THE OXIDATION OF COMPOUNDS RELATED TO THE TRICARBOXYLIC ACID CYCLE BY WHOLE CELLS AND ENZYME PREPARATIONS OF *MYCOBACTERIUM TUBERCULOSIS* VAR. *HOMINIS*[1]

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The effect of compounds related to the tricarboxylic acid cycle on the growth and rate of multiplication of the virulent strain H37Rv of *Mycobacterium tuberculosis* var. *hominis* has been reported previously (Youmans and Youmans, 1953). It was found that in certain concentrations lactic, pyruvic, acetic, oxalosuccinic, α-ketoglutaric, and oxalacetic acids supported the growth of small inocula of these organisms, but that no growth occurred in the presence of *cis*-aconitic, citric, isocitric, succinic, fumaric, malic, glutamic, aspartic acids, and alanine. It was suggested that the bacterial cells may have been impermeable to the substrates which had not supported growth, rather than that the cells lacked the specific enzymes.

Additional data on the oxidation of the intermediates of the tricarboxylic acid cycle by virulent tubercle bacilli have been obtained by showing the effect of these compounds on the respiration of whole cells, and upon enzyme preparations obtained by altering whole cells by acetone drying. Data from metabolic experiments using radioactive acetate also have been included.

MATERIALS AND METHODS

Cultures. The highly virulent strain H37Rv of *M. tuberculosis* var. *hominis* was employed in all experiments. The organisms were grown as surface pellicles on the modified Proskauer and Beck medium (Youmans and Karlson, 1947) in 250-ml Erlenmeyer flasks, and were used after two to three weeks of incubation at 37 C when growth was mature.

Preparation of Cells for the Manometric Studies

Whole cells. The cultures were harvested by pouring the contents of 8 to 10 flasks of cultures into a coarse sintered glass filter under suction, and the organisms washed using small aliquots of a total of not less than 500 ml of 0.01 M phosphate buffer at a pH 7.0. The cells, while quite moist, were scraped with a spatula from the surface of the filter, placed in a mortar, and ground by hand with a pestle for 10 to 15 minutes until a homogeneous suspension resulted. A small amount of buffer then was added, mixed with the suspension, and the contents of the mortar were poured into a large test tube. The suspension was standardized by centrifugation in a Hopkin's vaccine centrifuge tube as recommended by Youmans and Karlson (1947).

Because of the relatively slow metabolic rate of these organisms, heavy cell suspensions, either 40 or 100 mg per ml, wet weight, were employed in order to permit termination of the experiment after 3 to 5 hours.

Acetone dried cells. To limit the handling of the large number of virulent tubercle bacilli needed for such studies, a simple method was needed, either for altering the permeability or for making an enzyme preparation. Several methods which have been used successfully with other types of organisms, such as freezing and thawing, heat drying, drying over P_2O_5 and using cells of different ages, were tried without success.

Other enzyme preparations usually involve many more steps, and therefore a greater chance of accident, than the procedures described above. This risk appeared especially great in preparing cell-free extracts by using the ball mill grinding technique previously employed in this laboratory (Millman and Youmans, 1954). However, it was found that reproducible active enzyme preparations of virulent organisms could be made by drying the cells with acetone. The method followed was, in part, that described by Umbreit *et al.* (1951). Suspensions prepared as described above were diluted with sufficient 0.01 M phosphate buffer to make a fairly thick paste which could be poured in thick drops into 10 volumes of ice-cold dry acetone. The fluocculent material

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which appeared was shaken in the acetone intermittently for about 5 minutes and was collected on a coarse sintered glass filter under suction. It was washed with a small amount of phosphate buffer, removed with a spatula to another mortar, and again ground with a pestle to make a fine suspension. After the suspension had been poured into a large test tube, it was standardized so that each ml contained approximately 110.0 mg of cells, wet weight.

In contrast to suspensions of whole cells which appeared relatively stable metabolically when kept at 4 C for one to two weeks, suspensions of acetone dried cells had to be prepared the same day as used.

Preparation of the Substrates

Each of the substrates was dissolved in 0.01 M phosphate buffer, pH 7.0, to give a concentration of 1.0 M, the pH of the solutions was readjusted to 7.0, and tenfold dilutions were made in buffer from this concentration.

Manometric Methods

Standard Warburg procedure was followed as described by Umbreit *et al.* (1951) in measuring the oxygen uptake of the tubercle bacilli in air at 37 C. Employing whole cells, 1.0 ml of cells was placed in the Warburg vessel, 1.0 ml of the substrate in one of the side arms, and 0.2 ml of 10 per cent KOH in the center well. Employing acetone-dried cells, 0.9 ml of cells was placed in the vessel, 1.0 ml of substrate in a side arm, 0.1 ml of a solution containing 1×10^{-3} M methylene blue, 3.3×10^{-3} M Mg^{++}, and 1×10^{-5} M Mn^{++} in the other side arm, and 0.2 ml of 10 per cent KOH in the center well.

Phosphate buffer was substituted for the substrate in the control flasks. Moreover, to check the preparation of the acetone dried cells, 1.0 M lactic acid was used as a standard control substrate.

Since virulent organisms were used, it was necessary to use an adapter to join the Warburg vessel to the manometer so that the vessel and adapter could be removed as one unit for autoclaving before the flasks were washed. The adapter (kindly prepared by Mr. Lucas Van Orden) consisted of two standard taper joints joined by a short narrow tube in which a cotton plug was placed. The vessel and adapter were calibrated as a unit.

The whole cells were equilibrated 30 minutes before the substrate was tipped into the flask, and the manometers closed; the acetone dried cells were equilibrated 15 minutes, the contents of the sidearm tipped into the vessel, and equilibrated for 15 more minutes before closing the manometers. Readings were made every 15 or 30 minutes. Two or three vessels were run with each substrate and the average was taken as the final reading. Tests with each substrate were repeated two to six times.

The preparation of all cell suspensions and enzyme preparations, and the filling of the Warburg vessels, including the fitting of the adapters, was done in an exhaust safety hood. Other precautionary methods included the wearing of rubber gloves and the use of a mechanical pipetting device. Syringes and needles were used to transfer tubercle bacilli from rubber stoppered vaccine bottles covered with cotton to reduce the formation of aerosols.

Preparation of Cells Grown in the Presence of Radioactive Acetate

Radioactive sodium acetate-2-C^{14} was dissolved in water with sufficient sodium acetate to give a final acetate concentration of 0.01 M, and the material was sterilized by filtration through sintered glass after neutralization with KOH.

Forty-eight flasks of normal pellicle cultures of H37Rv were grown, as described above, for 2 weeks. At this time the cotton stoppers in the flasks were replaced with sterile rubber stoppers which held adapters containing barium hydroxide to adsorb the radioactive CO_2 produced by the metabolizing cells. One ml containing 2 μc of the sterile radioactive sodium acetate mixture was added then to each flask and the flask shaken for a few minutes to disrupt the pellicle growth and to distribute the acetate throughout the culture. The cultures were shaken while undergoing incubation at 37 C for an additional 24 hours. The metabolic reaction then was stopped and residual CO_2 was removed by adding, while shaking, 1.0 ml of 12 N HCl to each flask. The tubercle bacilli were killed by autoclaving for 30 minutes at 20 lbs pressure, harvested as described above, and ground in a ball mill for 72 hours by the method described by Millman and Youmans (1954).

The extract so obtained was centrifuged at 3,000 rpm for 1 hour, the supernatant was

collected and centrifuged again at 20,000 × G for 1 hour at 4 C and then put aside. The debris, representing whole cells and parts of cells, was taken up in sufficient isotonic NaCl to make a loose paste, and treated with one mg of lysozyme for 18 hours at 37 C. This material then was centrifuged and the supernatant combined with the first. The residue which remained was suspended in buffer at a pH 7.5 and 1.0 mg pancreatin added, and the mixture again incubated at 37 C for 18 hours, with 2 drops of toluene added as a preservative. This mixture was centrifuged in the same fashion, and all three supernatants were combined. The proteins in the supernatants were precipitated with tungstic acid and removed by filtration. The protein-free material was acidified with 12 N HCl to a pH of 1.0, and exhaustively extracted over 72 hours with anhydrous ether. The ether layers were combined and concentrated *in vacuo* to a volume of 100.0 ml.

Chromatographic Methods

To determine the presence of radioactive intermediates of the tricarboxylic acid cycle both paper partition chromatography using the descending procedure of Lugg and Overell (1948), and the continuous flow Dowex 1 anion exchange column procedure described by Busch *et al.* (1952) were used. After partitioning the unknown on paper, four strips were sprayed with bromphenol blue indicator to determine the effectiveness of separation and relative R_f values. Another four strips were placed in contact with X-ray film in the dark for 6 weeks. All R_f values were compared with those of known compounds of the Krebs' cycle.

For separation by anion exchange, 50 ml of the concentrated ether residue dissolved in water was forced through the Dowex 1 column. Samples in 2.5-ml amounts were collected by an automatic fraction collector and the contents of three tubes were pooled and dried in a 60 C oven. The residue of each was resuspended in 1.0 ml of distilled water, placed on a planchet, and again dried at 60 C. The activity (cpm) on each planchet then was determined by placing each planchet in a flow gas counter.

RESULTS

Table 1 gives the endogenous respiration of whole cells obtained in four experiments in which the numbers of cells were varied. In a single experiment there was a direct relationship between the number of cells present and the amount of oxygen consumed. However, in different experiments the endogenous respiration for a given number of cells varied from 0.6 μl to 1.57 μl of oxygen consumed per hour per mg of cells.

TABLE 1

The effect of the number of cells on the endogenous respiration of Mycobacterium tuberculosis var. hominis

Mg Cells per ml	Oxygen Uptake per hr per mg Cells Wet Weight			
	Experiment No.			
	1	2	3	4
20	0.60	0.85		1.4
40	0.70		1.17	1.3
60	0.67			
80	0.60			1.5
100		0.90	1.18	
120				1.5

TABLE 2

The effect of compounds related to Krebs' tricarboxylic acid cycle on the respiration of whole cells of Mycobacterium tuberculosis var. hominis

Compound	Oxygen Uptake per hr per mg Whole Cells, Wet Weight, Corrected for Endogenous Activity			
	Final molar concentration			
	0.5	0.05	0.005	0.0005
Lactic acid	2.0	0.98	0.12	0.08
Pyruvic acid	1.44	0.31	0	0
Acetic acid	0.65	1.11	0.39	0
cis-Aconitic acid	0*	0	0	0
Citric acid	−0.30*	−0.11	0	0
Isocitric acid	0*	0	0	0
Oxalosuccinic acid	−0.27*	0.50	0.08	0
α-Ketoglutaric acid	0.30	0.16	0	0
Succinic acid	0*	0	0	0
Fumaric acid	I†	0	0	0
L-Malic acid	−0.23*	0	0	0
Oxalacetic acid	0.83	0.28	0	0
DL-Alanine	0.07	0.22	0.08	0
L-Glutamic acid	I†	0.06	0	0
Aspartic acid	I†	0	0	0

* Slight stimulation of respiration for first 30 to 60 minutes.

† Insoluble at this concentration in the phosphate buffer solution.

TABLE 3

The effect of compounds related to Krebs' tricarboxylic acid cycle on the respiration of acetone dried cells of Mycobacterium tuberculosis var. hominis

Compound	Oxygen Uptake per hr per mg Acetone Dried Cells, Wet Weight, Corrected for Endogenous Activity Which Varied Between 0.12 and 0.62 μL	
	Final molar concentration	
	0.5	0.05
Lactic acid	0.96	0.62
Pyruvic acid	0.17	0.22
Acetic acid	0.03	0.19
cis-Aconitic acid	0.22	0.16
Citric acid	0.16	0.22
Isocitric acid	0.28	0.15
α-Ketoglutaric acid	0.35	0.12
Succinic acid	0.14	0.13
Fumaric acid	I†	0.10
L-Malic acid	0.19	0.11
Oxalacetic acid	0.15*	0.44
DL-Alanine	0.16	0
L-Glutamic acid	I†	0.05
Aspartic acid	I†	0.11

* Slight stimulation of respiration for first 60 minutes.

† Insoluble at this concentration in the phosphate buffer solution.

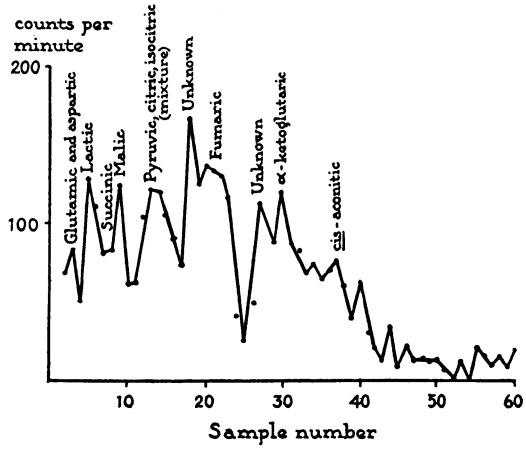

Figure 1. Counts per minute (C^{14}) of eluate (Dowex 1, 7.5 ml per sample). Labeled peaks represent intermediates and related intermediates of the tricarboxylic acid cycle.

Table 2 gives the amount of oxygen consumed by whole cells in the presence of different concentrations of substrates. In certain concentrations only lactic, pyruvic, acetic, oxalosuccinic, α-ketoglutaric, oxalacetic acids and DL-alanine stimulated the respiration of the mycobacterial cells with no lag period.

Table 3 shows the effect of these substrates on the respiration of the enzyme preparations obtained by acetone-drying the tubercle bacilli. With the possible exception of L-glutamic acid, all the substrates stimulated the oxygen uptake of these preparations. Moreover, these substrates all appeared to stimulate the oxygen uptake to a similar degree with the exception of lactic acid which had a greater stimulating effect.

In the radioactive acetate studies none of the acids of the tricarboxylic acid cycle could be identified by partitioning on paper and spraying with the indicator, bromphenol blue, due probably to insufficient quantity of the acids. However, X-ray film which had been placed in contact with the paper strips for 6 weeks showed a radioactive spot which was identified as citric acid.

The results of the experiment using the anion exchange chromatography are found in figure 1. The positions of the peaks of the individual acids were determined by the number of radioactive counts per planchet, and these peaks were identified by comparing with the known peaks obtained by Busch *et al.* (1952). As shown in the figure, glutamic and aspartic acids were eluted first, and then lactic acid. Succinic acid appears to be in a valley, but it may be that if three tubes of eluate had not been combined per planchet a peak might have occurred with one of the three tubes used. The radioactive count is quite high for this valley. Malic acid was eluted with the 9th sample. Samples 13 and 14 gave a flattened peak consisting of pyruvic, citric, and isocitric acids. The following peak, which was the highest, appears to be an unknown substance, and it is followed by fumaric acid, which was eluted in samples 20 to 22. The 7th peak, or sample 27, is unknown, and the 8th peak is identified as α-ketoglutaric acid. The last peak to be identified is *cis*-aconitic, sample 37, but it may include also the peak at sample 40, since Busch *et al.* found that *cis*-aconitic appeared as a long low peak which covered several samples.

DISCUSSION

The pathogenicity of the organism used is, in part, responsible for the qualitative nature of the data. Quantitative procedures such as those employed by Saz and Krampitz (1954) require enormous numbers of bacterial cells, and it was felt that these numbers would involve too great a health risk with the laboratory safeguards presently available. However, the data do show a relationship between cellular permeability, enhanced growth and oxidation. With the exception of alanine, only the substrates which supported the growth of small inocula stimulated the respiration of whole cells. However, enzyme preparations of whole cells, prepared by acetone drying, oxidized all the intermediates of the tricarboxylic acid cycle with the possible exception of glutamic acid. The latter observations are similar to those noted with saprophytic and avirulent mycobacteria (Geronimus, 1949; Blakley, 1951; Faine *et al.*, 1951; Millman and Youmans, 1954, 1955) with *M. avium* (Kusunose *et al.*, 1952) and with *M. tuberculosis* var. *hominis* strain H37 (Geronimus, 1949).

Experiments employing radioactive acetate showed that acetate was oxidized by whole cells through the Krebs' cycle, since glutamic, aspartic, lactic, malic, pyruvic, isocitric, citric, fumaric, α-ketoglutaric, *cis*-aconitic, and possibly succinic acids were identified by the method of Busch *et al.* (1952). In addition radioactive citrate was identified by paper partition chromatography. Citrate formation has been shown previously with *M. phlei* (Blakley, 1951) and with the H37Ra strain (Millman and Youmans, 1954).

In comparing the results obtained with the virulent H37Rv strain and its avirulent variant, H37Ra, virulence does not appear to be related to their terminal oxidative pathways. Both organisms contain enzymes which oxidize intermediates of the tricarboxylic acid cycle. However, whole cells of the H37Ra strain appear to be more permeable to these intermediates (Holmgren, Millman, and Youmans, 1954) than whole cells of the H37Rv strain (Youmans and Youmans, 1953).

SUMMARY

Substrates related to the tricarboxylic acid cycle were found to stimulate the respiration of enzyme preparations of the highly virulent strain H37Rv of *Mycobacterium tuberculosis* var. *hominis* prepared by drying cells with acetone. However, of these substrates only lactic, pyruvic, oxalosuccinic, α-ketoglutaric, acetic acids and DL-alanine stimulated the oxygen uptake of the whole mycobacterial cells.

Radioactive acetate was oxidized by way of this cycle, since on isolation by anion exchange chromatography, glutamic, aspartic, lactic, pyruvic, citric, isocitric, *cis*-aconitic, α-ketoglutaric, malic, fumaric, and possibly succinic contained radioactive carbon. By employing paper partition chromatography, radioactive citrate was found.

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