STUDIES ON THE METABOLISM OF MYCOBACTERIUM TUBERCULOSIS

VII. TERMINAL RESPIRATORY ACTIVITY OF AN AVIRULENT STRAIN OF MYCOBACTERIUM TUBERCULOSIS

IRVING MILLMAN AND GUY P. YOUmans

Department of Bacteriology, Northwestern University Medical School, Chicago, Illinois

Received for publication March 29, 1954

In a previous paper, Holmgren et al. (1954) reported that of the many Krebs’ cycle intermediates and precursors tested citrate and succinate were not oxidized by whole cells of the avirulent H37Ra strain of Mycobacterium tuberculosis var. hominis. Since these compounds are key constituents in either a di- or tri-carboxylic acid cycle, it was decided to reinvestigate the terminal respiratory mechanism of this organism using cell-free extracts.

Geronimus (1949) postulated that all previous failures to demonstrate a tri-carboxylic acid cycle in whole cells of the mycobacteria were due to the impermeable character of the cell membrane to many of the cycle intermediates. He was able to show oxygen uptake with most of these intermediates with cell extracts of Mycobacterium smegmatis, Mycobacterium phlei, Mycobacterium species 607, the bovine strain BCG, and strain H37 of M. tuberculosis var. hominis. Faine et al. (1951) confirmed this work with M. phlei extracts while Blakley (1951) produced some evidence for the existence of a Krebs’ cycle in mycobacteria by demonstrating citrate formation in cellular debris prepared by crushing M. phlei cells after acetone treatment. Citrate was produced anaerobically by condensation of oxalacetate with either acetyl phosphate or acetate and adenine triphosphate. Kusunose et al. (1952) reported the extraction of a number of enzymes related to the tri-carboxylic acid cycle from a strain of Mycobacterium avium. Among those isolated were succinic acid dehydrogenase, fumarase, L-malic acid oxidase, α-ketoglutaric acid dehydrogenase, and citric acid oxidase. Acetone dried powders of this organism were used to demonstrate citric acid synthesis. Sasakawa and Yamamura (1952) and Sasakawa and Fujii (1952) using extracts of this avian strain demonstrated the aerobic breakdown of citric acid and α-ketoglutaric acid in the presence of methylene blue, Mg++, and Mn++, and the decarboxylation of oxalacetic acid activated by Mg++ and Mn++. Yamamura et al. (1952) and Kusunose et al. (1952a) reported the isolation of two enzymes from an avian strain of tubercle bacilli capable of oxidizing lactic acid. One enzyme oxidatively decarboxylated lactic acid by directly taking up oxygen, and the other, a true dehydrogenase, required the presence of methylene blue as a carrier. Andrejew (1952) worked with extracts of sonically ruptured cells of the human avirulent H37Ra strain, the bovine strain BCG, and the avian strain A17 and reported that anaerobic dehydrogenation of succinic acid, as determined by the Thunberg technique, was ten times more intense with the avian than with the H37Ra and BCG strains. Aerobically in the absence of methylene blue the extracts did not stimulate oxygen uptake unless cytochrome C was added. Rosenberg and Andrejew (1952) demonstrated the presence of malic acid dehydrogenase and di-phosphopyridine nucleotide-cytochrome C reductase in strains H37Ra, BCG, and the avian strain A17. This work was done both aerobically in a Warburg vessel and under anaerobic conditions using methylene blue reduction as an indication of enzymatic activity.

In evaluating the available evidence for the existence of a tricarboxylic acid cycle in mammalian tubercle bacilli, it becomes evident that relatively little effort has been made to correlate the utilization of citric acid with the ability to synthesize this material. It is our belief that the existence of this cycle can be established with reasonable certainty only in this way. The work reported here was designed therefore to determine whether the H37Ra strain of M.

1 This investigation was aided, in part, by a research grant from Parke, Davis and Company, Detroit 32, Michigan.
2 Predoctorate Fellow of the U. S. Public Health Service.
*M. tuberculosis* var. *hominis* contains both the enzymatic equipment for full utilization of the Krebs' cycle intermediates and, for the synthesis of citric acid, the starting material in this cycle.

**MATERIALS AND METHODS**

**Organism.** The work was carried out with the avirulent H37Ra strain of *M. tuberculosis* var. *hominis* which was maintained by frequent transfer on the surface of modified Proskauer and Beck medium (Youmans and Karlson, 1947).

**Cell extract.** Organisms were grown on the surface of 400 ml of modified Proskauer and Beck medium contained in one liter Erlenmeyer flasks. Ten 1 liter flasks were required for approximately one g (dry wt) of cell harvest. After 30 days of incubation at 37 C the pellicles were removed by filtration through coarse sintered glass and washed with distilled water. The mass of cells was ground then either in a mortar with 0.01 M phosphate buffer, pH 7.0, to make a thick paste, or in a ball mill for 18 hr. In both cases the temperature of the cell paste was maintained at approximately 5 C with the aid of an ice bath. After grinding, the material was suspended in phosphate buffer, pH 7.0, and centrifuged at 4,000 rpm at 0 C for one hr. The crude supernatants were clear, straw colored liquids with a musty odor and were stored at 5 C until ready for use. Microscopic examination revealed an insignificant amount of whole cells and debris. Except where noted, no extracts stored longer than 5 days were employed. Extracts stored at 5 C for 5 days lost no activity. Nitrogen values were determined by the method of Ma and Zuazaga (1942). For citrate synthesis, two g of washed cell paste made with 0.1 M phosphate buffer, pH 6.4, containing 0.25 M sucrose were ground for 18 hr in a ball mill. The cell mass was suspended then in 60 ml of veronal-HCl buffer, pH 6.4, containing 0.25 M sucrose and centrifuged at 4,000 rpm for one hr at 0 C.

**Respiration.** The experiments were done by the conventional manometric methods at 37 C in air. Each flask contained 1.0 ml of crude bacterial cell extract (1.0 to 1.2 mg nitrogen); 1 mg diphosphopyridine nucleotide, adenosine triphosphate, coenzyme A concentrate (13 Lipmann units); 1 \( \times 10^{-4} \) M methylene blue; 3.3 \( \times 10^{-4} \) M Mg++; 1 \( \times 10^{-4} \) M Mn++; 0.5 ml of the respective substrates (0.25 M to 0.025 M).

1.0 g (dry wt) washed *M. tuberculosis* var. *hominis*, strain H37Ra, grown on modified P and B medium for 36 days.

Ground in a mortar to a thick paste with finely powdered glass and 0.01 M phosphate buffer, pH 7.0, for 6 hr.

Temperature maintained at approximately 5 C by immersing the mortar into an ice bath frequently.

Paste diluted with 0.01 M phosphate buffer, pH 7.0, to approximately 100 ml and centrifuged at 2,500 rpm for 20 minutes.

Residue no. 1

resuspended in 10 ml of 0.01 M phosphate buffer and stored 24 hr at 5 C; centrifuged at 5,000 rpm for 1 hour

Residue no. 3

discarded

40.0 ml centrifuged at 15,000 rpm (37,000 \( \times \) g) for 15 minutes

Supernatant no. 1

N\(_2\) 0.6342 mg/ml

Supernatant no. 3

N\(_2\) 0.031 mg/ml

Supernatant no. 2

Residue no. 2

resuspended in 40.0 ml phosphate buffer; N\(_2\) 0.124 mg/ml

Supernatant no. 2

N\(_2\) 0.2730 mg/ml
and sufficient buffer to give a final volume of 2.0 ml. All materials, except where otherwise indicated, were prepared with 0.01 M phosphate buffer, pH 7.0, and substrates were added as the potassium salts at the same pH. An equilibration period of 5 minutes was allowed before readings were recorded. \( Q_0 \), values were calculated from the initial linear portion of all curves.

**Centrifugal fractionation.** A preliminary centrifugal fractionation was attempted with the Servall SS-2 vacuum type angle centrifuge to determine whether enzymatic activity could be concentrated. The accompanying flow sheet describes the procedure used in the attempt to concentrate the enzymatic activity for both lactic and citric acids.

**Substrates.** All substrates and cofactors \(^6\) were commercial products. These were dissolved in 0.01 M phosphate buffer, pH 7.0, except in certain experiments when veronal-HCl buffer was used. All were neutralized, when necessary, with KOH.

**Identification of acids.** Following the final readings the material from the Warburg flasks was transferred to test tubes and the proteins precipitated with tungstic acid. The protein-free filtrates were treated with about 50 mg of activated charcoal to remove methylene blue and the colorless filtrate dried in an oven at 60 C. The dried material was dissolved then in 0.5 ml distilled water and chromatographed by the method of Lugg and Overell (1948) using \( n \)-butanol saturated with two per cent formic acid as solvent, and by the method of Denison and Phares (1952) using 13 parts ether, 3 parts acetic acid, and one part distilled water. Citric acid was further identified chemically by the method of Feigl (1946) and that of Ettinger et al. (1952).

**RESULTS**

The results of the addition of various substrates to cell extract are shown in figure 1 and table 1. They show that all the Krebs' cycle intermediates and their precursors which were tested were utilized. The highest rates of oxygen uptake were stimulated by substrates l-malic, lactic, and fumaric acids. While the oxygen uptake with these substrates plotted against time was linear, curves plotted for pyruvic, acetic, succinic, citric, \( \alpha \)-ketoglutaric, and oxalacetic acids leveled off after periods ranging from 10 to 30 minutes. With the exceptions of pyruvic and lactic acids the addition of cofactors diphosphopyridine nucleotide, adenosine tri-

---

\(^6\) We wish to thank Dr. L. Lachet of Armour and Company, Chicago, Illinois, for the generous supply of coenzyme A concentrate.
Oxidation of Krebs' cycle intermediates and precursors by cell-free extracts of Mycobacterium tuberculosis var. hominis, strain H37Ra

TABLE 1

<table>
<thead>
<tr>
<th>SUBSTRATES</th>
<th>(Qo) *</th>
<th>Molar concentration</th>
<th>With coenzymes</th>
<th>Without coenzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid...</td>
<td>0.25</td>
<td>72.0</td>
<td>76.0</td>
<td></td>
</tr>
<tr>
<td>Pyruvic acid...</td>
<td>0.25</td>
<td>45.0</td>
<td>74.0</td>
<td></td>
</tr>
<tr>
<td>Acetic acid...</td>
<td>0.025</td>
<td>50.0</td>
<td>20.0</td>
<td></td>
</tr>
<tr>
<td>Fumaric acid...</td>
<td>0.025</td>
<td>60.0</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>L-Malic acid...</td>
<td>0.25</td>
<td>100.0</td>
<td>60.0</td>
<td></td>
</tr>
<tr>
<td>Succinic acid...</td>
<td>0.25</td>
<td>22.5</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>Citric acid...</td>
<td>0.25</td>
<td>20.0</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>Oxalacetic acid...</td>
<td>0.25</td>
<td>8.3</td>
<td>-8.0</td>
<td></td>
</tr>
<tr>
<td>a-Ketoglutaric acid...</td>
<td>0.25</td>
<td>30.0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

\* Per hour per mg nitrogen corrected for endogenous activity.

Coenzymes adenosine triphosphate, 1.0 mg; coenzyme A concentrate, 1.0 mg (13 Lipmann units); and diphosphopyridine nucleotide, 1.0 mg.

All flasks contained methylene blue, 1.0 X 10^{-4} M; Mg^{++}, 3.3 X 10^{-2} M; Mn^{++}, 1.0 X 10^{-4} M; substrate dissolved in 0.01 M phosphate buffer and neutralized with KOH; and cell extract, 1.2 mg nitrogen. Total volume of flasks adjusted to 2.0 ml with 0.01 M phosphate buffer, pH 7.0. Center wells contained 0.2 ml of 10 per cent KOH.

Phosphate, and coenzyme A increased the rate of oxidation. This was particularly evident with fumaric, l-malic, and a-ketoglutaric acids. The effects of added cofactors are graphically expressed in figure 2.

In view of the work of Kusunose et al. (1952a) where two enzymes, lactic oxidase and lactic dehydrogenase, extracted from the avian strain of mycobacteria were described, it was of particular interest to determine whether these were present in strain H37Ra. Preliminary experiments with lactic acid showed, however, that in the absence of methylene blue notable increases in oxygen uptake above the endogenous control were not obtained.

The results of the centrifugal fractionation, described earlier, are shown in figures 3 and 4 and table 2. It will be noted that a greater than twofold concentration of enzymatic activity was obtained with the two substrates tested, lactic and citric acids. Residue 2 and supernatant 3 were completely without activity.

Figures 3 and 4 and table 2 also show the effect on enzyme activity of aging the cell extracts at -20 C for 20 days. While both citric acid and the endogenous activities were decreased with both soluble fractions (supernatants 1 and 2), the activity for lactic acid was slightly increased.
Figure 3. Rate of oxidation and effect of aging of lactic and citric acid dehydrogenases of supernatant no. 1. See text for details.

Curves 1 and 1a, endogenous activity of supernatant no. 1 before and after aging, respectively; curves 2 and 2a, citrate activity of supernatant no. 1 before and after aging, respectively; curves 3 and 3a, lactate activity of supernatant no. 1 before and after aging, respectively.

All flasks contained methylene blue $1.0 \times 10^{-4} \, \text{m}$; Mg$^{2+}$ $3.3 \times 10^{-4} \, \text{m}$; Mn$^{2+}$ $1.0 \times 10^{-4} \, \text{m}$; coenzymes adenosine triphosphate, diphosphopyridine nucleotide, and coenzyme A concentrate, 1.0 mg each; substrates dissolved in 0.01 m phosphate buffer, pH 7.0, and neutralised with KOH; and cell extract, 1.0 ml.

Total volume of flasks adjusted to 2.0 ml with 0.01 m phosphate buffer, pH 7.0. Center well contained 0.2 ml of 10 per cent KOH.

Citrate synthesis could not be demonstrated by the use of the specific aconitase inhibitors, fluorocacetate and Ba$^{2+}$. This probably was due to the fact that the small amount of citrate blocked by these inhibitors was beyond the limit of our means of detection. In order to demonstrate citrate synthesis, advantage was taken of an observation made in one of the preliminary experiments. It was noted that cells ground and extracted in veronal buffer, pH 6.4, contained no enzymatic activity for citrate while possessing good activity for fumarate and malate. Another attempt was made therefore to accumulate sufficient citrate for chromatographic detection by allowing these extracts, ground and extracted at pH 6.4, to oxidize fumarate and malate.

Into 5 Warburg flasks were pipetted 1.0 ml of
**TABLE 2**

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>Lactic acid</th>
<th>Citric acid</th>
<th>Endogenous</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant 1</td>
<td>31.5</td>
<td>39.4</td>
<td>12.6</td>
</tr>
<tr>
<td>Aged (-20°C for 20 days)</td>
<td>Fresh</td>
<td>Fresh</td>
<td>Fresh</td>
</tr>
<tr>
<td>Supernatant 2</td>
<td>87.9</td>
<td>87.9</td>
<td>27.4</td>
</tr>
<tr>
<td>Aged (-20°C for 20 days)</td>
<td>Fresh</td>
<td>Fresh</td>
<td>Fresh</td>
</tr>
<tr>
<td>Supernatant 3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>residue</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Per hour per mg nitrogen.

All flasks contained methylene blue, 1.0 \( \times 10^{-4} M; Mg^{++}, 3.3 \times 10^{-4} M; Mn^{++}, 1.0 \times 10^{-4} M; \) cell-free extract, 1.0 ml.

Substrates were dissolved in 0.01 M phosphate buffer, pH 7.0, neutralized with KOH, and added to the flasks so as to give final concentrations as follows: lactic acid, 0.005 M; citric acid, 0.01 M. The endogenous flasks received 0.01 M phosphate buffer. Total volume of flasks adjusted to 2.0 ml with 0.01 M phosphate buffer. Center wells contained 0.2 ml of 10 per cent KOH.

**DISCUSSION**

From the results showing utilization of Krebs' cycle intermediates and precursors and synthesis of citrate, it is evident that the avirulent strain H37Ra of *M. tuberculosis* var. *hominis* possesses a tricarboxylic acid terminal respiratory cycle. Moreover, Ochoa et al. (1951) have reported the isolation of the citric acid condensing enzyme from this same strain of *M. tuberculosis* among others.

It cannot be assumed, however, that the citric acid cycle is the sole terminal energy mechanism since the possibility of the simultaneous occurrence of a dicarboxylic acid cycle also must be kept in mind. Another possibility involving a 7-carbon unit, the condensation product of pyruvic and oxalacetic acids, as postulated by Umbreit (1949, 1953) and Umbreit et al. (1951) must be considered also.

Umbreit (1949) reported that streptomycin inhibited the condensation of pyruvic and oxalacetic acids in a streptomycin sensitive strain of *Escherichia coli*. His strain utilized citric acid slowly, and he suggested that citric acid accumulated only as a by-product of some process involved more directly in the terminal respiratory process. In our chromatograms, however, any evidence of the existence of an unknown acid was lacking. Moreover, the fact that Oginsky et al. (1950) were unable to demonstrate this streptomycin inhibition of the condensation of pyruvic and oxalacetic acids with a strain of *Mycobacterium avium* makes the possibility of the presence of a 7-carbon condensation unit in the mycobacteria more unlikely.

Previous respiratory studies (Holmgren et al., 1954) showed no oxygen uptake with whole resting cells using citric and succinic acids as substrates while high concentrations of these acids produced an inhibition of oxygen uptake. Two hypotheses were advanced to explain these results. One was the impermeability of the cell using the n-butanol, 2.0 per cent formic acid solvent system, and eluates were tested chemically for citric acid by the method described earlier. Citrate was identified by a brilliant blue fluorescence under ultraviolet light by the method of Feigl (1946) and by the presence of a pink to red pyridine layer using the method of Ettinger et al. (1952). All eluates were ninhydrin negative.
membrane to these substrates, and the second was the saturation of the enzymatic sites with intermediates of the high endogenous metabolism. The second hypothesis was deemed more likely since it could explain the inhibitions obtained with high concentrations. The results of experiments with cell extracts, which show that the avirulent H37Ra strain of *M. tuberculosis* var. *hominis* contains the enzymatic equipment for the utilization of many of the Krebs' cycle intermediates and precursors, including citric and succinic acids, unfortunately do not clear up the problem of which of the two hypotheses offered is correct. The activity of the extracts could still be attributed to either the destruction of the impermeable cell membrane or to the concentration of enzymes relatively free of oxidizable endogenous substrates. Starvation of cells as employed by Looebel et al. (1930, 1933a,b) to eliminate endogenous respiratory activity would fail to resolve the problem since it would be impossible to determine whether the starvation brought about an exhaustion of utilizable materials on the cell membrane, or closely associated with the cell membrane, thereby increasing permeability, or whether the starvation would merely free the enzyme sites which previously had been saturated with endogenous substrates.

All oxygen uptake curves, with the exception of those plotted for lactate, L-malate, and fumarate, leveled off after 30 minutes. This may have been due to destruction of apoenzyme or exhaustion of some needed coenzyme. This latter possibility appears improbable, however, since adenosine triphosphate, coenzyme A, and diphosphopyrididine nucleotide were present in high concentrations.

Geronimus (1949) stressed the need for the aging of extracts in order to obtain activity for many of the substrates he tested. We have found no increase in activity of extracts aged at 5°C over a period of one week and a decrease in activity of citrate dehydrogenase when stored at −20°C for 20 days.

**SUMMARY**

Lactic, pyruvic, acetic, fumaric, maleic, succinic, citric, oxalacetic, and α-ketoglutaric acids were oxidized by cell-free extracts of the avirulent H37Ra strain of *Mycobacterium tuberculosis* var. *hominis*. A centrifugal fractionation procedure produced a twofold increase in enzymatic activity for lactate and citrate. Cofactors adenosine triphosphate, diphosphopyridine nucleotide, and coenzyme A were found to increase the rate of oxygen uptake with all substrates except pyruvate and lactate. Plotting oxygen uptake against time, linear curves for lactate, fumarate, and L-malate were obtained while with the remainder of the substrates, pyruvate, acetate, oxalacetate, α-ketoglutarate, succinate, and citrate, curves were obtained which leveled off after 10 to 30 minutes.

Storage of extracts at 5°C for a period of one week had no effect on enzymatic activity. Freezing at −20°C for 20 days decreased the endogenous and citrate dehydrogenase activities while it increased lactate dehydrogenase activity slightly. The fact that a hydrogen carrier, methylene blue, was required for lactate activity indicated that lactate oxidase was absent from H37Ra extracts.

Citrate was synthesized from fumarate and malate by H37Ra extracts and was identified both chromatographically and chemically.

The utilization of the many Krebs' cycle intermediates and their precursors and the synthesis of citrate indicate that the avirulent H37Ra strain of *M. tuberculosis* var. *hominis* possesses a citric acid terminal respiratory cycle.

**REFERENCES**


IRVING MILLMAN AND GUY P. YOUMANS

GERONIMUS, L. H. 1949 Ph.D. dissertation, Studies on oxidative systems in mycobacteria with special emphasis upon the employment of cell-free extracts. Ohio State University.


