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VARIATIONS IN THE PATHWAYS OF MALATE OXIDATION AND PHOSPHORYLATION IN DIFFERENT SPECIES OF *MYCOBACTERIA*

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SUMMARY

Mycobacterium tuberculosis $H_{37}Rv$, the slow-growing human pathogenic strain of tubercle bacilli and *Mycobacterium smegmatis* and *Mycobacterium phlei*, the fast-growing saprophytes, have shown variations regarding the type of dehydrogenase that initiates malate oxidation in the respiratory chain.

M. tuberculosis $H_{37}Rv$ is characterized by having a malate oxidase system (designated MAL_{NAD} pathway) in which malate oxidation is mediated by the NAD^+-dependent malate dehydrogenase (EC 1.1.1.37) but not by FAD-dependent malate-vitamin K reductase. *M. smegmatis* possesses a different malate oxidase system (designated MAL_{FAD} pathway) in which malate oxidation is exclusively carried out by the FAD-dependent malate-vitamin K reductase because NAD^+-dependent malate dehydrogenase is absent in this organism. *M. phlei* has a mixed system of malate oxidase (designated $MAL_{NAD+FAD}$ pathways) in which both the NAD^+- and FAD-dependent dehydrogenases take part. In all the three systems, the rest of the electron transport chain is common.

INTRODUCTION

Recent studies in our laboratory have shown that there are qualitative and quantitative variations in the activities of malate dehydrogenases in different species of mycobacteria which grow at different rates. For example, *Mycobacterium tuberculosis* $H_{37}Rv$, the slow-growing human pathogenic strain of tubercle bacilli, contains the usual NAD^+-dependent malate dehydrogenase (EC 1.1.1.37) which is either absent or present in negligible amounts in the fast-growing nonpathogenic

Abbreviations: MTT, 3-(4,5 dimethyl thiozolyl-2)-2,5 diphenyl tetrazolium bromide; HQNO, 2-*n* heptyl 4-hydroxyquinoline-*N*-oxide; DCIP, 2,6-dichlorophenolindophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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strain, *Mycobacterium smegmatis*. On the other hand, *M. smegmatis* possesses malic enzyme (malate dehydrogenase (decarboxylating), EC 1.1.1.40) which is absent in *M. tuberculosis* $H_{37}Rv$. Both the enzymes are present in *Mycobacterium phlei* [1]. These variations in the occurrence of malate dehydrogenases in the three species of *Mycobacterium* have been interpreted in relation to their lipid content.

Malate-vitamin K reductase, a malate dehydrogenase which does not require NAD^+ but requires FAD and phospholipids, capable of mediating malate oxidation has been demonstrated in *M. phlei* [2, 3] although its functional significance is not yet completely understood. There are also differences in the composition of cytochromes in different species of mycobacteria [4]. It is not known as to how these differences affect the mechanisms of oxidative phosphorylation in these species of mycobacteria with malate as substrate. While detailed studies on the mechanisms of electron-transport in *M. phlei* (ATCC 354) have been carried out by Brodie and his co-workers (recently reviewed by Ramakrishnan et al. [5]), there are no studies which throw light on these differences in other species of mycobacteria. Furthermore, *M. phlei* (ATCC 354) is not a true representative of all the other species of mycobacteria at least with regard to oxidation and phosphorylation. We have demonstrated that the overall energy synthesis is greatest in *M. phlei* followed by *M. smegmatis* and *M. tuberculosis* $H_{37}Rv$ [6]. Therefore, comparative studies on the electron-transport mechanisms with particular reference to malate as substrate have been undertaken in *M. smegmatis*, *M. phlei* and *M. tuberculois* $H_{37}Rv$.

MATERIALS AND METHODS

FAD, NAD^+, MTT, DCIP, ADP, phenazine methosulphate and hexokinase were obtained from Sigma Chemical Co., U.S.A. Casamino acids were purchased from Difco Laboratories, Detroit, Mich., U.S.A. Asolectin was the product of Associated Concentrates Inc., New York, U.S.A. Vitamin K-1 was obtained from Mann Research Laboratories, U.S.A. HQNO and CCCP were kindly supplied to us by Dr C. R. Krishna Murti of Central Drug Research Institute, Lucknow, India.

M. smegmatis (NCTC, London) and *M. phlei* (ATCC 354) were grown in Brodie and Gray's medium [7] on a rotary shaker at 37 °C for 18 and 20 h respectively. *M. tuberculosis* $H_{37}Rv$ (NCTC, 7416) was, however, grown in the same medium supplemented with glycerol at a final concentration of 2 % for 5–6 days.

The method followed for the isolation of membranes from *Mycobacterium* species was essentially that of Brodie [8] with slight modifications. Cells were harvested by centrifugation in a refrigerated centrifuge (International Equipment Co., B-20A) and washed three times with ice-cold glass-distilled water, each time suspending them by a magnetic stirrer. All the subsequent operations were carried out at 0–4 °C unless specified otherwise. Cells were suspended in 0.1 M Tris · HCl buffer (pH 7.4) to give a concentration of 400 mg (wet wt) per 1 ml. Aliquots of cell suspensions (10 ml) of *M. smegmatis* and *M. phlei* were sonicated in a Raytheon sonic oscillator (9 KHz) for 4 min and that of *M. tuberculosis* $H_{37}Rv$ for 5 min. The sonicate was centrifuged at $20000 \times g$ for 25 min. The cell-free extracts from each organism were separated into membranes and supernatant fraction by centrifugation at $144000 \times g$ for 60 min in a L2-65B Beckman preparative ultracentrifuge. After removal of the supernatant, the membranes were suspended in 0.1 M Tris · HCl

buffer (pH 7.4) and washed free of supernatant by centrifugation at 144000 × *g* for 30 min. Finally, the membranes were suspended in the same buffer at a concentration of approx. 15 mg protein/ml. These membranes are also referred to as "electron-transport particles".

The cell-free extract and the high-speed supernatant were rapidly dialyzed for 4–6 h against cold glass-distilled water to reduce or remove the endogenous activity.

Malate dehydrogenase [9], malate-vitamin K reductase [3], and succinate dehydrogenase [10] (EC 1.3.99.1) were estimated spectrophotometrically. Oxygen uptake was measured by the conventional manometric technique. The method of Fiske and SubbaRow [11] was used to determine the phosphorus content. Protein was estimated by the method of Stadtman et al. [12]. Inhibitors were solubilized as described by Asano and Brodie [2].

RESULTS

The optimal time required for disrupting the cells of each species of *Mycobacterium* was ascertained by studying the efficiency of the cell-free extracts to yield maximum P:O ratios with malate as substrate (Table I). Oxidation of malate was not affected appreciably even with up to 7 min of sonication, whereas phosphorylation was sensitive to sonication after 4 min in the case of *M. smegmatis* and *M. phlei* and 5 min in the case of *M. tuberculosis* $H_{37}Rv$. The sharp decline in P:O ratio after 4–5 min sonication probably indicates the dependence of coupling activity on the structural integrity of the membranes.

Using conditions which gave maximum P:O ratios, the distribution of the activity of malate dehydrogenase and malate-vitamin K reductase was studied in the

TABLE I

EFFECT OF TIME OF SONICATION ON OXIDATIVE PHOSPHORYLATION WITH MALATE IN MYCOBACTERIA

The main compartment of the Warburg flask contained in 3 ml: inorganic phosphate, 10 μmol; $MgCl_2$, 15 μmol; Tris · HCl buffer (pH 7.4), 100 μmol; glucose, 20 μmol; hexokinase 2 mg and cell-free extracts, 12 mg protein. The side arm of the flask received ADP, 2.5 μmol; KF, 25 μmol and potassium malate, 50 μmol. In the experiments with *M. smegmatis*, 25 nmol FAD and in those with *M. phlei* and *M. tuberculosis* $H_{37}Rv$, 0.5 μmol NAD^+ were added. After equilibration for 15 min the reaction was stopped by the addition of 1.0 ml of 10 % cold trichloroacetic acid and the deproteinized supernatant analysed for phosphorus content. Results represent the average of three experiments.

Time of sonication (min)	*M. smegmatis*		*M. phlei*		*M. tuberculosis* $H_{37}Rv$	
	μatoms O_2 consumed	P : O	μatoms O_2 consumed	P : O	μatoms O_2 consumed	P : O
2	7.4	0.46	6.2	0.90	2.6	0.80
3	7.5	0.48	6.4	1.10	3.4	0.80
4	8.2	0.55	6.5	1.24	3.6	0.86
5	8.3	0.52	6.1	1.15	3.7	0.90
6	8.2	0.46	6.0	0.80	3.8	0.82
7	8.0	0.30	5.7	0.64	3.0	0.62

TABLE II

LOCALIZATION AND SPECIFIC ACTIVITY OF MALATE AND SUCCINATE DEHYDROGENASES IN MYCOBACTERIA

The assay system for malate dehydrogenase contained in 3 ml: 180 μmol glycine buffer (pH 10.0); 1.0 μmol NAD^+ and enzyme (0.2–1.0 mg protein). The reduction of NAD^+ was followed at 340 nm after the addition of 25 μmol of L-malate. The reaction mixture for the assay of malate-vitamin K reductase contained in 3 ml: 200 μmol Tris · HCl buffer (pH 7.4), 100 μmol KCl, 30 μmol $MgCl_2$, 0.35 mg asolectin 0.5 μmol vitamin K-2, 25 nmol FAD, 250 nmol MTT and enzyme (0.01–0.1 mg protein). The reaction was started by the addition of 25 μmol L-malate and MTT reduction was followed at 565 nm. Succinate dehydrogenase was assayed by the phenazine methosulphate-mediated reduction of DCIP. The assay system contained in 3 ml: 50 μmol Tris · HCl buffer (pH 7.4), 10 μmol potassium cyanide, 20 nmol phenazine methosulphate, 100 nmol DCIP and enzyme (0.5–1.0 mg protein). Reaction was initiated with 25 μmol succinate and the reduction of DCIP was followed at 600 nm. Results, the average of three experiments, are specific activities expressed as nmol cofactor or dye reduced per min per mg protein.

Enzyme	*M. smegmatis*		*M. phlei*		*M. tuberculosis* $H_{37}Rv$	
	Membranes	Supernatant	Membranes	Supernatant	Membranes	Supernatant
Malate dehydrogenase	—	0.2	—	166.0	—	345.0
Malate-vitamin K reductase	145.0	21.0	110.0	87.0	1.4	1.5
Succinate dehydrogenase	6.6	0.7	23.1	1.9	6.0	1.1

membranes and supernatant fractions of *M. smegmatis*, *M. phlei* and *M. tuberculosis* $H_{37}Rv$, (Table II). The distribution of succinate dehydrogenase was also studied in order to assess whether the membranes are intact or not. 90 % of the total succinate dehydrogenase was found to be localized in the membranes indicating that the membranes are probably relatively intact.

The results in Table II show that *M. tuberculosis* $H_{37}Rv$ had NAD^+-dependent malate dehydrogenase which was exclusively found in the supernatant fraction, but it had negligible activity of malate-vitamin K reductase. On the other hand, *M. smegmatis* is characterized by highly active malate-vitamin K reductase most of which was localized in the membranes. *M. phlei* contained equal amounts of both types of dehydrogenases. Malate dehydrogenase was found in the supernatant fraction while malate-vitamin K reductase was distributed in both the membranes and soluble fraction. The presence of higher amounts of malate-vitamin K reductase in the supernatant fraction of *M. phlei* than in *M. smegmatis* probably indicates that the enzyme is more firmly bound to the membranes in the latter organism than in the former.

Oxidative phosphorylation with malate was studied in the three species of mycobacteria and results presented in Table III. In *M. smegmatis*, oxidation and coupled phosphorylation were elicited by the membranes alone i.e., even without added supernatant or cofactors. Addition of either FAD or NAD^+ as cofactors did not influence significantly either the oxidation or phosphorylation indicating that oxidative phosphorylation was due to membrane-bound cofactor(s) which appeared to be FAD for reasons mentioned later. Supplementation of membranes with

TABLE III

EFFECT OF COFACTORS ON OXIDATIVE PHOSPHORYLATION WITH MALATE BY SUBCELLULAR FRACTIONS OF MYCOBACTERIA

The experimental details were the same as described under Table I. However, the cell-free extracts were replaced by membranes and supernatant fraction. Membranes; 5, 7 and 10 mg protein from *M. smegmatis*, *M. phlei* and *M. tuberculosis* $H_{37}Rv$, respectively; and supernatant, 10 mg protein from each organism were added as indicated below. 25 nmol of FAD and 0.5 μmol of NAD^+ were added wherever indicated. Results are the average of three experiments.

Organism and system	Cofactor					
	None		FAD		NAD^+	
	μatoms O_2 consumed	P : O	μatoms O_2 consumed	P : O	μatoms O_2 consumed	P : O
M. smegmatis						
Membranes	8.5	0.70	9.0	0.72	7.7	0.68
+supernatant	7.0	0.49	7.0	0.50	6.5	0.55
M. phlei						
Membranes	—	—	1.5	0.42	—	—
Membranes +supernatant	—	—	5.2	0.71	7.0	1.12
M. tuberculosis $H_{37}Rv$						
Membranes	—	—	—	—	—	—
Membranes +supernatant	—	—	—	—	3.6	0.98

the supernatant fraction resulted in slight inhibition of malate oxidation instead of showing any stimulation. As expected from the data on the localization of dehydrogenases (Table II), the membrane fraction of *M. tuberculosis* $H_{37}Rv$ could not oxidize malate unless the supernatant fraction which contains the malate dehydrogenase and NAD^+ as cofactor were added. P:O ratio associated with NAD^+-pathway (1.0) was slightly higher than that with FAD - pathway (0.7).

In order to see whether the observed differences in malate oxidation were in anyway related to the differences in the composition of cytochromes reported earlier by Kusaka et al. [4], difference spectra were taken (Fig. 1) with malate as substrate using the systems from *M. smegmatis* (Curve A) and *M. tuberculosis* $H_{37}Rv$ (Curve B). In both the species, all the cytochromes of the type *b*, *c* and *a* were reduced by malate as indicated by their α-peaks at 562, 551 and 602 nm, respectively. The participation of flavoprotein in malate oxidation, by both the species is evident from its characteristic trough at 460 nm.

In order to gain an insight into the nature and sensitivity to uncouplers of the respiratory chain associated with malate oxidation in *M. smegmatis* and *M. tuberculosis* $H_{37}Rv$, the effect of some inhibitors and uncouplers was studied (Table IV). Atebrin was a potent inhibitor of oxidative phosphorylation, confirming the participation of flavoprotein in malate oxidation by both the species. The inhibition by dicumarol of malate oxidation is an indication of the probable role of vitamin K in malate oxidation. The involvement of terminal oxidases of *M. smegmatis* and *M. tuberculosis* $H_{37}Rv$ in malate oxidation is evident from the inhibition of oxidation by cyanide. 2,4-Dinitrophenol was not an effective uncoupler of oxidative phosphoryla-

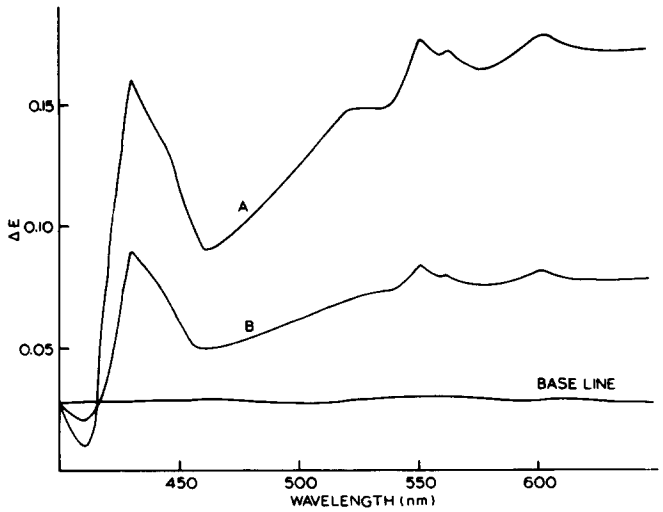

Fig. 1. Malate reduced minus oxidized difference spectra. Curve A: Malate reduced minus oxidized difference spectrum by *M. smegmatis*. The cuvettes with 1-cm light path contained in 3.0 ml the following: 100 μmol of Tris · HCl buffer (pH 7.4), 10 μmol of inorganic phosphate, 15 μmol of $MgCl_2$, 5 μmol of ADP and 15 mg protein of membranes. 50 μmol of malate were added to the sample cuvette and the reference cuvette was kept in the oxidized state by bubbling O_2. After the contents of the sample cuvette reached the anaerobic state (approx. 15 min) the spectrum was recorded. Curve B: Malate reduced minus oxidized difference spectrum by *M. tuberculosis* $H_{37}Rv$. The experimental conditions were the same as described for Curve A. In addition, the cuvettes contained 1.0 μmol NAD^+ and 10 mg protein of supernatant fraction.

TABLE IV

EFFECT OF INHIBITORS AND UNCOUPLERS ON OXIDATIVE PHOSPHORYLATION WITH MALATE BY *M. SMEGMATIS* AND *M. TUBERCULOSIS* $H_{37}Rv$

The system was similar to that described in Table I. However, 6 mg protein of membranes from *M. smegmatis* and 8 and 12 mg protein of membranes and supernatant fraction, respectively, from *M. tuberculosis* $H_{37}Rv$ were added in place of cell-free extracts. Membranes and supernatant fraction were incubated with the inhibitors or uncouplers for at least 12 min before the addition of malate (50 μmol). Reaction was run for 15 min at 30 °C. The results with *M. smegmatis* are the average of four experiments and those with *M. tuberculosis* $H_{37}Rv$ represent the mean of duplicates.

Inhibitor or uncoupler	Concentration (M)	*M. smegmatis* % inhibition of:		*M. tuberculosis* $H_{37}Rv$ % inhibition of:	
		Oxidation	Phosphorylation	Oxidation	Phosphorylation
Atebrin	$3 \cdot 10^{-3}$	98	100	95	100
Dicumarol	$1 \cdot 10^{-3}$	98	96	90	100
KCN	$3 \cdot 10^{-2}$	97	100	100	100
DNP	$5 \cdot 10^{-4}$	29	43	25	50
CCP	$1 \cdot 10^{-5}$	25	100	15	95

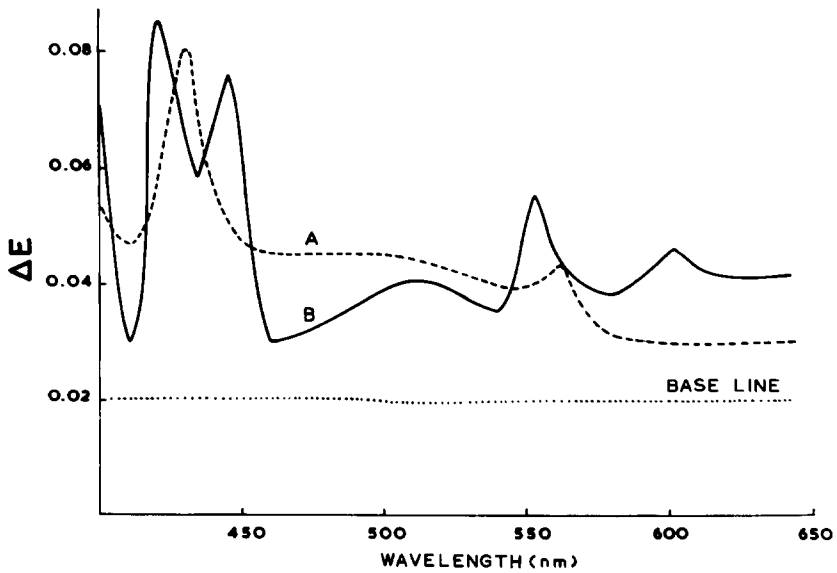

Fig. 2. Sequential reduction of cytochromes in *M. smegmatis*. Curve A: The system consisted of 100 μmol of Tris · HCl buffer (pH 7.4), 10 μmol of inorganic phosphate, 15 μmol of $MgCl_2$, 5 μmol of ADP and membranes (13 mg of protein) in a total volume of 3.0 ml. HQNO (2 μg/mg of protein) was added to both sample and reference cuvettes. After 10 min of incubation with HQNO, 50 μmol of malate were added to the sample cuvette. The contents of the reference cuvette were saturated with oxygen. Reduced minus oxidized difference spectrum was recorded after 12 min. Curve B: The system was similar to that described for Curve A. 25 μmol of ascorbate and 100 nmol of phenazine methosulphate were added to the sample cuvette and the reference cuvette was kept in an oxidized state by supplying a stream of oxygen. Difference spectrum was recorded as for Curve A.

tion in both the species. Many microbial systems are known to be less sensitive to 2,4-dinitrophenol [13]. CCCP, a potent uncoupling agent with mitochondrial systems [14], was found to effectively uncouple oxidative phosphorylation in both the species without appreciably affecting the oxidation rate.

The order of cytochromes in the respiratory chain of *M. smegmatis* was then studied (Fig. 2). In presence of HQNO which blocks the electron-transport at cytochrome *b* level, the addition of malate to membranes resulted in the reduction of only cytochrome *b* which showed α- and γ-peaks at 562 and 430 nm respectively (Fig. 2A). There was no reduction of cytochromes *c* and *a*; thus showing that cytochrome *b* is before cytochromes *c* and *a*.

It is known that electron donors like ascorbate plus TMPD [15] and ascorbate plus phenazine methosulphate [16] enter the respiratory chain at cytochrome level and then reduce cytochrome *a*. Upon the addition of ascorbate plus phenazine methosulphate [16] to the membranes of *M. smegmatis*, absorption bands appeared at 551 and 420 nm corresponding to the α- and γ-peaks of cytochrome *c* and at 602 and 445 nm representing those of cytochrome *a* (Fig. 2B). Under these conditions cytochrome *b*, which is present before cytochromes *c* and *a*, was not reduced and consequently absorption peaks were not observed. Similar results were obtained with the preparations of *M. tuberculosis* $H_{37}Rv$.

DISCUSSION

Malate oxidation in different species of mycobacteria is not initiated by the same dehydrogenase. In view of the fact that the NAD^+-dependent malate dehydrogenase is either absent or present in negligible amounts (Table I) in *M. smegmatis*, the pathway of malate oxidation in this organism seems to be exclusively by the FAD-dependent malate-vitamin K reductase. NAD^+, although present in the membranes of *M. smegmatis* (fluorometrically estimated), does not appear to function in malate oxidation because no absorption was observed at 340 nm after the addition of malate. There is no other report of an organism having malate oxidation and associated phosphorylation exclusively by the FAD-pathway although there are reports of the presence of malate dehydrogenase requiring FAD in other microorganisms like *Mycobacterium avium* [17], *Micrococcus lysodeikticus* [18], *Pseudomonas ovalis* Chester [19] and *Moraxella lwoffi* [20].

In *M. phlei*, malate-vitamin K reductase has been shown for the first time to participate in the electron transport chain in addition to the usual NAD^+-dependent malate dehydrogenase [2, 3]. The results in Tables II and III with *M. phlei* confirm this observation. However, in slow growing *M. tuberculosis* $H_{37}Rv$, malate oxidation and coupled phosphorylation are by the usual NAD^+-pathway only.

In these three species of mycobacteria, full complement of cytochromes of the type *b*, *c* and *a* are reduced by malate (Fig. 1 and ref. 2). Oxidative phosphorylation with malate has been lost on irradiation with ultraviolet light (360 nm) and is restored by the addition of vitamin K-1 (results not shown). In view of the extensive studies carried out with *M. phlei* by Brodie and his co-workers (reviewed by Brodie and Gutnick [13]), this observation has been taken as evidence for the participation of vitamin K in the electron transport pathways of *M. smegmatis* and *M. tuberculosis* $H_{37}Rv$. It thus appears that the observed differences in the pathways of oxidative phosphorylation with malate lie probably in the dehydrogenases present in the three species of mycobacteria i.e., before vitamin K and the cytochromes (Fig. 3).

The three species of mycobacteria investigated here represent three different systems involved in malate oxidation and phosphorylation. The first type of malate oxidase system (designated as MAL_{NAD} pathway) is characterized by *M. tuberculosis* $H_{37}Rv$ in which malate oxidation is initiated by NAD^+-dependent malate dehydrogenase only but not by malate-vitamin K reductase. The second type of malate oxidase system (designated MAL_{FAD} pathway) is present in *M. smegmatis* in which malate oxidation is exclusively by the FAD-pathway but not by DAN^+-pathway. The third type of malate oxidase system (designated $MAL_{NAD+FAD}$ pathways) is represented by *M. phlei* which possesses a mixed system in which the NAD^+- and FAD-dependent

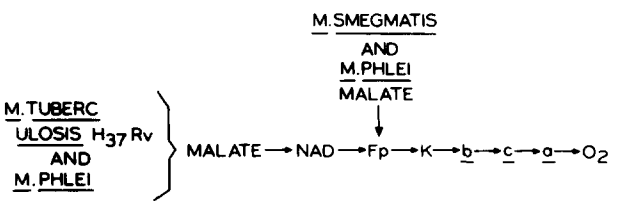

Fig. 3. Pathways of malate oxidation in mycobacteria.

malate dehydrogenases share the responsibilities of generating ATP associated with malate oxidation. Fig. 3 shows the respiratory chains applicable for malate oxidation in different species of mycobacteria. It is expected that all the other species of mycobacteria or microorganisms in general may belong to one of the three types mentioned above as far as malate oxidation is concerned.

Brodie and Gutnick [13] postulated that oxaloacetate formed from the particulate malate-vitamin K reductase pathway could be reduced to malate by extraparticulate malate dehydrogenase and NADH. However, our studies do not support this view since *M. smegmatis* does not have NAD^+-dependent malate dehydrogenase. It is quite possible that these two malate dehydrogenases have significance from an evolutionary point of view, at least in the case of mycobacteria. In *M. smegmatis*, which lacks NAD^+- dependent malate dehydrogenase FAD-pathway is the only source of generation of ATP from malate oxidation. During the evolutionary process, species like *M. tuberculosis* $H_{37}Rv$ might have found it economical to oxidize malate also by the same pathway as that for the other NAD^+-linked substrates. The situation with *M. phlei* probably represents an intermediate stage in which both the pathways are present.

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