

- Cook, J. W., Blake, J. R. & Yip, G. (1958). *J. Ass. off. agric. Chem.* **41**, 399.
- Cook, J. W. & Yip, G. (1958). *J. Ass. off. agric. Chem.* **41**, 407.
- Dixon, M. & Webb, E. C. (1958). *The Enzymes*, p. 118. London: Longmans, Green and Co.
- Frawley, J. P., Fuyat, H. N., Hagan, E. C., Blake, J. R. & Fitzhugh, O. G. (1957). *J. Pharmacol.* **121**, 96.
- Gaines, T. B. (1960). *Tox. appl. Pharmacol.* **2**, 88.
- Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 450. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Main, A. R. (1960). *Biochem. J.* **75**, 188.
- Main, A. R., Miles, K. E. & Braid, P. E. (1961). *Biochem. J.* **78**, 769.
- Murphy, S. D., Anderson, R. L. & Dubois, K. P. (1959). *Proc. Soc. exp. Biol., N.Y.*, **100**, 483.
- Murphy, S. D. & Dubois, K. P. (1957). *Proc. Soc. exp. Biol., N.Y.*, **96**, 813.
- Myers, D. K. & Mendel, B. (1953). *Biochem. J.* **53**, 16.
- Seume, F. W. & O'Brien, R. D. (1960). *Agric. & Fd Chem.* **8**, 36.
- Strelitz, F. (1944). *Biochem. J.* **38**, 86.
- Wilkinson, G. N. (1961). *Biochem. J.* **80**, 324.
- Yip, G. & Cook, J. W. (1959). *J. Ass. off. agric. Chem.* **42**, 405.

Biochem. J. (1962) **84**, 263

Tricarboxylic Acid Cycle and Related Enzymes in Cell-Free Extracts of *Mycobacterium tuberculosis* H₃₇R_v

BY P. SURYANARAYANA MURTHY, M. SIRSI AND T. RAMAKRISHNAN
Pharmacology Laboratory, Indian Institute of Science, Bangalore 12, India

(Received 27 November 1961)

It has been fairly well established that several bacteria possess the tricarboxylic acid cycle for the terminal oxidation of carbohydrates. Youmans & Youmans (1953) could not demonstrate the growth of *Mycobacterium tuberculosis* H₃₇R_v from small inocula in the presence of the intermediates of the tricarboxylic acid cycle. They suggested that this might be due rather to the impermeability of the bacterial cells to these compounds than to the fact that the cells lacked the corresponding enzymes. In support of this theory, Youmans, Millman & Youmans (1956) presented qualitative data on the oxidation of all the intermediates of the tricarboxylic acid cycle by acetone-dried cells of the organism. However, no correlation was made between the amount of substrate disappearing and of oxygen consumed, nor were the oxidation products identified. Indira & Ramakrishnan (1959) have already shown that *M. tuberculosis* utilizes both the glycolytic and pentose phosphate pathways for the catabolism of glucose to the stage of pyruvic acid. The present study was undertaken to detect the individual enzymes of the tricarboxylic acid cycle in cell-free extracts of *M. tuberculosis* H₃₇R_v.

MATERIALS AND METHODS

Chemicals. Citric acid, α -oxoglutaric acid, fumaric acid, sodium hydrogen malate, 2,6-dichlorophenol-indophenol and triphenyltetrazolium chloride were obtained from British Drug Houses Ltd.; NADP⁺, NADH, NADPH, CoA, horse-heart cytochrome *c*, phenazine methosulphate,

glyoxylic acid (sodium salt), thiamine, pyrophosphate, acetyl phosphate and FAD were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A.; oxaloacetic acid, GSH, DL-isocitric acid lactone and lipoic acid were from California Corp. for Biochemical Research, Calif., U.S.A.; *cis*-aconitic acid, DL-isocitric acid (trisodium salt) and FMN were from Nutritional Biochemicals Corp., Ohio, U.S.A.; NAD⁺, ATP and ADP were from Pabst Laboratories, Milwaukee, Wis., U.S.A.; sodium succinate, sodium acetate and sodium pyruvate were from E. Merck A.-G., Darmstadt, Germany; vitamin K₃ (menadione), vitamin K₁ and α -tocopherol acetate were from Hofmann-La Roche and Co. Ltd., Basle, Switzerland; bovine serum albumin fraction V was from Armour and Co., Chicago, Ill., U.S.A.; [2-¹⁴C]acetate was from The Radiochemical Centre, Amersham, Bucks. Acetyl-CoA was prepared according to the method of Stadtman (1957).

Test organism. *Mycobacterium tuberculosis* H₃₇R_v, originally obtained from the National Collection of Type Cultures, London, was maintained on Petrik medium (Gradwohl, 1948). It was cultured in the liquid synthetic medium (Youmans & Karlson, 1947).

Whole-cell suspensions. The procedure used was essentially that of Youmans *et al.* (1956). After 14 days of incubation at 37°, the cells were harvested by filtration, washed with water, dried between several folds of filter paper and ground in a mortar with phosphate buffer (0.05M, pH 7.0) to make a homogeneous suspension containing 100 mg. of cell material/ml.

Cell-free extracts. Whole-cell suspensions prepared as described above, except that the pH of the suspending buffer was 6.0, were subjected to oscillation in a 10 kcyc. Raytheon sonic oscillator for 20 min. at 3–6°. The preparation was centrifuged at 10 000g for 60 min. at 0–5° in a Servall high-speed centrifuge and the supernatant was used as the source of the crude enzyme. Alternatively, cells were

ground with glass powder as described by Ramasarma & Ramakrishnan (1961), with the modification that phosphate buffer (0.05M, pH 6.0) was used to extract the enzyme.

Protein content. The amount of protein was determined by the biuret method (Gornall, Bardawill & David, 1957).

Enzyme assays. Aconitase was determined by the increase in extinction values at 240 m μ with citrate (Racker, 1950); isocitrate dehydrogenase by NADP⁺ reduction at 340 m μ in the presence of isocitrate (Goldman, 1956a); succinate dehydrogenase by reduction of 2,6-dichlorophenol-indophenol (Kusunose, Nagai, Kusunose & Yamamura, 1956) at 600 m μ or ferricyanide (as for pyruvate and α -oxoglutarate dehydrogenase); fumarase by decrease in extinction values at 300 m μ in the presence of fumarate (Massey, 1955); malate dehydrogenase by NAD⁺ reduction (Goldman, 1956b); α -oxoglutarate and pyruvate dehydrogenases by NADP⁺ reduction at 340 m μ and also by reduction of ferricyanide (Stumpf, Zarundnaya & Green, 1947; Slater & Bonner, 1952); condensing enzyme by estimating the amount of citric acid formed by the method of Natelson, Pincus & Lugovoy (1948) as modified by Eisenberg (1953), the phosphotransacetylase from green gram (*Phaseolus radiatus*) being used (Ramakrishnan, 1959); diaphorase by reduction of 2,6-dichlorophenol-indophenol in the presence of NADH or NADPH (Mahler, 1955b); transhydrogenase by the method of Hochster & Katznelson (1958); oxidases of NADH and NADPH by decrease in extinction values at 340 m μ (Dolin, 1959); NADH-cytochrome *c* reductase and NADPH-cytochrome *c* reductase by following the reduction of cytochrome *c* at 550 m μ in the presence of NADH or NADPH respectively (Horecker, 1955; Mahler, 1955a); malic enzyme by NADP⁺ reduction in the presence of Mn²⁺ ions (Ochoa, 1955); isocitratase by estimation of glyoxylate (Friedmann & Haugen, 1943) formed from isocitrate (Smith & Gunsalus, 1954); cytochrome oxidase by the decrease in extinction at 550 m μ in the presence of dithionite-reduced cytochrome *c*; malate synthetase by the synthesis of radioactive malate from glyoxylate and labelled acetate (Kornberg & Madsen, 1958); NADH- and NADPH-menadione reductases by menadione-catalysed oxidation of NADH and NADPH (Wosilait & Nason, 1954).

Measurement of radioactivity. The organic acids were separated by the ascending chromatographic technique as outlined by Maitra & Roy (1961) with, as solvent, butan-1-ol-acetic acid-water (4:1:1, by vol.) and containing 0.05% of sodium acetate to prevent the formation of acid bands on the chromatogram (Lawson & Hartley, 1958). The chromatograms after complete drying were sprayed with 0.04% (w/v) bromocresol green in ethanol adjusted to pH 7.0. The acids, appearing as yellow spots against a blue background, were eluted with ethanol containing 0.05N-HCl. All the samples were plated on aluminium planchets and counted in a windowless gas-flow counter and the counts were corrected for self-absorption.

Measurement of oxidation. Aerobic oxidations were studied by the conventional Warburg manometric method (Umbreit, Burris & Stauffer, 1957) as modified by Youmans *et al.* (1956), with an adapter with a plug of cotton wool used between the Warburg flask and the manometer because of the pathogenicity of the organism. Appropriate blank values were subtracted from all figures shown in this paper. When the effect of cyanide was tested 0.2 ml. of 2N-NaCN in 0.5N-KOH was added to the centre well.

RESULTS

Studies with whole-cell suspensions

Pyruvate, oxaloacetate, acetate and lactate significantly stimulated the oxygen uptake above the endogenous value; the consumption of oxygen in μ l./hr./mg. wet wt. of cells (corrected for endogenous respiration) was 1.2, 0.31, 1.5 and 1.9 respectively in a typical experiment. Succinate, fumarate, α -oxoglutarate, malate and glyoxylate were either not effective or increased the oxygen uptake to only a very small extent. Citrate, *cis*-aconitate and isocitrate often showed inhibition of the oxygen uptake. The oxygen uptake varied considerably from preparation to preparation; for example q_{O_2} wet-weight values (μ l. of oxygen consumed/hr./mg. wet wt. of cells) obtained with pyruvate ranged from 0.8 to 1.6.

Studies with cell-free extracts

Oxidation of substrates. Lactate, citrate, *cis*-aconitate, isocitrate and oxaloacetate were utilized by cell-free extracts only in the presence of added artificial electron acceptors such as methylene blue, menadione, phenazine methosulphate etc. The results with extracts prepared either by grinding or by sonic treatment were similar. Hence in all subsequent experiments sonic extracts were used. The oxidation of citrate, *cis*-aconitate and isocitrate was observed only when NADP⁺ was added in addition to the other electron acceptors mentioned above. With boiled enzyme there was no activity with any of these substrates. Succinate, malate, fumarate, acetate, pyruvate and α -oxoglutarate were not oxidized to any significant extent. The addition of lipoic acid (reduced), thiamine pyrophosphate, CoA, bovine serum albumin fraction V, α -tocopherol and boiled yeast extract, with phenazine methosulphate or menadione as the electron acceptor, did not promote the oxidation of these substrates.

Oxidation of lactate. Crude cell-free extracts (with or without dialysis against distilled water overnight) oxidized lactate to a significant extent. The results in Table 1 show the relative abilities of various electron acceptors to promote the oxygen uptake with lactate as the substrate. Triphenyl-tetrazolium chloride showed no effect as acceptor, and K₃Fe(CN)₆, cytochrome *c*, vitamin K₁ and ascorbic acid stimulated the oxidation to a very small extent. Dichlorophenol-indophenol nearly doubled the oxygen uptake, whereas methylene blue increased it fourfold. Menadione (in 0.05 ml. of ethanol) and phenazine methosulphate, however, enhanced the lactate oxidation considerably, by about 9 and 13 times respectively. The omission from the complete system of NAD⁺, NADP⁺,

Mg²⁺ ions, nicotinamide (included in the complete system to prevent the destruction of NAD⁺ by nicotine-adenine dinucleotidase) did not decrease the oxygen uptake significantly.

Dicoumarol (10 μM) inhibited by about 20% the menadione-promoted lactate oxidation, and cyanide (1 mM) stimulated it by about 40%.

Oxidation of citrate, isocitrate and cis-aconitate. The cofactors required for the oxidation of citrate by crude cell-free extracts were determined with phenazine methosulphate as the electron acceptor. NAD⁺, CoA and ATP had no significant effect, but NADP⁺ was essential. *cis*-Aconitate and isocitrate were also oxidized by the extracts in the presence of NADP⁺ and phenazine methosulphate. The rates of oxidation of these substrates were: citrate, 68; *cis*-aconitate, 64; isocitrate, 50 μl. of oxygen/hr./mg. of protein.

Enzymes of the tricarboxylic acid cycle

Since very few of the substrates of the tricarboxylic acid cycle were oxidized either by the intact cells or the cell-free extracts, attempts were directed towards demonstrating the individual enzymes of the tricarboxylic acid cycle and of the electron-transport chain (Tables 2 and 3).

The crude extracts reduced only NADP⁺ but not NAD⁺ in the presence of lactate and α-oxoglutarate, demonstrating the presence of NADP⁺-dependent lactate and α-oxoglutarate dehydrogenase (Fig. 1). For pyruvate-dehydrogenase activity, both NADP⁺ and CoA were essential. There was no reduction of NAD⁺ even with added

CoA (Fig. 1). Isocitrate also catalysed the reduction of NADP⁺. However, the sonic extracts exhibited NAD⁺-linked malate-dehydrogenase activity at alkaline pH values. The reduction of NAD⁺ in the presence of malate was negligible at pH 7.0 and increased with increase in pH up to 10.0. The activity of malic enzyme was tested by NADP⁺ reduction in the presence of Mn²⁺ ions and with malate as the substrate at pH 7.0; at this pH malate dehydrogenase does not interfere, since the latter requires high pH values for its activity (Ochoa, 1955). There was no reduction of NADP⁺ under these conditions, indicating the absence of malate enzyme.

Evidence for the presence of aconitase was already indicated by stimulation of oxidation of citrate, *cis*-aconitate and isocitrate by NADP⁺. Its presence was further confirmed by the rapid increase in the extinction values at 240 mμ in the presence of citrate as the substrate. Succinic dehydrogenase and fumarase were also present.

The sonic extracts had condensing-enzyme activity because citric acid was synthesized from oxaloacetate and acetyl-CoA. There was also phos-

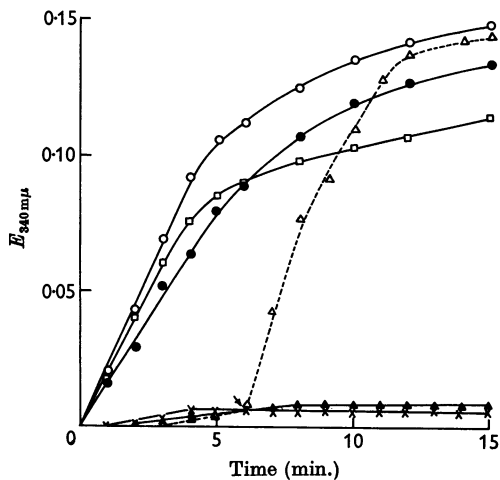


Fig. 1. Lactate, pyruvate and α-oxoglutarate dehydrogenases in cell-free extracts of *M. tuberculosis* H₃₇R_v. The cuvettes contained (in 3 ml.): phosphate buffer (pH 7.0), 100 μmoles; NADP⁺, 0.28 μmole; NAD⁺, 0.5 μmole; lactate or pyruvate, 20 μmoles; α-oxoglutarate, 50 μmoles; enzyme, 2.5 mg. of protein. For pyruvate dehydrogenase the system contained, in addition: MgSO₄, 10 μmoles, and CoA (where added), 0.15 μmole. ○, Lactate + NADP⁺; ▲, lactate + NAD⁺; ●, pyruvate + NADP⁺ + CoA; ×, pyruvate + NAD⁺ + CoA; △, pyruvate + NADP⁺ (CoA omitted); at the point indicated by the arrow CoA was added; □, α-oxoglutarate + NADP⁺. With NAD⁺ there was no reduction. Reaction was started with NADP⁺ or NAD⁺. Correction was applied for the endogenous reduction of NADP⁺ in the absence of substrate.

Table 1. *Effect of various electron acceptors on the oxidation of lactate by sonic extracts of Mycobacterium tuberculosis H₃₇R_v*

The Warburg flasks contained, in 3 ml.: phosphate buffer (pH 7.0), 50 μmoles; nicotinamide, 30 μmoles; cysteine, 10 μmoles; MgSO₄, 10 μmoles; electron acceptor, 3 μmoles, final concn. 1 mM (except cytochrome *c*, 0.03 μmole, final concn. 0.01 mM); ATP, 5 μmoles; NAD⁺, 0.5 μmole; NADP⁺, 0.14 μmole; enzyme, 2.5 mg. of protein. Lactate (75 μmoles) was tipped from the side arm after equilibration for 10 min.; temperature 37°; gas phase air; the centre well contained 0.1 ml. of 20% KOH.

Acceptor	Oxygen consumed (μl./hr./mg. of protein)
None	4
Phenazine methosulphate	53
Menadione (vitamin K ₃)	36
Methylene blue	17
Dichlorophenol-indophenol	9
Ascorbic acid	6
Vitamin K ₁	6
Cytochrome <i>c</i>	6
Ferricyanide	5
Triphenyltetrazolium chloride	4

Table 2. *Enzymes of the tricarboxylic acid cycle in sonic extracts of Mycobacterium tuberculosis H₃₇R_v*

Assay systems: (a) Pyruvate, α -oxoglutarate and lactate dehydrogenase: by NADP⁺ reduction as described in Fig. 1. (b) Isocitrate dehydrogenase (in 3 ml.): tris buffer (pH 7.0), 50 μ moles; MnSO₄, 15 μ moles; NADP⁺, 1 μ mole; DL-isocitrate, 3 μ moles; enzyme, 0.5 mg. of protein. (c) Malate dehydrogenase (in 3 ml.): glycine buffer (pH 10), 100 μ moles; NAD⁺, 1 μ mole; malate, 30 μ moles; enzyme, 0.3 mg. of protein. (d) Succinate, pyruvate and α -oxoglutarate dehydrogenases (by reduction with K₃Fe(CN)₆), in 3 ml.: phosphate buffer (pH 7.0), 50 μ moles; KCN, 10 μ moles; K₃Fe(CN)₆, 1 μ mole; MgSO₄, 5 μ moles; substrate, 30 μ moles; for pyruvate and α -oxoglutarate dehydrogenases, 1 μ mole of thiamine pyrophosphate; enzyme, 2.5 mg. of protein. (e) Aconitase (in 3 ml.): phosphate buffer (pH 7.0), 220 μ moles; citrate, 30 μ moles; enzyme, 0.4 mg. of protein. (f) Fumarase (in 3 ml.): phosphate buffer (pH 7.0), 100 μ moles; fumarate, 75 μ moles; enzyme, 0.9 mg. of protein. (g) Condensing enzyme (in 3 ml.): phosphate buffer (pH 7.2), 60 μ moles; oxaloacetate, 5 μ moles; cysteine, 10 μ moles; acetyl-CoA (where added), 0.15 μ mole; lithium acetyl phosphate, 5 μ moles; CoA, 0.15 μ mole; enzyme, 5 mg. of protein. (h) Isocitratase (in 3 ml.): tris buffer (pH 7.5), 100 μ moles; MgSO₄, 10 μ moles; cysteine, 5 μ moles; DL-isocitrate, 5 μ moles; enzyme, 5 mg. of protein; incubated under N₂ for 10 min. at 37°. (i) Malate synthetase (in 2 ml.): phosphate buffer (pH 7.7), 100 μ moles; ATP, 10 μ moles; MgSO₄, 10 μ moles; labelled acetate, 10 μ c; glyoxylate, 10 μ moles; enzyme, 5 mg. of protein.

Enzyme	Substrate	Measurement made	Cofactors added	Activity (μ moles/hr./mg. of protein)
Aconitase	Citrate	Increase in <i>E</i> at 240 m μ	—	9.22
Isocitrate dehydrogenase	Isocitrate	NADP ⁺ reduction (at 340 m μ)	NADP ⁺ , Mn ²⁺ ions	5.22
α -Oxoglutarate dehydrogenase	α -Oxoglutarate	NADP ⁺ reduction (at 340 m μ)	NADP ⁺ , Mg ²⁺ ions	0.21
		K ₃ Fe(CN) ₆ reduction (at 410 m μ)	Mg ²⁺ ions, thiamine pyrophosphate	0.42
Succinate dehydrogenase	Succinate	K ₃ Fe(CN) ₆ reduction (at 410 m μ)	—	0.42
Fumarase	Fumarate	Fall in <i>E</i> at 300 m μ	—	38.00
Malate dehydrogenase	Malate	NAD ⁺ reduction (at 340 m μ)	NAD ⁺	2.58
Condensing enzyme	Oxaloacetate and acetyl-CoA	Citrate formation	—	0.37
Condensing enzyme and phosphotransacetylase	Oxaloacetate and acetyl phosphate	Citrate formation	CoA	1.02
Pyruvate dehydrogenase	Pyruvate	NADP ⁺ reduction (at 340 m μ)	Mg ²⁺ ions, NADP ⁺ , CoA	0.18
		K ₃ Fe(CN) ₆ reduction (at 410 m μ)	Mg ²⁺ ions, thiamine pyrophosphate	0.42
Lactate dehydrogenase	Lactate	NADP ⁺ reduction (at 340 m μ)	NADP ⁺	0.24
Isocitratase	Isocitrate	Glyoxylate (at 420 m μ)	Mg ²⁺ ions, cysteine	0.3
Malate synthetase	Glyoxylate and [¹⁴ C]acetate	Radioactivity in malate	CoA	2981 counts/min. (total in malate)

phosphotransacetylase activity, since citrate was formed from oxaloacetate, acetyl phosphate and CoA. Inclusion of green-gram phosphotransacetylase resulted in only a slight increase in the amount of citric acid formed, from 1.02 to 1.20 μ moles/hr./mg. of protein.

The cell-free extracts catalysed the formation of glyoxylate from DL-isocitrate in the presence of Mg²⁺ ions and cysteine. The crude extracts also contained malate-synthetase activity, as shown by the observation that labelled malate was formed from [2-¹⁴C]acetate and glyoxylate. From 10 μ c of radioactive acetate, the malate spot contained 2981 counts/min. in the presence of glyoxylate, compared with 844 in its absence.

Enzymes of the electron-transport chain

Since the NADH-oxidase and NADPH-oxidase and transhydrogenase have a great controlling effect on the respiration by regulating the amounts of the available oxidized coenzymes, the presence of these enzymes in *M. tuberculosis* H₃₇R_v was sought. The sonic extracts have a very low NADPH-oxidase activity but a relatively high NADH-oxidase activity, which was not inhibited by mm-cyanide (Table 3). The method of Hochster & Katznelson (1958), which is applicable for this type of enzyme preparation, did not show transhydrogenase activity (Table 3).

It is evident from Table 3 that diaphorase is

Table 3. *Enzymes of the electron-transport chain in sonic extracts of Mycobacterium tuberculosis H₃₇R₆*

Assay systems: (a) NADH- and NADPH-oxidase and transhydrogenase (in 3 ml.): phosphate buffer (pH 7.0), 300 μ moles; MgSO₄, 10 μ moles; NADH or NADPH, approx. 0.1 μ mole; NAD⁺, 0.5 μ mole; enzyme, 1.5 mg. of protein; after the rate of auto-oxidation of NADH or NADPH was followed for 3 min. the reaction was started with enzyme. (b) Diaphorase (in 3 ml.): phosphate buffer (pH 7.2), 300 μ moles; MgSO₄, 10 μ moles; NADH or NADPH, 0.1 μ mole; dichlorophenol-indophenol, 0.1 ml. of aqueous 0.05% solution; enzyme, 0.8 mg. of protein. (c) NADH- and NADPH-cytochrome *c* reductase (in 1 ml.): phosphate buffer (pH 7.2), 80 μ moles; MgSO₄, 5 μ moles; cytochrome *c*, 0.02 μ mole; NADH or NADPH, 0.1 μ mole; FMN, FAD, vitamin K₃ or K₁, 0.01 μ mole; enzyme, 0.8 mg. of protein; correction for non-enzymic reduction (if any) of cytochrome *c* was applied. (d) Menadione reductase (in 3 ml.): tris buffer (pH 8.0), 30 μ moles; NADH or NADPH, 0.15 μ mole; vitamin K₃ or K₁, 0.1 μ mole (in 0.05 ml. of ethanol); enzyme, 0.8 mg. of protein; correction for the absorption due to vitamin K₃ or K₁ was applied.

Enzyme	Substrate	Measurement made	Cofactors added	Activity (μ moles/hr./mg. of protein)
NADH-oxidase	NADH	NADH oxidation (at 340 m μ)	Mg ²⁺ ions	0.90
NADPH-oxidase	NADPH			0.17
Transhydrogenase	NADPH and NAD ⁺			0.17
Diaphorase	NADH	Dichlorophenol-indophenol reduction (at 600 m μ)	Mg ²⁺ ions	0.88
	NADPH			0.48
NADH-cytochrome <i>c</i> reductase	NADH and cytochrome <i>c</i>	Cytochrome <i>c</i> reduction (at 550 m μ)	Mg ²⁺ ions	0.80
NADPH-cytochrome <i>c</i> reductase	NADPH and cytochrome <i>c</i>			0.27
NADH-menadione reductase	NADH and menadione	NADH oxidation (at 340 m μ)	—	5.46
NADPH-menadione reductase	NADPH and menadione	NADPH oxidation (at 340 m μ)	—	0.24

present in crude extracts. NADH and NADPH caused rapid reduction of dichlorophenol-indophenol. This reduction was not affected by mm-cyanide.

The fresh cell-free extracts exhibited both NADH- and NADPH-cytochrome *c*-reductase activities. Externally added FAD, FMN and vitamins K₃ and K₁ had little effect on the reduced nicotinamide-adenine nucleotide-cytochrome *c* reductases. However, when the extract was dialysed, both NADH- and NADPH-cytochrome *c*-reductase activities were considerably decreased. With dialysed enzyme the NADH-cytochrome *c*-reductase activity was restored by externally added vitamin K₃ and FMN, but not by vitamin K₁ or FAD. These results are in agreement with those of Weber & Brodie (1957) obtained with *Mycobacterium phlei*. The NADPH-cytochrome *c* reductase was stimulated only by menadione but not by FMN, FAD or vitamin K₁. The absence of stimulatory effect of FMN was in contrast with that observed with NADH-cytochrome *c* reductase.

Sonic extracts were unable to re-oxidize reduced cytochrome *c* in a system containing phosphate buffer, pH 7.2, and Mg²⁺ ions, thus indicating the lack of cytochrome oxidase in the extracts. Rat-liver mitochondria served as a positive control.

The results presented in Table 3 show that NADH was rapidly oxidized in the presence of

menadione. In contrast, vitamin K₁ did not enhance the rate of oxidation of NADH above that of the endogenous activity. The rate of menadione catalysed NADH oxidation was 5.46, as against an endogenous value of 0.76 μ mole/hr./mg. of protein. The slight NADPH-oxidase activity of the extracts was stimulated to a minor extent by menadione but vitamin K₁ did not affect it.

DISCUSSION

The cell-free extracts of *M. tuberculosis* oxidized only a few intermediates of the tricarboxylic acid cycle. However, the fact that citrate, *cis*-aconitate and isocitrate, which were not utilized and which often showed slight inhibition of the endogenous respiration of the intact cells, were oxidized by cell-free extracts in the presence of an artificial electron acceptor offers definite evidence that the cells of this bacillus are impermeable to these substrates. The inability of even the cell-free extracts to consume oxygen in the presence of these substrates may be due to the inactivation of cytochrome oxidase, which has been shown (Frei, Riedmuller & Almasy, 1934) to be present in the H₃₇R₆ strain during the extraction procedure. In the present work, the activity of the enzymes of the tricarboxylic acid cycle in the cell-free extracts has been detected by the standard assay procedures

for the individual enzymes. The high activity of aconitase and fumarase as compared with other enzymes (shown in Table 2) may be due to the ease with which these enzymes are known to be released from particles. All the respiratory enzymes, other than cytochrome oxidase, have been shown to be present in the cell-free extracts. The presence of cytochrome *a*, *b* and *c* has already been reported (Frei *et al.* 1934).

Though the cell-free extracts of *M. tuberculosis* have all the normal components of the tricarboxylic acid cycle, the dehydrogenases of the cycle, unlike most other biological systems, are all NADP⁺-dependent, with the exception of malate dehydrogenase, which shows activity with NAD⁺ at a high pH. The inability to detect the reduction with the other dehydrogenases may be due to the presence of the potent NADH-oxidase in the extracts, which apparently prevents even the NAD⁺ reduction with malate unless it is carried out at a pH unfavourable to the activity of the oxidase. By analogy with mammalian organs that are highly active biosynthetically (Lowenstein, 1961), it is probable that the presence of NADP⁺-requiring dehydrogenases in *M. tuberculosis* guarantees a constant high concentration of NADPH as a reducing agent for its metabolic processes, and the presence of NADH-oxidase in the organism ensures that sufficient NAD⁺ is always available as an oxidizing agent in these processes.

In addition to the normal constituents of the tricarboxylic acid cycle, *M. tuberculosis* also possesses phosphotransacetylase activity and the enzymes of the glyoxylate bypass. Isocitratase, which is generally associated with bacteria grown on C₂ compounds such as acetate, and whose synthesis is in fact suppressed by the presence of glucose and glycerol in the growth media (Smith & Gunsalus, 1955), is found in glycerol-grown cells of *M. tuberculosis* H₃₇R_v. The glyoxylate cycle acts as a link in the formation of carbohydrates from fatty acids and occurs in seedlings rich in oil such as castor bean (*Ricinus communis*) (Kornberg & Beevers, 1957) and peanut (*Arachis*) (Bradbeer, 1958). It is therefore significant that the enzymes of this cycle are present in *M. tuberculosis*, which has a very high lipid content.

The cell-free extracts also appear to contain all the enzymes of the respiratory chain with the exception of cytochrome oxidase. Those present include NADH-oxidase, NADH- and NADPH-cytochrome *c* reductase, diaphorase and menadione reductase. Since the rate of NADH oxidation is not affected by cyanide it is apparent that cytochrome oxidase is not involved in this process. This activity, as well as the slow oxidation of lactate in the absence of any added electron acceptor, may be explained either by the presence of an auto-

oxidizable natural electron acceptor, released during disruption of the cells, or by assuming that a peroxidase-catalysed reaction (Dolin, 1954) is taking place in both cases.

The presence of menadione reductase in the extracts and the stimulation by menadione of lactate oxidation and NADH- and NADPH-cytochrome *c* reductase are of interest in the light of the reported occurrence of vitamin K in *M. tuberculosis* (Fieser, Campbell & Fry, 1939; Francis, Madinavertia, Mac Turc & Snow, 1949) and the recent reports of the implication of vitamin K in electron transport (Martius, 1955; Weber & Brodie, 1957). The possibility that vitamin K may function in electron-transport reactions of *M. tuberculosis* is as yet based on circumstantial evidence. However, it is likely that the very potent menadione-reducing activity of the extracts, similar to that found in heart muscle (Colpa-Boonstra & Slater, 1957) and in *Streptococcus faecalis* (Dolin & Wood, 1960), may reflect a physiological function of vitamin K in the organism. The inhibition by dicoumarol of the menadione-stimulated oxidation of lactate in the extracts also suggests that this vitamin plays a role in the electron transport. Though dicoumarol has also been shown to inhibit in the region of cytochrome oxidase (Cooper & Lehninger, 1956), this region appears to be absent in the sonic extracts of *M. tuberculosis* H₃₇R_v, as confirmed by the lack of cyanide inhibition; hence dicoumarol may act at the vitamin K region of the electron transport in this organism.

SUMMARY

1. Washed whole-cell suspensions of *Mycobacterium tuberculosis* H₃₇R_v oxidized only pyruvate, acetate, oxaloacetate and lactate to a significant extent. Sonic extracts of the organism utilized lactate and oxaloacetate in the presence of a suitable artificial electron acceptor, such as phenazine methosulphate or menadione, and citrate, *cis*-aconitate and isocitrate only after the addition of NADP⁺ as well as the electron acceptor.

2. All the individual enzymes of the tricarboxylic acid cycle and of the electron-transport chain, with the exception of cytochrome oxidase, were demonstrated to be present in the crude cell-free extracts.

3. Enzymes of the glyoxylate bypass were also present in the cell-free extracts. Isocitratase was present in extracts of the glycerol-grown organisms.

4. Except for malate dehydrogenase, all the other dehydrogenases investigated were found to be NADP⁺- but not NAD⁺-dependent. Malate dehydrogenase was dependent on NAD⁺ only at high pH values, where NADH-oxidase activity is very low.

5. The oxidation of NADH, which was very rapid compared with that of NADPH, was insensitive to cyanide, presumably because cytochrome oxidase is absent.

6. Vitamin K₃, but not vitamin K₁, considerably stimulated the oxidation of NADH. NADH-cytochrome *c*-reductase activity, which was lost on dialysis, was restored by vitamin K₃ or FMN, but not by vitamin K₁ or FAD. But NADPH-cytochrome *c*-reductase activity, which was decreased after dialysis, was restored by vitamin K₃ alone, but not by vitamin K₁ or FAD or FMN.

The authors are grateful to the Indian Council of Medical Research for financial assistance during the course of this investigation and to the Rockefeller Foundation for a grant towards equipment. They are also thankful to Dr T. Ramasarma for helpful discussions.

REFERENCES

- Bradbeer, C. (1958). *Nature, Lond.*, **182**, 1429.
- Colpa-Boonstra, J. P. & Slater, E. C. (1957). *Biochim. biophys. Acta*, **27**, 122.
- Cooper, C. & Lehninger, A. L. (1956). *J. biol. Chem.* **219**, 519.
- Dolin, M. I. (1954). *Arch. Biochem. Biophys.* **45**, 247.
- Dolin, M. I. (1959). *J. Bact.* **77**, 383.
- Dolin, M. I. & Wood, N. P. (1960). *J. biol. Chem.* **235**, 1809.
- Eisenberg, M. A. (1953). *J. biol. Chem.* **203**, 815.
- Fieser, L. F., Campbell, W. P. & Fry, E. M. (1939). *J. Amer. chem. Soc.* **61**, 2206.
- Francis, J., Madinavertia, J., Mac Ture, H. M. & Snow, G. A. (1949). *Nature, Lond.*, **163**, 365.
- Frei, W., Riedmuller, L. & Almsy, F. (1934). *Biochem. Z.* **274**, 253.
- Friedmann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
- Goldman, D. S. (1956*a*). *J. Bact.* **71**, 732.
- Goldman, D. S. (1956*b*). *J. Bact.* **72**, 401.
- Gornall, A. G., Bardawill, C. S. & David, M. M. (1957). In *Methods in Enzymology*, vol. 3, p. 450. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Gradwohl, R. B. H. (1948). In *Clinical Laboratory Methods and Diagnosis*, 4th ed., vol. 2, p. 1356. St Louis: C. V. Mosby and Co.
- Hochster, R. M. & Katznelson, H. (1958). *Canad. J. Biochem. Physiol.* **36**, 669.
- Horecker, B. L. (1955). In *Methods in Enzymology*, vol. 2, p. 704. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Indira, M. & Ramakrishnan, T. (1959). In *Biology and Biochemistry of Micro-organisms*, Golden Jubilee Symp., p. 26. Bangalore: Indian Institute of Science.
- Kornberg, H. L. & Beevers, H. (1957). *Nature, Lond.*, **180**, 35.
- Kornberg, H. L. & Madsen, N. B. (1958). *Biochem. J.* **68**, 549.
- Kusunose, M., Nagai, S., Kusunose, E. & Yamamura, Y. (1956). *J. Bact.* **72**, 754.
- Lawson, G. J. & Hartley, R. D. (1958). *Biochem. J.* **69**, 3*p*.
- Lowenstein, J. M. (1961). *J. biol. Chem.* **236**, 1213.
- Mahler, H. R. (1955*a*). In *Methods in Enzymology*, vol. 2, p. 688. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Mahler, H. R. (1955*b*). In *Methods in Enzymology*, vol. 2, p. 707. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Maitra, P. K. & Roy, S. C. (1961). *Biochem. J.* **79**, 446.
- Martius, C. (1955). *Angew. Chem.* **67**, 161.
- Massey, V. (1955). In *Methods in Enzymology*, vol. 1, p. 729. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Natelson, S., Pincus, J. B. & Lugovoy, J. R. (1948). *J. biol. Chem.* **175**, 745.
- Ochoa, S. (1955). In *Methods in Enzymology*, vol. 1, p. 739. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Racker, E. (1950). *Biochim. biophys. Acta*, **4**, 211.
- Ramakrishnan, T. (1959). *Arch. Biochem. Biophys.* **81**, 525.
- Ramasarma, T. & Ramakrishnan, T. (1961). *Biochem. J.* **81**, 303.
- Slater, E. C. & Bonner, W. D. (1952). *Biochem. J.* **52**, 185.
- Smith, R. A. & Gunsalus, I. C. (1954). *J. Amer. chem. Soc.* **76**, 5002.
- Smith, R. A. & Gunsalus, I. C. (1955). *Nature, Lond.*, **175**, 174.
- Stadtman, E. R. (1957). In *Methods in Enzymology*, vol. 3, p. 931. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Stumpf, P. K., Zarundnaya, K. & Green, D. E. (1947). *J. biol. Chem.* **167**, 817.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques*, p. 7. Minneapolis: Burgess Publishing Co.
- Weber, M. M. & Brodie, A. F. (1957). *Biochim. biophys. Acta*, **25**, 447.
- Wosilait, W. D. & Nason, A. (1954). *J. biol. Chem.* **208**, 785.
- Youmans, A. S., Millman, I. & Youmans, G. P. (1956). *J. Bact.* **71**, 565.
- Youmans, A. S. & Youmans, G. P. (1953). *J. Bact.* **65**, 96.
- Youmans, G. P. & Karlson, A. G. (1947). *Amer. Rev. Tuberc.* **55**, 529.