STUDIES ON THE METABOLISM OF MYCOBACTERIUM TUBERCULOSIS

II. The Effect of Compounds Related to the Kreb's Tricarboxylic Acid Cycle on the Growth of Mycobacterium tuberculosis var. hominis[1]

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During the course of studies concerned with the effect of carbon sources on the growth or the respiration of tubercle bacilli, investigators in the past have examined compounds of the Kreb's tricarboxylic acid cycle and occasionally have speculated on the possibility that the intermediary carbohydrate metabolism of these organisms might be through such a pathway. Furthermore, Boissevain (1943*b*) demonstrated chemically that one of these intermediate compounds, pyruvic acid, was formed during the growth of virulent human type tubercle bacilli from the glycerol in Long's medium.

Long (1919), employing the human strain H37 of *Mycobacterium tuberculosis* and a basal synthetic medium containing 2.5 per cent glycerol, observed excellent growth at the end of a three week incubation period in the presence of ammonium succinate and ammonium malate, but no growth in the presence of ammonium pyruvate or ammonium lactate. He suggested the possibility that the dibasic acids arise from the breakdown of the constituents of the "usual glycerol-peptone medium". Merrill (1931*a*) observed that citrate, acetate, and lactate, when available as a single carbon source, were utilized by the majority of the 14 strains of mycobacteria but not by the one known human strain H37. Merrill (1931*b*) felt that acetate and lactate were utilized possibly more readily than glucose, and that these acids might be intermediate products in glucose metabolism. Nakamura (1938) found no increase in the oxygen consumption during a 30 minute period by a human type strain in the presence of 1.0 per cent concentrations of citric acid, succinic acid, or malic acid. Steinbach (1940) investigated the effect of the substrates lactate, pyruvate, succinate, and glycerophosphate on the respiration of *M. tuberculosis* and found they did not serve as hydrogen donators. Bernheim (1941) reported that citric, succinic, malic, and pyruvic acids had no effect on the oxygen consumption of a bovine strain although he gave no experimental data. Two years later, Boissevain (1943*a*) observed that the growth of 5 bovine strains was stimulated by the addition of 0.1 per cent pyruvate to Long's medium which normally is not a suitable medium for growth of these organisms, but that higher concentrations of this acid inhibited growth. Furthermore, pyruvic acid plus sodium phosphate added to distilled water permitted multiplication of human type bacilli.

Edson and Hunter (1943) employed both growth and respiration studies to determine the carbon requirements of a number of strains of mycobacteria. They found that 8 nonvirulent strains exhibited differences in their ability to oxidize the same carbon source although none of the strains oxidized citrate, and that generally there appeared to be a correlation between the growth and respiration of 7 of these strains. Moreover, they observed that a 10^{-2} mg inoculum frequently would not grow in the presence of certain carbon compounds while growth would occur if 1.0 mg of the organisms was used as an inoculum. Growth of *Mycobacterium phlei*, *M. smegmatis*, and *M. karlinski* occurred in the presence of 1.0 per cent concentrations of lactate and pyruvate during a 7 day incubation period employing a 10^{-2} mg inoculum. After 5 weeks of incubation, *M. smegmatis* also grew in succinate, and after 6 weeks of incubation, *M. karlinski* grew in acetate. The presence of L-lactic, pyruvic, L-malic, fumaric, and α-ketoglutaric acids, in 0.01 per cent concentrations, was ineffectual in stimulating the growth of *M. phlei* during a 7 day incubation period. Edson and Hunter concluded from this finding that "citric acid is not oxidized as a source of energy, nor does it catalyze the oxidation of carbohydrate after the manner of the citric acid cycle, since the intermediates of

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the cycle, such as α-ketoglutaric, succinic, fumaric, and L-malic acids, did not stimulate growth."

Gerundo (1947) felt that the asparagin in Long's medium was metabolized by tubercle bacilli to succinic acid and thereby utilized as a source of energy for growth. He therefore substituted sodium succinate for the asparagin and obtained more abundant growth of the tubercle bacilli than in Long's medium.

Edson and Hunter (1947) observed that the oxygen consumption of *M. ranae* was augmented by the presence of lactate, pyruvate, L(−)malate, and glucose. Since lactate and glucose increased the oxygen consumption fivefold, they felt that lactate might possibly be an intermediate in glucose metabolism. In the same year, Edson (1947) reported on the oxidation of lactic acid by an enzyme preparation obtained from *M. phlei*. He found, using methylene blue as the hydrogen acceptor, that L(+)lactate was oxidized anaerobically to pyruvate. Aerobically, lactic acid was oxidized to acetate and carbon dioxide. The enzyme was specific for the aerobic reaction and was insensitive to cyanide inhibition. From these results Edson concluded that aerobic oxidation is independent of a cytochrome system.

Lindsay, O'Donnell, and Edson (1950), in further studies with *M. ranae*, observed that the addition of pyruvate or acetate increased respiration approximately fourfold. Acetate was not found as an intermediate product during the metabolism of pyruvate. The authors suggested that perhaps 75 per cent of the acetate formed was burned to completion and the remainder was assimilated for growth of the organisms. Cell extracts and broken cells were unable to oxidize pyruvate or acetate.

In the same year, Oginsky, Smith, and Solotorovsky (1950) reported that the avian strain, Kirchberg, oxidized pyruvic and oxalacetic acids, but oxidized pyruvate plus oxalacetic acid only slightly. Moreover, this strain did "not oxidize to an appreciable state" *cis*-aconitate, citrate, α-ketoglutarate, succinate, fumarate, aspartate, asparagin, glutamate, lactate, acetate, and glucose. With this strain the investigators were "able to obtain no positive evidence for a citric acid cycle."

According to Edson (1951), many enzymes concerned with the metabolism of intermediates of the Kreb's cycle have been found in cell-free extracts of mycobacteria, and he stated that "at the present time there is sufficient evidence to suggest that the glycolytic and citric acid cycles both play part in the metabolism of the acid fast bacteria, but it is not possible to see how far the details of the cycles may need modification."

Unfortunately, most of the previous studies have been made on saprophytic strains. There is no assurance that the metabolism of highly virulent strains would be identical to that of saprophytic mycobacteria.

The present study is concerned with the quantitative measurement of the rate of growth of a highly virulent strain of *M. tuberculosis* var. *hominis* in a synthetic medium containing, as single sources of carbon, compounds which are related to the Kreb's tricarboxylic acid cycle. It was hoped from such an investigation that some information might be gained concerning the intermediate metabolism of carbohydrates by virulent tubercle bacilli.

METHODS

The small inocula method used to determine the rate of growth of the virulent H37Rv strain of *M. tuberculosis* and all other technical procedures were the same as described in the first paper of this series (Youmans and Youmans, 1953).

RESULTS

The results are given in table 1. Listed are the generation times, in hours, of the tubercle bacilli which grew in the various media. In certain concentrations of some of the compounds, growth occurred only in those tubes inoculated with 10^{-2} mg. This finding is so indicated in the table. In all cases where a generation time is recorded, growth of the tubercle bacilli occurred in all 5 inocula, and growth increased during the 5 week incubation period although the maximal growth present at the end of that period was not always as great as that obtained with glycerol.

Growth was obtained in the presence of certain concentrations of glycerol, lactic acid, pyruvic acid, acetic acid, oxalosuccinic acid, α-ketoglutaric acid, and oxalacetic acid.[2] With the exception of acetic acid and oxalosuccinic acid, these compounds stimulated the growth of the organisms to the same degree as did the higher concentrations of glycerol. However, the generation time obtained with acetic and oxalosuccinic acids was similar to the generation time found with the lower concentrations of glycerol. The importance of the concentration in which the substrate was

employed also is evident from the data in the table. It is of interest that none of the amino acids which, by oxidative deamination, are thought to be able to enter the Kreb's cycle could serve as a carbon source for growth.

DISCUSSION

The evidence obtained from these studies lends support, perhaps, to the theory that a tricarboxylic acid or a similar cycle may be involved in the carbohydrate metabolism of *M. tuberculosis* var. *hominis* since pyruvic, acetic, oxalacetic,[2] oxalorate obtained in the presence of the best carbon source, glycerol; and (3) these compounds stimulated the growth of the smallest inoculum (10^{-6} mg) employed. Edson and Hunter (1943) have pointed out the importance of the size of the inoculum in experiments in bacterial nutrition since many substances will stimulate the growth of large, but not of small, inocula.

Although citric acid, which, according to current belief, belongs in the Kreb's cycle, and the other acids of the Kreb's cycle, *cis*-aconitic, isocitric, succinic, fumaric, and malic, did not allow

TABLE 1

The effect of compounds related to the Kreb's tricarboxylic acid cycle on the growth of Mycobacterium tuberculosis var. hominis

COMPOUNDS	GENERATION TIME IN HOURS								
	Concentration of compound in per cent								
	16.0	4.0	2.0	1.0	0.25	0.06	0.016	0.004	0.0
Glycerol	0	21.7	21.7	21.7	21.7	24.0†	24.0†		
Lactic acid		0		0	22.7	22.7	22.7		
Pyruvic acid		0		0	20.5	20.5	21.7		
Acetic acid		0		0	(10^{-2})*	25.2†	25.2†	0	
cis-Aconitic acid						0	0	0	
Citric acid		0		0	0	0	0	0	
Isocitric acid						0	0	0	
Oxalosuccinic acid					0	(10^{-2})*	25.2	(10^{-2})*	
α-Ketoglutaric acid				21.7	21.7	21.7	21.7	0	
Succinic acid		0		0	0	0	0	0	
Fumaric acid				0	0	0	0	0	
L-Malic acid		0		0	0	0	0	0	
Oxalacetic acid				0	21.7	21.7	21.7		
DL-Alanine				0	0	0	0	0	
Glutamic acid				0	0	0	0	0	
Aspartic acid				0	0	0	0	0	
None									0

0 = no growth
* = smallest inoculum (in milligrams) which grew
† = slight growth.

succinic, and α-ketoglutaric acids supported the growth of the virulent H37Rv strain. These results are even more striking when one considers (1) the very few carbon sources which will support the growth of this strain (Youmans and Youmans, 1953); (2) that the rates of growth obtained with these compounds were similar to the growth, these results do not rule out the operation of such a cycle since the problem of the penetration of these substances into the bacterial cell must be considered. Clarification of this problem is difficult since there are, at present, no data available on the permeability of virulent mycobacterial cells to these substrates. However, Edson (1951) quotes Geronimus as having found that clarified extracts of the H37 strain would consume oxygen in the presence of citrate or isocitrate when methylene blue was employed as a hydrogen carrier. Edson (1951) also reported

[2] Since oxalacetic acid readily may undergo spontaneous decarboxylation to form pyruvic acid and CO_2, the growth promoting activity of this compound actually may be due to pyruvic acid.

that Geronimus obtained fumarase and malic dehydrogenase in soluble preparations from the H37 strain, but succinic dehydrogenase appeared to be absent, weakly developed, or remained adherent to the insoluble debris. If these enzymes are present and if they actually function in the metabolism of the H37Rv strain, many of the negative results obtained in the present study may have been due to the inability of the substrates to penetrate the bacterial cell.

Edson (1951) also listed a number of enzymes of the Kreb's cycle which appear to be present in mycobacteria, and these are: aconitase, isocitric dehydrogenase, oxalosuccinic carboxylase, α-ketoglutaric dehydrogenase, malic dehydrogenase, fumarase, and succinic dehydrogenase. Most of these enzymes, however, apparently have been isolated from nonvirulent or saprophytic strains.

In growth studies such as this one, one might expect intermediates in the metabolism of a given substrate to produce the same rate of growth as that obtained with the parent compound. Of the substrates which stimulated growth, this was found to be true with the exception of acetic and oxalosuccinic acids. The slower rate of growth produced by these compounds again possibly may have been due to a slower rate of penetration of the bacterial cell.

Of added interest is the finding that the tubercle bacilli grew at a similar rate in both glycerol and lactic acid. This suggests that the two compounds may be metabolized in a similar manner.

SUMMARY

By employing quantitative growth studies and a basal synthetic medium from which the normal carbon source, glycerol, was omitted, it was found that pyruvic, acetic, oxalacetic, oxalosuccinic, α-ketoglutaric acids, lactic acid, and glycerol supported the growth of the virulent human type strain H37Rv of *Mycobacterium tuberculosis*.

cis-Aconitic, isocitric, succinic, fumaric, L-malic, and citric acids did not support growth; nor did the three amino acids, glutamic acid, aspartic acid, and DL-alanine.

The relation of these findings to the operation of the Kreb's tricarboxylic acid cycle in the intermediary carbohydrate metabolism of *M. tuberculosis* var. *hominis* was discussed.

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