Extracellular Acids Produced by Mycobacterium ranae and Mycobacterium tuberculosis H37Rv*

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The subject of extracellular organic acids produced by mycobacteria became of interest more than fifty years ago when Theobald Smith (1) found that human and bovine strains of tubercle bacilli differ in their manner of developing an acid reaction in glycerol broth. Smith (1) assumed that degradation of glycerol to acidic products accounted for increases of acidity in the mycobacterial cultures, but Merrill (2) and most subsequent investigators concluded from their studies of carbohydrate metabolism in mycobacteria that glycerol is completely oxidized to carbon dioxide and water and that intermediates do not accumulate in the culture medium. In more recent years, on the other hand, acetic acid (5), succinic acid (6), malic acid (5, 6) citric acid (6), oxalic acid (5, 5), and pyruvic acid (7) have been demonstrated in culture filtrates derived from various typical virulent and nonvirulent strains of Mycobacterium. These conflicting findings suggest that mycobacteria may produce a wide variety of extracellular acids intermediates, but that the accumulation of these compounds except at relatively low concentrations is precluded by rapid further metabolism. The isolation and identification of such substances would be of considerable interest since it might be anticipated that they would represent not only immediate degradation products of glycerol, but also intermediates in biosynthetic pathways leading to specialized mycobacterial structures such as the unusually branched and hydroxylated lipids. The primary objective of the present investigation was to determine whether methods applied previously to Lactobacillus culture filtrates in this laboratory (9, 10) would yield evidence in support of these views.

EXPERIMENTAL

Cultures of Mycobacterium ranae and Mycobacterium tuberculosis H37Rv* were maintained on Peiser’s egg medium (Difeo)

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1 Detailed discussions with literature references may be found in recent books (3, 4).

2 Succinic, a-ketoglutaric, and pyruvic acids have been demonstrated in culture filtrates of Mycobacterium butyricum, a metabolically anomalous organism (8).

3 Olive View Sanitorium cultures numbered 15 (M. ranae) and 797 (M. tuberculosis H37Rv) obtained through the courtesy of Dr. S. Froman. All work with M. tuberculosis H37Rv up to and including final sterilization of the cultures was carried out at Olive View under Dr. Froman’s supervision. The authors are indebted to Dr. Froman and Olive View Sanitorium for this kind cooperation.

RESULTS AND DISCUSSION

Countercurrent distributions in ethyl acetate over n acetic acid of the extracellular nonvolatile acids produced by M. ranae and M. tuberculosis H37Rv under comparable conditions are described on pages 226 to 232 of Block (13).
shown by Curves R(A) and Tb.(A), respectively, in Fig. 1. These data suggest that largely the same extracellular acids are produced by the two mycobacterial species, although considerable differences in relative proportions of some of the components are readily apparent. That significant changes in composition of the extracellular acid mixtures may have been brought about by autoclaving the cultures before filtration (a necessary precaution with M. tuberculosis H37Rv) is indicated by Curves R(A) and R(N), which represent acids from autoclaved and nonautoclaved cultures, respectively, of M. ranae. These changes do not appear to be of a disturbingly large magnitude, however. It was anticipated that acids with partition ratios either lower than 0.1 or higher than 10 would not be resolved by the countercurrent distribution procedure described under Fig. 1. Resolution of the low partition ratio acids was therefore carried out by paper chromatography. The first distribution peak (cells 0–10) of Curve R(A), Fig. 1, yielded five chromatographically distinct components (RF values: 0.07, 0.12, 0.20, 0.28, 0.38), whereas the first distribution peak (cells 0–4) of Curve Tb.(A) yielded only three components (RF values: 0.07, 0.11, 0.15). The second distribution peak (cells 13–20) of Curve Tb.(A) yielded five components (RF values: 0.07, 0.13, 0.36, 0.55, 0.73), whereas the corresponding region (cells 13–20) of Curve R(A) yielded only four components (RF values: 0.20, 0.38, 0.55, 0.73). These results suggest possible qualitative as well as quantitative differences in the extracellular acids produced by M. ranae and M. tuberculosis H37Rv. Substances with intermediate partition ratios appeared to be resolved adequately by the countercurrent distribution procedure described under Fig. 1. Thus the distribution peak centered near cell 90 in all three curves, Fig. 1, yielded crystalline succinic acid, and that centered near transfer number 150 yielded crystalline fumaric acid. Acetic acid (crystallized as the sodium salt) was similarly obtained from the extracellular acids of both M. ranae and M. tuberculosis H37Rv after countercurrent distribution in equal parts of butanol and heptane over water. dl-5-Carboxymethylhydantoin was crystallized from a fraction of the M. ranae acids after countercurrent distribution in butanol over water. Data concerning the isolation and identification of these mycobacterial products are given in the following paragraphs. Isolation and identification of additional extracellular acids produced by these bacteria are in progress and will be described in future reports.

Succinic Acid—A component with partition ratios approximating 0.02 in diisopropyl ether over water, 0.40 in ethyl acetate over aqueous n acetic acid, and 0.94 in butanol over water was isolated in crystalline form from culture filtrates of both M. ranae and M. tuberculosis H37Rv. It melted at 180.0–182.3°. Authentic succinic acid (Eastman Kodak) melted at 182.3–184.3° and yielded partition ratios identical with those found for the isolated material. A mixture of the authentic sample with the isolated product melted at 181.0–183.0°. Analyses of the isolated material yielded C 40.68%, H 5.23%, and equivalent weight 59.1. The corresponding values calculated for C2H4(COOH)2 are C 40.68%, H 5.12%, and equivalent weight 59.04. The infrared absorption spectra of the mycobacterial products may be seen to correspond to that of authentic succinic acid (Fig. 2). Succinic acid has previously been isolated in crystalline form from culture filtrates of various mycobacteria grown in a glucose-glutamic acid medium (6).

Fumaric Acid—A component with partition ratios approximating 0.27 in diisopropyl ether over water, 1.9 in ethyl acetate over aqueous n acetic acid, and 1.8 in equal mixture of butanol and heptane over water was isolated in crystalline form from culture filtrates of both M. ranae and M. tuberculosis H37Rv. It sublimed at temperatures in excess of 200° and readily re-

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**Fig. 1.** Countercurrent distributions (300 transfers through a 100 cell, 10 ml per phase apparatus in ethyl acetate over n acetic acid) of extracellular acids produced in modified Sauton's medium by M. ranae and M. tuberculosis H37Rv under comparable conditions. Curve R(A) represents 18.2 meq of nonvolatile acids derived from 9.15 liters of autoclaved M. tuberculosis H37Rv culture. Curve R(N) represents 38.8 meq of nonvolatile acids derived from 20.8 liters of autoclaved M. ranae culture. Curve R(N) represents 14.2 meq of nonvolatile acids derived from 8.67 liters of nonautoclaved M. ranae culture. Distributions in cells 0–40 and in withdrawn samples corresponding to transfers 100–120 are plotted separately (left- and right-hand sections, respectively) to allow for the extended vertical scale in these parts of the curves. Vertical scale values represent μeq of titratable acid per sample (cell contents or withdrawn material) per meq of total (nonvolatile) distributed acid.

**Fig. 2.** Infrared absorption spectra of mycobacterial products and corresponding authentic reference compounds in potassium bromide pellet preparations. Spectra designated Mlb and Mrs represent compounds isolated from M. tuberculosis H37Rv and M. ranae culture filtrates, respectively. Those designated Ref represent authentic reference compounds.
duced potassium permanganate in aqueous solution to manganese. Authentic fumaric acid (Eastman Kodak) sublimed in like manner, similarly reduced potassium permanganate, and yielded partition ratios identical with those found for the isolated material. Analyses of the isolated material yielded C 41.16%, H 3.57%, and titration equivalent 58.8. The corresponding values calculated for C\textsubscript{4}H\textsubscript{6}O\textsubscript{4}(COOH)\textsubscript{2} are C 41.39%, H 3.47%, and equivalent weight 58.04. The infrared absorption spectra of the isolated products may be seen to correspond to that of authentic fumaric acid (Fig. 2). Previous reports of fumaric acid as a product of mycobacteria were not followed.

Acetic Acid—A volatile component with partition ratios approximating 0.17 in diisopropyl ether over water and 0.48 in an equal mixture of butanol and heptane over water was isolated in crystalline form as its sodium salt from culture filtrates of both \textit{M. ranae} and \textit{M. tuberculosis} H37Rv. Authentic acetic acid (Baker’s reagent) yielded identical partition ratios, and the infrared absorption spectrum of authentic sodium acetate (Baker’s reagent) corresponded to those of the isolated salts (Fig. 2). Acetic acid has previously been reported in mycobacterial cultures according to a literature review of Lassota et al. (6), but the original paper (5) describing the means by which acetic acid was identified has not as yet been available to the present authors.

\textit{mL-5-Carbomethylhydantoin—}A component with partition ratios of approximately 0.08 in ethyl acetate over aqueous Na acetate and 0.33 in butanol over water was isolated in crystalline form from \textit{M. ranae} culture filtrates. The crystalline material melted at 218.5-219.8° (uncorrected), and its analysis yielded C 38.61%, H 4.40%, N 15.95%, O 40.39% (by difference; tests for S, P, and halides were negative), titration equivalent 161 (pH 7 titration value was 3.84; additional acidic functions were not revealed by back-titrating a solution of the compound in alcoholic sodium hydroxide after heating it for 1 hour under reflux), and specific rotation [α\textsubscript{D} +4° (c = 1 in water). The empirical formula C\textsubscript{6}H\textsubscript{5}O\textsubscript{3}N\textsubscript{2} is indicated by the composition analysis, was matched by that of 5-carboxymethylhydantoin (C\textsubscript{6}H\textsubscript{12}O\textsubscript{4}N\textsubscript{2}), and the infrared absorption spectrum of the isolated acid (Fig. 2) seen to show essentially all of the characteristics of that for L-5-carboxymethylhydantoin given by Lieberman and Kornberg (14). The product of Lieberman and Kornberg, however, was apparently optically pure (specific rotation [α\textsubscript{D} -99°) and was spectrographed in a Nujol suspension (14), whereas the compound produced by \textit{M. ranae} was presumably nearly completely racemic (α\textsubscript{D} +4°) and was spectrographed in a potassium bromide pellet (Fig. 2). These differences evidently accounted for the lack of complete agreement between the spectra of Lieberman’s and Kornberg’s L-5-carboxymethylhydantoin and of the \textit{M. ranae} acid since the latter was identified with synthetic \textit{mL-5-carboxymethylhydantoin (Sigma Chemical Company product) by the following criteria:} (a) both the isolated and synthetic compounds reacted slowly with m-dihydroorotic acid and L-ureidosuccinic acid (14), and it has therefore been supposed to be concerned in the pyrimidine metabolism of that organism. The presence of this substance in mycobacterial culture filtrates suggests that it may have a similar function in mycobacteria.

SUMMARY

Countercurrent distribution and paper chromatography data have revealed that culture filtrates from \textit{Mycobacterium ranae} and \textit{Mycobacterium tuberculosis} H37Rv contain a considerably more complex array of acid products than indicated by previously published results. The same acids in similar proportions seem generally to be produced by the two mycobacterial species, although some marked differences are also apparent. Three of the acids have been isolated in crystalline form from both organisms and unequivocally identified as succinic acid, fumaric acid (not previously identified as a mycobacterial product) and acetic acid (isolated as sodium acetate). A fourth acid isolated in crystalline form from \textit{M. ranae} was identified as \textit{mL-5-carboxymethylhydantoin}. It appears also to occur in culture filtrates from \textit{M. tuberculosis} H37Rv, but does not appear to have been described previously as a mycobacterial product.

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