium was discarded. After addition of pentane and shaking in the presence of glass beads, the suspension was placed in ice. Equal volumes of the supernatant, free of aggregates, were used as inocula.

M. aurum A+ (from the Institut Pasteur, Paris, France) and type strain ATCC 23366 were grown at 37°C in shaking cultures as previously described (10, 14).

M. fortuitum ATCC 6841 was grown on Sauton medium at 30°C as shaking cultures (G24 rotary incubator; New Brunswick).

MIC determinations and drug treatment of cultures. Growth curves and MICs for *M. tuberculosis* (Canetti strain), *Mycobacterium* sp. strain CR 21, and *M. fortuitum* were established by weighing dry cells after autoclaving, filtration, and drying (under vacuum in a desiccator), while growth curves and MICs for both *M. aurum* strains were determined by A_{650} measurements.

In all of the experiments, ETH was added at the beginning of the exponential phase. Because its solubility in water is very low, stock solutions were prepared in dimethylformamide (DMF) after checking that up to 0.2% (vol/vol) DMF

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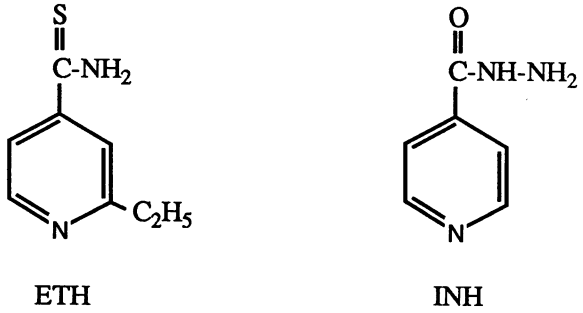

ETH INH

FIG. 1. Structures of ETH and INH.

had no effect on the cell growth of these bacteria. We sterilized solutions by passing them through a 0.22-μm-pore-size filter membrane (Millex GS; Millipore). Adequate quantities of ETH were directly added from the stock solution to the shaking cultures. For pellicle cultures, the drug was first diluted into 5 ml of Sauton medium for better spreading. The MIC was defined as the lowest drug concentration able to stop cell growth definitively after 1 doubling time.

Drug effect on mycolic acid synthesis by whole cells. The bacteria studied in this work were either slowly growing, such as *M. tuberculosis* Canetti and *Mycobacterium* sp. strain CR21, or fast growing, like *M. aurum* (two strains). The experimental conditions used to study acetate incorporation into mycolic acids of whole cells were therefore adapted to each case. Different researchers have noted (15, 19) that differences in contact time in the presence of a given concentration of the drug were more important than the actual drug concentration and produced more pronounced effects on bacterial metabolism. Various incubation times in the presence of the drug were analyzed for *M. aurum* A+ and *Mycobacterium* sp. strain CR21, which both presented a high susceptibility to ETH (MIC, 3 μg/ml for each) but for which the inhibitory effect on mycolic acid synthesis seemed to be weaker than for *M. tuberculosis*. As already pointed out (10), acetate must be added during the exponential phase to be incorporated. Various quantities of ETH were introduced into cultures at the beginning of the exponential growth phase, and after various times of contact, [1-^{14}C]acetate (2 nmol/ml; specific activity, 1.9 GBq/mmol) was added. The reaction was stopped after various times by adding HCl (final concentration, 0.1 M). Table 1 summarizes the exact experimental conditions.

After collection by decantation (for *M. tuberculosis* and *Mycobacterium* sp. strain CR21) or by centrifugation (*M. aurum*), cell pellets were saponified and fatty acid extraction was carried out as already described (10, 14).

CE. CE were prepared from the two strains of *M. aurum* by breaking cells twice in a cold French pressure cell as previously described (8, 14) and using the "fluffy layer" as the coarse enzyme fraction.

The CE was preincubated for various times (as specified in Table 4 and Fig. 2) in the presence of increasing concentrations of ETH. The incubation media used for the two strains of *M. aurum* have already been described (8, 14).

In all of the experiments, the ETH solution was prepared in DMF, and it was verified that up to 0.13% (vol/vol) the solvent had no effect on CE activity.

CE prepared from ETH-treated bacteria. A mid-log-phase (40-h) *M. aurum* A+ culture was treated for 8 h with 3 μg of ETH per ml, and the CE was prepared and washed twice by centrifugation (40,000 × *g*) to eliminate free ETH, as previously described for INH (14). A control was submitted to the same protocol without ETH treatment.

Lipid extraction. Lipids were extracted by diethyl ether after saponification and analyzed by radioscanner on thin-layer chromatography plates and by radio-gas chromatography. Total radioactivities were determined by scintillation counting of aliquots as previously described (8).

Oxidative and pyrolytic cleavages of unsaturated mycolic acids. Oxidative and then pyrolytic cleavages of unsaturated mycolic acids were performed, as previously described (9), after isolation of the acids on preparative thin-layer chromatography plates. After purification, the fragments were further analyzed by radio-gas chromatography and pyrolytic radio-gas chromatography.

Chemicals. ETH was obtained from Sigma. [1-^{14}C]acetate was from Amersham.

RESULTS

MIC determinations and selection of ETH-susceptible species. It is difficult to find reliable data in the literature concerning MICs, because experimental conditions are so different that it could be erroneous to compare published values. Therefore, MICs were evaluated under the conditions described in Materials and Methods, with species selected to have most of the known mycolic acid molecular types (2). Table 2 presents the INH and ETH MICs for slowly and fast-growing species of mycobacteria. It must be noted that susceptibilities to INH and ETH were close and that ETH-susceptible species were also susceptible to INH.

TABLE 1. Experimental conditions used to study mycolic acid synthesis with whole cells of various strains of mycobacteria

Organism	Doubling time (h)	Preincubation time(s) (h) with ETH (in DMF)	Incubation time (h) with [1-^{14}C]acetate
M. tuberculosis Canetti[a]	114	6	24
Mycobacterium sp. strain CR21[a]	72	6, 24	24
M. aurum strains			
A+[b]	21	1, 8	0.5
ATCC 23366[b]	8	0.5	0.5

[a] ETH was added in DMF after dilution in Sauton medium for pellicle cultures.
[b] ETH was added in DMF directly.

TABLE 2. Comparison of ETH and INH MICs for slowly and fast-growing species of mycobacteria[a]

Drug	MIC (μg/ml)				
	Slowly growing species[b]		Fast-growing species[c]		
	M. tuberculosis Canetti	*Mycobacterium* sp. strain CR21	*M. fortuitum*	*M. aurum*	
				A+	ATCC 23366
ETH	0.5	3	>250	3	60
INH	ND[d]	1	ND	1	>300

[a] See Materials and Methods for experimental conditions.
[b] ETH was added in DMF after dilution in Sauton medium for pellicle cultures.
[c] ETH was added in DMF directly.
[d] ND, not done.

TABLE 3. Influence of ETH on labelling distribution between different types of mycolic acid synthesized by whole cells of *M. aurum* A+

ETH concn (μg/ml)[a]	Inhibition of total mycolic acids (%)	Mycolic acids (relative % of total mycolic acids)[b]	
		Oxygenated	Unsaturated
0	0	68 ± 4	32 ± 4
3	7	70.5	29.5
10	28	64 ± 3	36 ± 3
100	72	53 ± 2	47 ± 2

[a] Preincubation time in the presence of ETH, 1 h; labelling time, 30 min.
[b] Relative percentages of oxygenated and unsaturated mycolic acids are means of several experiments, expressed with standard deviations.

TABLE 4. Comparison of ETH effects on inhibition levels and on labelling distribution between oxygenated (oxo and dicarboxylic esters) and unsaturated mycolic acids of both strains of *M. aurum* in whole cells and CE

M. aurum strain and portion tested	Inhibition of total mycolic acids (%)	Relative labelling distribution in mycolic acid types (%)[a]			
		Control		ETH[b]	
		Oxygenated	Unsaturated	Oxygenated	Unsaturated
A+					
Whole cells	72	68 ± 4	32 ± 4	53 ± 2	47 ± 2
CE	71	63 ± 3	37 ± 3	51.5 ± 3	48.5 ± 3
ATCC 23366					
Whole cells	15	75 ± 3	25 ± 3	35 ± 2	65 ± 2
CE	15	40 ± 2	60 ± 2	10 ± 4	90 ± 3

[a] Results are means ± standard deviations of several experiments.
[b] ETH concentrations applied to whole cells were 100 μg/ml (1 h) for *M. aurum* A+ and 60 μg/ml (30 min) for *M. aurum* ATCC 23366. In CE, concentrations 30 times the MIC (100 μg/ml) (preincubation time, 90 min; labelling time, 90 min) and 15 times the MIC (850 μg/ml) (preincubation time, 60 min; labelling time, 60 min) were used for strains A+ and ATCC 23366, respectively.

The assertion that INH-resistant species are more susceptible to ETH (15) was not confirmed. It is worth noting that the same MIC (3 μg/ml) was found for both a slowly growing (*Mycobacterium* sp. strain CR21) and a fast-growing (*M. aurum* A+) species.

M. tuberculosis Canetti, *Mycobacterium* sp. strain CR 21, and *M. aurum* A+ can therefore be considered to have similar susceptibilities to ETH, and they were chosen to study mycolic acid synthesis in whole cells to determine whether this metabolism represents a target for ETH.

Effect of ETH on mycolic acid synthesis in whole cells. Increasing quantities of ETH (in DMF) from 0 to 0.5 μg/ml (MIC) were added to the culture medium and left in contact of cells for different times (Table 1), and then mycolic acid synthesis was monitored by addition of a labelled precursor, [1-^{14}C]acetate, as reported in Table 1. Inhibition of mycolic acid synthesis reached 100% at the MIC in *M. tuberculosis*. These data are in good agreement with previous results obtained with *M. bovis* BCG (20), a very susceptible species close to *M. tuberculosis*. A slight imbalance (from 5 to 10%) between syntheses of unsaturated and oxygenated types of mycolic acids was recorded at lower concentrations (<MIC).

With the two other highly ETH-susceptible bacteria (*M. aurum* A+ and *Mycobacterium* sp. strain CR21), it was confirmed that long contact times in the presence of the same quantity of the drug are more efficient than short ones for inhibition of mycolic acid synthesis. With long preincubation times (Table 1) for *Mycobacterium* sp. strain CR21 (24 h) and *M. aurum* A+ (8 h) which correspond to one-third of their respective doubling times, mycolic acid synthesis inhibition reached 34 and 85%, respectively (at the MIC of 3 μg/ml), whereas with shorter contact times (6 and 1 h [Table 1]) the inhibition level was scarcely significant in either case (about 8%). It must be noted that in *M. aurum* A+ it was possible to reach 75 to 85% inhibition either by using the MIC for a long time or by using a higher ETH dose (100 μg/ml) for a short time (1 h), while in *Mycobacterium* sp. strain CR21 it was necessary to use a high dose (three times the MIC) simultaneously with a long incubation time (24 h) to reach 75% inhibition of mycolic acid synthesis. Such drug concentrations are probably toxic and provoke nonspecific metabolic disturbances. The effect on mycolic acid synthesis, therefore, could be just secondary.

With higher ETH doses, the decrease of oxygenated-type mycolic acids was greater than that of unsaturated acids in *M. aurum* A+ (80 and 58%, respectively, with 100 μg/ml for 1 h [Table 3], as already noted in *M. tuberculosis* subjected to 0.1 μg/ml for 6 h.

In the case of ETH treatment of a resistant strain (*M. aurum* ATCC 23366), total acetate incorporation was only slightly decreased (15%) in ETH-treated cells (at the MIC) but the effect on the imbalance between oxygenated and unsaturated mycolic acids was strongly accentuated: oxo and dicarboxylic mycolic acids almost completely disappeared. In contrast, mycolic acid synthesis was not affected by INH treatment in resistant species (14).

The effect of ETH was examined in the cell-free system synthesizing mycolic acids, since in intact cells too many processes interfere and one can observe only the result of the sum of various activities.

Comparison of ETH and INH effects on CE from ETH-susceptible and -resistant strains of *M. aurum*. CE able to synthesize mycolic acids from acetate as a precursor were previously described for the two *M. aurum* strains (8, 14). INH inhibited all of the kinds of mycolic acid with the CE prepared from susceptible strain A+, while no inhibition was noticed with the CE from the resistant strain (14).

(i) ETH-susceptible strain. In the presence of ETH, an effect similar to that observed with INH was obtained in *M. aurum* A+ CE (14): 71% inhibition of mycolic acids synthesized by the CE from *M. aurum* A+ was reached for an ETH concentration equivalent to 30 times the MIC (i.e., 100 μg/ml) (Table 4). With INH, a value corresponding to 15 times the MIC had to be used with the same CE to reach 90% inhibition of mycolic acid synthesis, and this concentration was considered to correspond to the accumulation of INH inside cells when it is used (at its MIC) on whole bacteria (1).

It must be noted that in the presence of ETH, oxygenated mycolic acid synthesis in the CE was inhibited more (80%) than that of the unsaturated acids (56%) (Table 4), as already mentioned for whole cells.

(ii) ETH-resistant strain. *M. aurum* ATCC 23366 can be considered ETH resistant (MIC, 60 μg/ml), and the cell-free system was also ETH resistant, since at between 60 and 800 μg/ml there was only 15% inhibition of mycolic acid synthesis. However, Table 4 and Fig. 2 show an important modification of mycolic acid distribution, as noted in ETH-treated cells. Figure 2 illustrates the absence of oxygenated mycolic acids after ETH treatment at the MIC (peaks 3 and 4), while

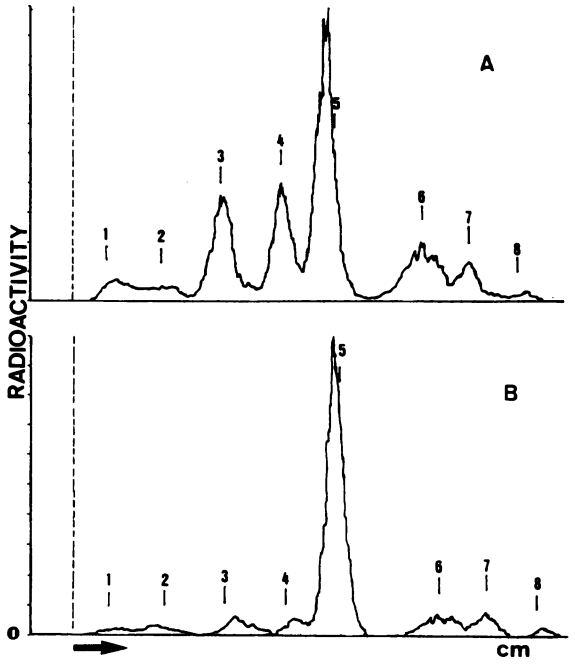

FIG. 2. Profiles of methyl esters on thin-layer chromatography plates (solvent, CH_2Cl_2) after incubation of CE of *M. aurum* ATCC 23366 with [1-^{14}C]acetate. Experimental conditions are given in Materials and Methods and reference 8. Panels: A, standard conditions; B, in the presence of ETH (60 μg/ml). Peaks: 1 and 2, unknown polar compounds; 3, dicarboxylic mycolic ester; 4, oxo-mycolic ester; 5, diunsaturated mycolic ester; 6, nonhydroxylated fatty esters; 7, meromycolic esters; 8, nonpolar compounds (ketones). cm, 20-cm migration.

the unsaturated acid (peak 5) increased, as shown by the total acetate incorporation. This result constitutes a fundamental difference from the action of INH.

Since the length of contact time with ETH seemed to be important for whole cells, different incubation times of the drug with CE were tested. Figure 3 clearly shows the simultaneous increase of unsaturated and decrease of oxygenated mycolic acids, but there is only a little influence of the time of contact between the CE and the drug.

Thus, ETH-resistant and -susceptible strains show similar discrepancies between the drug susceptibilities of oxygenated and unsaturated mycolic acid syntheses.

(iii) Is there any covalent linkage between ETH and the enzymatic system of the susceptible strain? A covalent modification by INH of the cell-free system was demonstrated earlier (14) with *M. aurum* A+, so the presence of a similar effect was checked with ETH. A mid-log-phase *M. aurum* A+ culture was treated with ETH, and a CE was prepared (Materials and Methods). Mycolic acid labelling was then determined, and only 6% inhibition was recorded, demonstrating that ETH was eliminated by the washings and not covalently linked to the enzymatic system. This, then, is another divergence from the mode of action of INH.

(iv) Inhibition of oxygenated mycolic acid synthesis. A possible filiation, as already proposed 30 years ago (4), between the two types of mycolic acids (unsaturated and oxygenated) could explain the simultaneous increase of the former and decrease of the latter in the presence of ETH with subsequent accumulation of the nontransformed unsaturated ones.

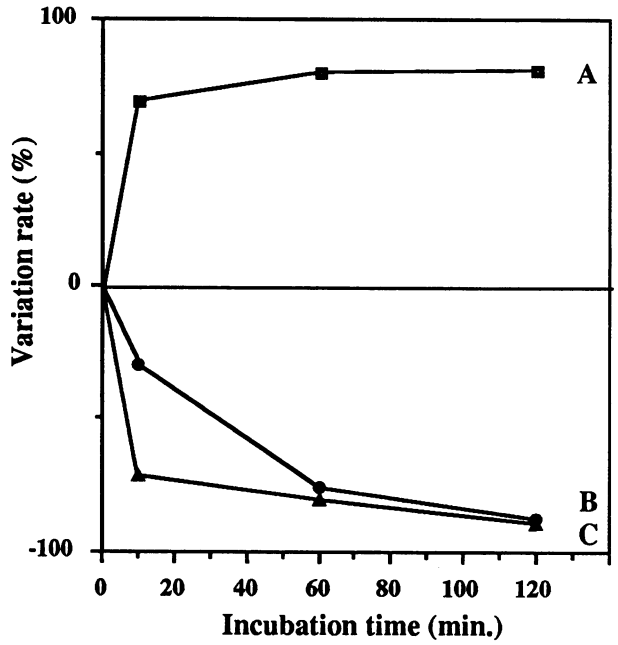

FIG. 3. Influence of time of preincubation of CE with ETH on synthesis of the different kinds of mycolic acid produced by *M. aurum* ATCC 23366. The ETH concentration was 60 μg/ml. A, unsaturated mycolic ester; B, dicarboxylic mycolic ester; C, oxo-mycolic ester.

The diunsaturated mycolic acids synthesized in the presence of ETH in the CE prepared from *M. aurum* ATCC 23366 were structurally analyzed to see whether all of the homologs were identical to those found in the control experiment and to those previously identified (9). After oxidative and pyrolytic cleavages, the fragments present in the extract (Fig. 4) were analyzed as already described (9). By pyrolytic gas chromatographic analysis it was shown (Fig. 5) with whole extracts that the two profiles (in the presence and in the absence of ETH) were different. The mixtures were complex and composed of monoesters (M), diesters (D), aldehyde esters (A), and side-chain esters (C22) (Fig. 4). In the ETH-treated extract, the presence of two major peaks at the positions of C_{16} and C_{18} monoesters (Fig.

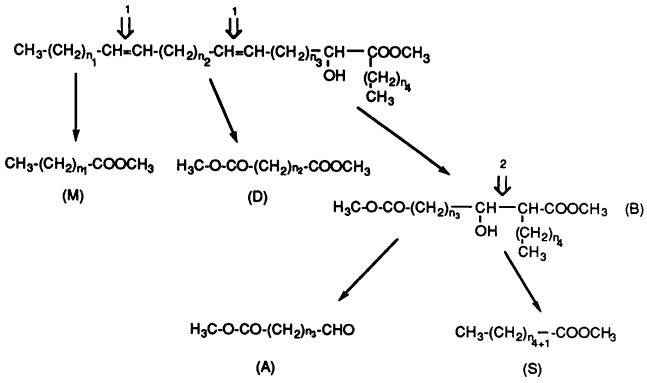

FIG. 4. Schematic representation of oxidative and pyrolytic cleavages of diunsaturated mycolic acids. 1, Oxidation; 2, pyrolysis; M, monoester; D, diester; B, branched ester; A, aldehyde ester; S, side-chain ester.

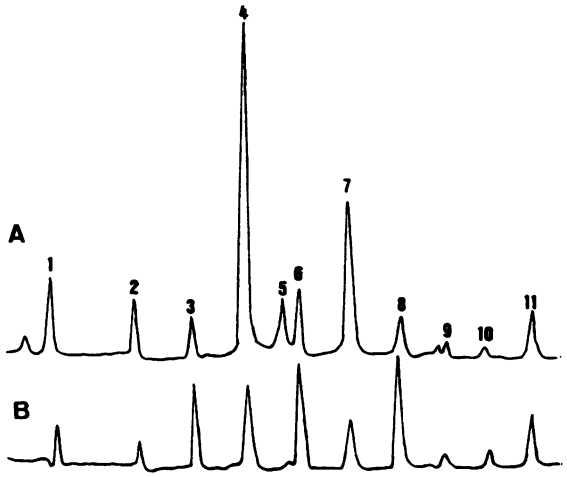

FIG. 5. Comparison of profiles of methyl ester mixtures after oxidative and pyrolytic fragmentation of diunsaturated mycolic ester in the presence or absence of ETH (with CE). (A) With 60 μg of ETH per ml. Peaks: 1, unknown; 2, C_{11} diester and/or C_{14} monoester; 3, C_{12} diester and/or C_{15} monoester; 4, C_{13} diester and/or C_{16} monoester; 5, unknown; 6, C_{17} monoester; 7, C_{15} diester and/or C_{18} monoester; 8, C_{19} monoester, 9 and 10, aldehyde ester (pyrolysis); 11, C_{22} (pyrolysis). (B) Standard conditions.

5A, peaks 4 and 7) was surprising. They were also present in the standard extract in smaller amounts (Fig. 5B), and they were attributed to the C_{13} and C_{15} diesters which were, respectively, superimposed on the C_{16} and C_{18} monoesters. To elucidate whether the two major peaks corresponded to monoesters or diesters, the cleavage fragments (M, D, and B in Fig. 4) were separated on thin-layer chromatography plates, eluted, and separately analyzed by radio-gas chromatography. Figure 6 represents the monoester profile corresponding to fragment M of the molecule: C_{16} and C_{18} monoesters were the major peaks (4 and 7), while C_{15}, C_{17}, and C_{19} (peaks 3, 6, and 8), which were the only entities found in the absence of ETH (9), were still present but in smaller amounts. The other fragments (diesters D and branched esters B) gave profiles similar to that of the control (data not shown). An analogous experiment was performed on mycolic acids from intact cells in the presence of ETH, and the same conclusions were drawn.

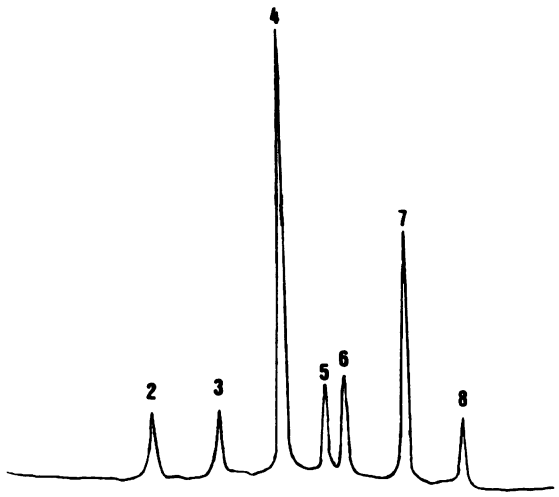

FIG. 6. M (methyl-terminal fragment) monoesters isolated after oxidation cleavage of unsaturated mycolic acid synthesized in the presence of ETH. Peaks: 2, C_{14}; 3, C_{15}; 4, C_{16}; 5, unknown; 6, C_{17}; 7, C_{18}; 8, C_{19}.

Thus, unsaturated mycolic acid molecular species synthesized in the presence of ETH present important differences in their methyl end part. Instead of a methyl-terminal fragment M containing an odd number of C atoms, i.e., 15, 17, or 19, we found even chain lengths of 16 and 18 C atoms, which correspond to the number of carbon atoms present between the methyl group and the oxygenated function in the corresponding mycolic acids. These findings indicate that disturbances provoked by the drug must intervene early in the pathway that leads to oxygenated mycolic acid.

DISCUSSION

A direct relationship between ETH inhibition of growth and mycolic acid synthesis has been postulated by Winder et al. (20).

In the present study, 100% inhibition at the MIC was observed only with *M. tuberculosis* Canetti. In the other highly susceptible species (*M. aurum* A+ and *Mycobacterium* sp. strain CR21), a long preincubation time was necessary to inhibit mycolic acid synthesis. With short times of contact between the drug and the bacteria, the inhibition level was scarcely significant, and this observation is in favor of the importance of factors such as cell wall permeability and transport or chemical transformation of the ETH molecule for its inhibitory activity. So there is no parallel between bacteriostatic activity and mycolic acid inhibition in the presence of ETH that allows the conclusion that this metabolism is not a primordial target of ETH.

A more general effect is the imbalance between the synthesis of unsaturated and oxygenated mycolic acid types: very important in a resistant strain (*M. aurum* ATCC 23366), it remains observable in either whole cells or CE of the other species.

It was previously demonstrated (14) that INH acts at a very early step of mycolic acid synthesis, resulting in complete inhibition of all of the types of mycolic acid synthesized both by whole cells and by the cell-free system from susceptible strains, but it is not possible to go further in the analogy between the effects of INH and ETH, and the latest data explain, to some degree, the lack of cross-resistance between INH and ETH (15, 19).

Structural analysis of the unsaturated types of mycolic acid synthesized either by whole cells or by CE in the presence of ETH has shown important differences in the methyl end part (fraction M in Fig. 4) of the molecule. The presence of these new unsaturated mycolic acid molecular species gave additional experimental support to previous structural and stereochemical analyses (10) and metabolic studies (7) which have cast some doubt over the hypothesis of a possible filiation between the two kinds of mycolic acid. Moreover, according to our findings (12), fragment M constitutes the primer in the metabolic process that leads to mycolic acids and it is not entirely synthesized by the cell-free system. These unusual unsaturated mycolic acids synthesized in the presence of the drug show that oxygenated mycolic acids did not stem from the major unsaturated mycolic acids produced in the absence of ETH, since there was no accumulation of normal unsaturated mycolic acids. Oxygenated acids must derive from a specific precursor which cannot be transformed into oxo compound and gives

unusual unsaturated acids in the presence of ETH. In conclusion, oxygenated and unsaturated mycolic acids could be synthesized from a common precursor by parallel ways, one of which is susceptible to ETH early in the process.

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