# SUCCINIC DEHYDROGENASE IN THE PARTICULATE FRACTION OF *MYCOBACTERIUM AVIUM*[1]

MASAMICHI KUSUNOSE, SADAMU NAGAI, EMI KUSUNOSE, AND YUICHI YAMAMURA

*Research Laboratory of National Sanatorium, Toneyama Hospital, and the Toneyama Institute of Tuberculosis, University of Osaka City Medical School, Toyonaka, Osaka, Japan*

Received for publication May 10, 1956

Previously, all the enzymes involved in the tricarboxylic acid cycle were extracted in the cell-free state from *Mycobacterium avium*, and some properties of these enzymes were described (Kusunose *et al.*, 1952; Yamamura *et al.*, 1954). More recently, these enzymes were separated into particulate and soluble fractions by differential centrifugation, and it was found that the former fraction contained L-malic dehydrogenase, aconitase, fumarase, and oxalacetic decarboxylase (Yamamura *et al.*, 1955). However, it was noticed that succinoxidase activity was not shown in either particulate or soluble fraction alone, although the combination of the two fractions showed the apparent activity.

Therefore, the present study was undertaken in order to determine which fraction contained succinic dehydrogenase and to elucidate the mechanism of the combined effect.

## MATERIALS AND METHODS

*Preparation of particulate and soluble fractions.* *Mycobacterium avium* strain Takeo was grown in glycerol-bouillon medium for 4 days, as described previously (Yamamura *et al.*, 1952). About 100 g of wet cells were ground with 100 g of sea-sand in a chilled mechanical mortar for 40 min, mixed with 700 ml of 0.25 M sucrose, and centrifuged at 3,000 rpm for 15 min. The supernatant fluid was centrifuged in the Spinco model L preparative centrifuge at 12,000 × G for 60 min. The precipitate formed was discarded, and the supernatant fluid was then centrifuged at 100,000 × G for 40 min. The reddish pellet formed was washed twice with 0.25 M sucrose by centrifugation at 100,000 × G for 30 min., and finally suspended in 20 ml of 0.25 M sucrose. This insoluble fraction was designated as $R_{38}$;[2] the supernatant fluid containing soluble enzymes was designated as $S_{38}$. The former could be stored in a refrigerator at 2 C for a few days with little loss of activity; the latter could be stored in a frozen state for 2 weeks.

*Assay method.* Succinoxidase activity was measured by the conventional Warburg manometric procedure at 30 C.

Succinic dehydrogenase activity was measured spectrophotometrically by following the reduction of 2,6-dichlorophenol indophenol (DCPP) at 600 mμ in a reaction mixture containing 60 μmoles of phosphate buffer, pH 7.3, 10 μmoles of potassium cyanide, 0.1 ml of 0.1 per cent dye, enzyme preparations, and 50 μmoles of succinate in 4.0 ml.

Succinate-cytochrome c reductase activity was measured spectrophotometrically by following the reduction of cytochrome c at 550 mμ in a similar reaction mixture containing cytochrome c instead of DCPP.

Fumarase activity was determined spectrophotometrically by following the decrease in optical density at 300 mμ with time, using fumarate as substrate, according to the method of Racker (1950) modified by Massey (1955).

Oxalacetic decarboxylase activity was determined manometrically by measuring $CO_2$ evolution according to the procedure of Herbert (1955).

*Materials.* Fumarase was crystallized from pig heart muscle, according to the method of Massey (1955), and recrystallized before use.

Oxalacetic decarboxylase was prepared from *Micrococcus lysodeikticus* according to the method of Herbert (1955). The cells were lysed by lysozyme followed by acetone and acid precipitation. The precipitate obtained between the limits 40 to

[1] A preliminary report of this work was presented at the 27th annual meeting of the Japanese Biochemical Society, Kyoto, April 2, 1955.

[2] For biochemical properties of $R_{38}$ see previous report (Yamamura *et al.*, 1955).

50 per cent acetone was dissolved in acetate buffer, then poured into several volumes of cold acetone. Finally, the resulting precipitate was dried and stored in a vacuum desiccator. Forty mg of this powder contained the activities of 22 units of oxalacetic decarboxylase and 3 units of fumarase, but did not include malic and succinic dehydrogenase activities.

Cytochrome c was prepared from beef heart muscle by the method of Keilin and Hartree (1952).

## RESULTS

From figure 1 it is evident that neither $R_{38}$ nor $S_{38}$ oxidizes succinate aerobically, but the combination of $R_{38}$ and $S_{38}$ shows oxygen uptake at an appreciable rate. Little information has as yet been given about such absolute requirements of two different fractions for succinoxidase activity. Methylene blue, cytochrome c, adenosine triphosphate (ATP), heated crude extract of *M. avium*, glutathione (GSH), glutamate, or metals such as $Mn^{++}$, $Mg^{++}$, $Al^{+++}$, or calcium phosphate gel cannot at all replace either $R_{38}$ or $S_{38}$. Accordingly, this combined action can be explained neither by deficiency of any electron carriers, nor by alteration of colloidal state of the succinoxidase system (Keilin and Hartree, 1949).

*Reduction of DCPP by succinate.* We first investigated whether or not such combined effect was observed in succinic dehydrogenase. Using DCPP as an electron acceptor, succinic dehydrogenase was investigated independently from cytochrome systems which linked succinic dehydrogenase to molecular oxygen. As shown in figure 2, rapid reduction of DCPP by succinate as well as aerobic oxidation were observed only in the presence of $R_{38}$ and $S_{38}$. No activity was observed in the presence of $S_{38}$ alone. However, it was found that $R_{38}$, without the addition of $S_{38}$, could catalyze slow reduction of the dye, although the reaction rate was only one-fiftieth of the rate in the combined system. The change in optical density of the $R_{38}$ controls varies with different preparations of $R_{38}$. However, this variation does not affect the experimental results since the values are relative. It was therefore suggested that a moiety of succinic dehydrogenase was associated with $R_{38}$, and an additional factor or factors other than succinic dehydrogenase was present in $S_{38}$. Since this activation effect was found to be destroyed completely by heating $S_{38}$ in a boiling water bath for 2 minutes, it appears that some enzymic mechanism was involved in this effect. Further, a preparation obtained by fractionation

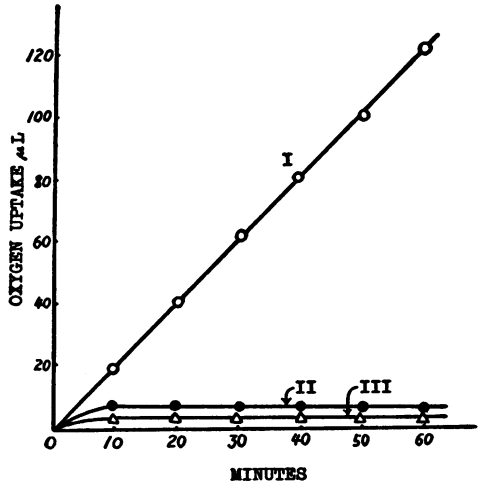


*Figure 1.* Oxidation of succinate by $R_{38}$ and $S_{38}$ from *Mycobacterium avium*. Curve I indicates oxygen uptake when vessel contains 0.5 ml of $R_{38}$, 2.0 ml of $S_{38}$, 140 μmoles of phosphate buffer (pH 7.0), and 100 μmoles of succinate. Curve II: no $R_{38}$. Curve III: no $S_{38}$. Gas phase, air. 30 C.

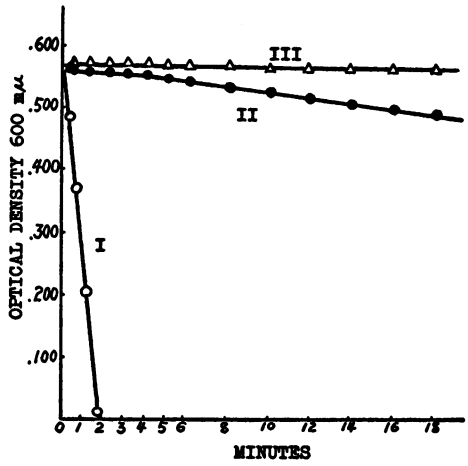


*Figure 2.* Reduction of 2,6-dichlorophenol indophenol by succinate. The standard assay system (see Methods) was employed. Curve I indicates change of optical density when vessel contains 0.2 ml of $R_{38}$ and 0.3 ml of $S_{38}$ (dialyzed against distilled water for 12 hr). Curve II: 0.2 ml of $R_{38}$ alone. Curve III: 0.3 ml of dialyzed $S_{38}$ alone. Each curve was corrected for endogenous optical density change without succinate.

TABLE 1

*Activation of succinic dehydrogenase assayed with 2,6-dichlorophenol indophenol by "purified" preparation of $S_{38}$ or pig heart muscle*

| Conditions | Optical Density Change at 600 mμ per 2.5 min |
|---|---|
| $R_{38}$ | 0.004 |
| "Purified" $S_{38}$ | 0.010 |
| $R_{38}$ + "purified" $S_{38}$* | 0.334 |
| Fumarase† | 0.004 |
| $R_{38}$ + fumarase† | 0.331 |

* Eluate from calcium phosphate gel.

† Crystalline fumarase from pig heart muscle containing 20 units of fumarase activity.

Each cuvette contained 0.3 ml of $R_{38}$ (nitrogen content 1.66 mg per ml). Other conditions as in Methods.

of $S_{38}$ with ammonium sulfate from 0.2 to 0.6 saturation, followed by adsorption of calcium phosphate gel and elution with phosphate buffer, pH 7.3, still showed a pronounced activation effect (table 1). No pigment was detected spectrophotometrically in this preparation. On the other hand, it was noted that this preparation contained considerable amounts of fumarase activity. Therefore, the effect of mammalian fumarase on succinic dehydrogenase was examined. As shown in table 1, fumarase crystallized from pig heart can replace satisfactorily "purified" $S_{38}$ for the activation effect of succinic dehydrogenase. In the presence of a given amount of $R_{38}$, the reaction rate with crystalline fumarase is actually the same as the rate with "purified" $S_{38}$ containing the same units of fumarase activity, as illustrated in figure 3. Figure 4 shows that the initial rates of dye reduction by succinate are strictly proportional to the concentration of $R_{38}$ in the presence of pig heart fumarase.

From these results it is apparent that succinic dehydrogenase is exclusively located in $R_{38}$, but the succinate oxidation by $R_{38}$ when DCPP is used as an electron acceptor requires the cooperative action of fumarase. In fact, as shown in table 2, fumarate at one-tenth of the final concentration of succinate inhibits the reaction about 30 per cent, whereas malate has no effect.

In order to determine whether any additional factor was stimulatory, various substances were added in the presence of excess amounts of crystalline fumarase. Table 3 summarizes the influences of various substances. Cyanide has a pronounced stimulating effect. This effect may be due to the inhibition of the cytochrome system associated with $R_{38}$. For this reason cyanide was usually added to the assay system of succinic dehydrogenase.

Crude flavin adenine dinucleotide (FAD) was shown to stimulate the reaction rate, especially when cyanide was omitted from the system. This fact is worthy of note, since Basford *et al.* (1955)

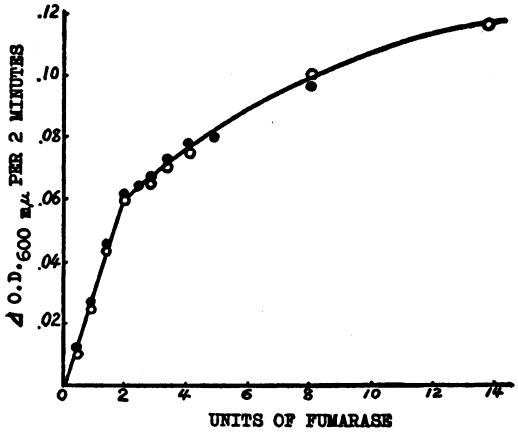


*Figure 3.* Comparison between the effects of "purified" $S_{38}$ and pig heart fumarase on succinic dehydrogenase activity assayed with dichlorophenol indophenol. Each cuvette contains 0.1 ml of $R_{38}$ and the reaction mixture described in Methods. ●: "purified" $S_{38}$; the same preparation as described in table 1. ○: fumarase crystallized from pig heart muscle.

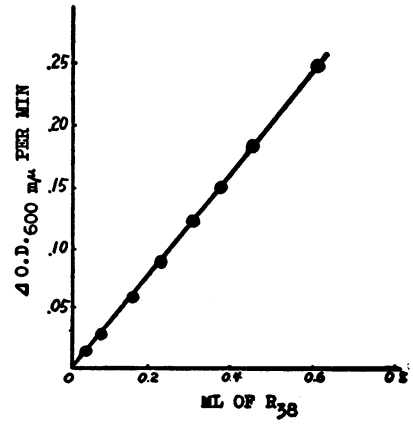


*Figure 4.* Effect of concentration of $R_{38}$ on succinic dehydrogenase activity assayed with dichlorophenol indophenol. Each cuvette contains 20 units of fumarase.

TABLE 2

*Effect of fumarate on succinic dehydrogenase*

| Addition | Optical Density Change at 600 mμ per 8 min |
|---|---|
| None | 0.049 |
| Fumarate, 25 μmoles | 0.035 |
| Fumarate, 50 μmoles | 0.021 |

Each value was corrected for control optical density change without succinate. The cuvette contained 0.3 ml of $R_{38}$, 250 μmoles of succinate, 1.0 ml of phosphate buffer (pH 7.68), 10 μmoles of cyanide and 0.1 mg of dye.

TABLE 3

*Effect of various substances on succinic dehydrogenase assayed with 2,6-dichlorophenol indophenol*

| Exp. No. | Addition | Optical Density Change at 600 mμ per 2 Min |
|---|---|---|
| 1 | None | 0.082 |
| | Cyanide, 10 μmoles | 0.140 |
| | FAD*, 42 μmoles | 0.160 |
| | Cyanide + FAD | 0.196 |
| 2 | None | 0.135 |
| | $Mg^{++}$ | 0.173 |
| | $Mn^{++}$ | 0.129 |
| | $Fe^{++}$ | 0.165 |
| | $Al^{+++}$ | 0.144 |

Each cuvette contained 1.0 ml of $R_{38}$ and 20 units of fumarase. Other conditions as in Methods.

* Flavin adenine dinucleotide.

have recently reported that the mammalian "succinic dehydrogenase complex" contained one mole of FAD. Among several metals tested, $Mg^{++}$ or $Fe^{++}$ was often observed to stimulate the activity. This property resembles somewhat that of the succinic dehydrogenase prepared from *Azotobacter vinelandii* by Repaske (1954).

The effect of various inhibitors is given in table 4. It is quite unexpected that malonate, oxalacetate, and pyrophosphate, all of which have long been known to be potent competitive inhibitors of succinic dehydrogenases from animal and other sources (Dixon and Elliott, 1929; Pardee and Potter, 1948), cannot inhibit the activity of this system at all, even at a high concentration. *p*-Chloromercuribenzoate (PCMB) at a final concentration of $7.5 \times 10^{-4}$ M considerably inhibits

TABLE 4

*Effect of various inhibitors on succinic dehydrogenase assayed with 2,6-dichlorophenol indophenol*

| Exp. No. | Addition | Optical Density Change at 600 mμ per 90 sec | Inhibition |
|---|---|---|---|
| | | | % |
| 1 | None | 0.343 | — |
| | Malonate, 100 μmoles | 0.356 | 0 |
| | Oxalacetate, 20 μmoles | 0.338 | 0 |
| | Pyrophosphate, 10 μmoles | 0.397 | 0 |
| 2 | None | 0.287 | — |
| | PCMB,* $7.5 \times 10^{-4}$ M | 0.029 | 90 |
| | EDTA,† $2.5 \times 10^{-2}$ M | 0.289 | 0 |

Conditions as in table 3.

* *p*-Chloromercuribenzoate.

† Ethylenediaminetetraacetate.

the activity. Optimum pH was found to be around 7.6, as illustrated in figure 5.

*Succinate-cytochrome c reductase activity.* $R_{38}$ is also able to use mammalian cytochrome c as an electron acceptor for the oxidation of succinate. Figure 6 shows that the condition necessary for observing the reduction of cytochrome c is similar in the reduction of DCPP. Malonate again has no inhibitory effect. In view of the finding that a factor sensitive to dimercaptopropanol (BAL) is linked between succinic dehydrogenase and cytochrome c in animal tissues, $R_{38}$ was treated with BAL essentially according to the procedure described by Slater (1949). No inhibition was observed for the reduction of cytochrome c by succinate.

*Aerobic oxidation of succinate.* From the results obtained above, when DCPP or cytochrome c was used as an electron acceptor, it was expected that the effect of $S_{38}$ on the aerobic oxidation of succinate could also be interpreted as due to a similar mechanism. Nevertheless, it was found that added fumarase from pig heart muscle could not substitute for $S_{38}$ in the aerobic oxidation as given in table 5. This fact strongly indicated that one or more factors other than fumarase would be needed for the succinoxidase activity of $R_{38}$. In an earlier communication (Yamamura *et al.*, 1954), we found that the aerobic oxidation of succinate by crude cell-free extracts from *M. avium* was remarkably stimulated by the addition of

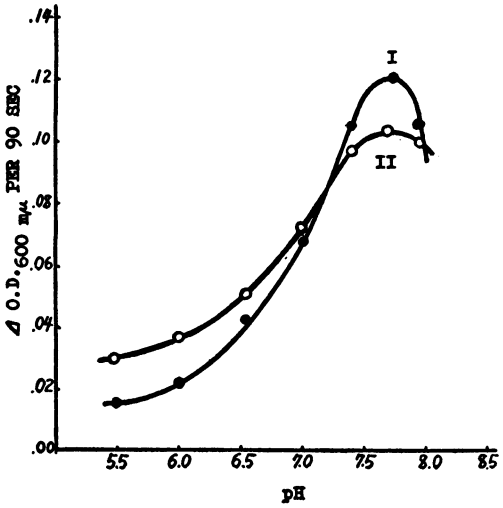


*Figure 5.* Effect of pH on succinic dehydrogenase activity. Each cuvette contains 0.1 ml of $R_{38}$ and 120 units of fumarase. Excess amounts of fumarase were added in order to prevent the pH dependence of fumarase activity. Curve I shows the curve of observed optical density change for 90 sec. Curve II shows the curve corrected for the effect of pH on the dye.

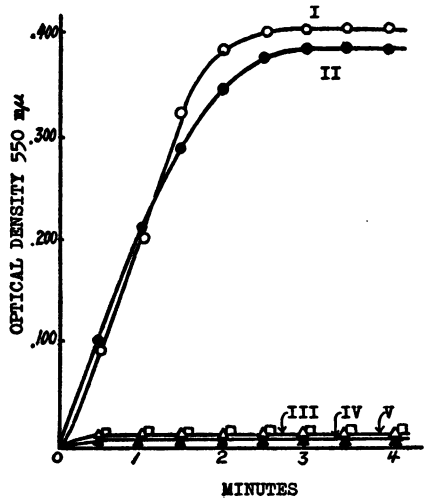


*Figure 6.* Reduction of mammalian cytochrome c. Conditions as in table 1 except for cytochrome c instead of dichlorophenol indophenol. Curve I: 0.1 ml of $R_{38}$ and 0.3 ml of "purified" $S_{38}$. Curve II: 0.1 ml of $R_{38}$ and 16 units of fumarase. Curve III: 0.1 ml of $R_{38}$. Curve IV: 0.3 ml of "purified" $S_{38}$. Curve V: 16 units of fumarase.

glutamate. This phenomenon was previously shown in the succinate oxidation of Azotobacter extract by Stone and Wilson (1952), who demonstrated that transaminase was necessary to remove oxalacetate formed after the oxidation of succinate. As described previously (Yamamura *et al.*, 1955), oxalacetic decarboxylase was contained in significant amounts in $S_{38}$, and $R_{38}$ contained powerful L-malic oxidase. Thus there remained the possibility that the requirement of $S_{38}$ might be due to the presence of oxalacetic decarboxylase besides fumarase. If so, the addition of oxalacetic decarboxylase purified from *M. lysodeikticus* with fumarase should be able to induce the succinate oxidation by $R_{38}$.

A definite oxygen uptake was caused by the addition of fumarase and oxalacetic decarboxylase (table 5). Thus, there is some evidence supporting the above possibility. However, $S_{38}$ was able to cause succinate oxidation to a much greater ex-

TABLE 5

*Effect of fumarase and oxalacetic decarboxylase on aerobic oxidation of succinate*

| Exp. No. | | Oxygen Uptake (μL) | | |
|---|---|---|---|---|
| | | 20 min | 40 min | 60 min |
| 1 | None | 13.0 | 16.4 | 14.7 |
| | Fumarase* | 6.7 | 10.1 | 8.5 |
| | Oxalacetic decarboxylase† | 18.9 | 39.7 | 55.7 |
| | Fumarase* + oxalacetic decarboxylase† | 31.4 | 57.7 | 77.0 |
| 2 | None | 6.0 | 9.0 | 11.7 |
| | $S_{38}$‡ (without $R_{38}$) | 3.2 | 6.4 | 8.1 |
| | $S_{38}$‡ | 11.4 | 25.9 | 42.2 |
| | Fumarase* + oxalacetic decarboxylase† | 7.6 | 15.2 | 21.0 |
| | Fumarase* + oxalacetic decarboxylase† + $S_{38}$‡ | 8.8 | 27.8 | 43.7 |

Each value was corrected for a corresponding blank without succinate. Each cup contained 0.3 ml of $R_{38}$, 200 μmoles of phosphate buffer (pH 6.6), 3 μmoles of $MnSO_4$, 100 μmoles of succinate and other additions in a final volume of 3.0 ml.

* Recrystallized from pig heart, contained 38 units of fumarase activity.

† Prepared from *Micrococcus lysodeikticus*, contained 6.6 units of oxalacetic decarboxylase and 0.9 units of fumarase activities.

‡ Contained 4 units of fumarase and 0.33 units of oxalacetic decarboxylase activities.

tent than the combined system of fumarase and oxalacetic decarboxylase, in spite of the fact that $S_{38}$ contained much smaller amounts of fumarase and oxalacetic decarboxylase activities than those of the combined system. These results suggest that a factor or factors necessary for maximal activity of succinoxidase other than fumarase or oxalacetic decarboxylase were involved in $S_{38}$. But almost nothing is known about the nature of these factors. It seems likely that it is a protein or proteins, because heated $S_{38}$ was quite inactive even in the presence of added fumarase and oxalacetic decarboxylase.

TABLE 6

*Effect of various substances on succinoxidase*

| Exp. No. | Addition | Oxygen Uptake at 60 Min |
|---|---|---|
| | | μL |
| 1 | None | 79.8 |
| | $MnSO_4$, 4 μmoles | 123.0 |
| | $MgSO_4$, 4 μmoles | 93.6 |
| 2* | None | 57.3 |
| | Malonate, 50 μmoles | 34.4 |
| | Malonate, 100 μmoles | 29.9 |
| 3 | None | 53.1 |
| | Oxalacetate, 5 μmoles | 11.6 |
| | Oxalacetate, 10 μmoles | 7.5 |

Each cup contained 0.3 ml of $R_{38}$, 1.0 ml of $S_{38}$, 200 μmoles of phosphate buffer (pH 6.6), 100 μmoles of succinate, and other additions in a final volume of 3.0 ml.

* Added 3 μmoles of $MnSO_4$.

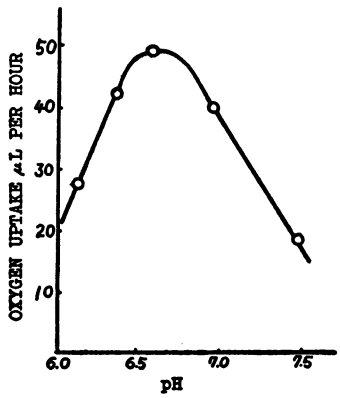


*Figure. 7* Effect of pH on succinoxidase activity.

In the presence of $S_{38}$, other properties of the succinoxidase were studied (table 6). It was noted that $Mn^{++}$ stimulated the oxidation. Either malonate or oxalacetate was shown to inhibit the oxidation considerably. Maximal activity was attained at pH 6.6, as shown in figure 7.

## DISCUSSION

From cytological studies on mycobacteria, Mudd and associates published evidence suggesting that granules found in *Mycobacterium thamnopheos* and *Mycobacterium tuberculosis* var. *hominis* are mitochondria (Mudd *et al.*, 1951; Mudd and Winterscheid, 1953; Winterscheid and Mudd, 1953). Recently, Millman and Youmans (1955) reported that the ground extract of *M. tuberculosis* var. *hominis* strain H37Ra was divided into soluble and particulate fractions, and the latter was suggested to be composed of bacterial mitochondria. This particulate fraction contained almost all enzymes related to the tricarboxylic acid cycle. The authors independently reported on the isolation of a particulate fraction from the cell-free extract of *Mycobacterium avium* by differential centrifugation (Yamamura *et al.*, 1955). It was elucidated that succinic dehydrogenase, accompanied by L-malic dehydrogenase, was located in this particle. More recently Millman and Darter (1956) reported on quantitative studies of the distributions of enzymes in *Mycobacterium tuberculosis* var. *hominis* H37Ra and it was found that cytochrome oxidase, DPNH oxidase and succinic dehydrogenase were associated mainly with the two largest particulate fractions.

These results strongly suggest that the granules or particles in mycobacteria are mitochondria, or active subunits thereof, indicating the similarity of cytological and enzymochemical characteristics of bacteria to higher organisms.

As described above, it is very peculiar that a fumarase requirement was established for the succinate oxidation by *M. avium*. This fact is considered to be responsible for the product inhibition, although the extremely high sensitivity to added fumarate was not indicated. Recently, Kearney and Singer (1954) pointed out that the succinate oxidation in *Proteus vulgaris* extract was inhibited competitively not only by oxalacetate but also by fumarate.

With regard to oxalacetate inhibition, several reports have appeared on the succinoxidase of

animal tissues (Das, 1937; Pardee and Potter, 1948; Tyler, 1955). *M. lysodeikticus* oxalacetic decarboxylase, used as a means for the removal of oxalacetate, was shown to be less effective than $S_{38}$. Therefore, the possibility for the presence of one or more factors besides oxalacetic decarboxylase would remain; however, the possibility could not yet be rejected that another more efficient mechanism for the removal of oxalacetate was actually operating in $S_{38}$.

There are some discrepancies between the results assayed with DCPP (or cytochrome c) reduction and oxygen uptake. In particular, the lack of malonate inhibition when the dye is used is unusual,[3] and this problem awaits further investigation.

In the latest work in our laboratory, the combined effect was observed in the succinate oxidation by *M. phlei*. Moreover, it was of interest that the $S_{38}$ of *M. avium* could substitute for the corresponding fraction prepared from *M. phlei*, *M. smegmatis* or a mycobacterium No. 607. Accordingly, it appeared that such combined effect was a common phenomenon for succinate oxidation by mycobacteria.

ACKNOWLEDGMENTS

The authors wish to express their gratitude to Professor Stuart Mudd, Department of Microbiology, University of Pennsylvania, School of Medicine, for his advice and interest in our work. They are also grateful for the help of Miss T. Nagasuga in the preparation of crystalline fumarase.

SUMMARY

Succinic dehydrogenase is localized in the particulate fraction from *Mycobacterium avium*. This result supports the concept that this particulate fraction contains mitochondria.

The reduction of 2,6-dichlorophenol indophenol (DCPP) or cytochrome c by succinate with the particulate fraction requires the addition of the soluble fraction which can be replaced satisfactorily by heart muscle fumarase. Succinic dehydrogenase assayed with DCPP or cytochrome c is quite insensitive to either malonate or oxalacetate.

The aerobic oxidation of succinate with the particulate fraction also requires the soluble fraction. The latter fraction cannot be replaced by heart muscle fumarase, but can be partially replaced by oxalacetic decarboxylase from *Micrococcus lysodeikticus*. Succinoxidase is inhibited by either malonate or oxalacetate in a manner similar to animal enzymes.

[3] A similar result was obtained when ferricyanide was used as electron acceptor.

REFERENCES

BASFORD, R. E., MII, S., AND GREEN, D. E. 1955 Succinic dehydrogenase. Federation Proc., **14**, 178.

DAS, N. B. 1937 Studies on the inhibition of the succinic and lactic-malic dehydrogenases. Biochem. J. (London), **31**, 1124–1130.

DIXON, M. AND ELLIOTT, K. A. C. 1929 The effect of cyanide on the respiration of animal tissues. Biochem. J. (London), **23**, 812–830.

HERBERT, D. 1955 Oxalacetic carboxylase of *Micrococcus lysodeikticus*. *Methods in Enzymology*, pp. 753–757, Vol. I, edited by S. P. Colowick and N. O. Kaplan. Academic Press Inc., New York.

KEARNEY, E. B. AND SINGER, T. P. 1954 The coupled oxidation of succinate and L-cysteine sulfinate by soluble enzymes. Biochim. et Biophys. Acta, **14**, 572–573.

KEILIN, D. AND HARTREE, E. F. 1952 Cytochrome c. *Biochemical Preparations*, p. 753, Vol. I, edited by E. G. Ball. John Wiley & Sons, Inc., New York.

KEILIN, D. AND HARTREE, E. F. 1949 Activity of the succinic dehydrogenase-cytochrome system in different tissue preparations. Biochem. J. (London), **44**, 205–218.

KUSUNOSE, M., KUSUNOSE, E., AND YAMAMURA, Y. 1952 Studies on the tricarboxylic acid cycle in tubercle bacillus. J. Biochem. (Japan), **39**, XXXIV.

MASSEY, V. 1955 Fumarase. *Methods in Enzymology*, pp. 729–735, Vol. I, edited by S. P. Colowick and N. O. Kaplan. Academic Press Inc., New York.

MILLMAN, I. AND DARTER, R. W. 1956 Intracellular localization of enzymes in *Mycobacterium tuberculosis* var. *hominis*. Proc. Soc. Exptl. Biol. Med., **91**, 271–274.

MILLMAN, I. AND YOUMANS, G. P. 1955 The characterization of the terminal respiratory enzymes of the H37Ra strain of *Mycobacterium tuberculosis* var. *hominis*. J. Bacteriol., **69**, 320–325.

MUDD, S. AND WINTERSCHEID, L. C. 1953 A note concerning bacterial cell walls, mitochondria and nuclei. Exptl. Cell Research, **5**, 251–254.

MUDD, S., WINTERSCHEID, L. C., DELAMATER, E. D., AND HENDERSON, H. J. 1951 Evi-

dence suggesting that the granules of Mycobacteria are mitochondria. J. Bacteriol., **62**, 459–475.

PARDEE, A. B. AND POTTER, V. R. 1948 Inhibition of succinic dehydrogenase by oxalacetate. J. Biol. Chem., **176**, 1085–1094.

RACKER, E. 1950 Spectrophotometric measurements of the enzymatic formation of fumaric and *cis*-aconitic acids. Biochim. et Biophys. Acta, **4**, 211–214.

REPASKE, R. 1954 Succinic dehydrogenase of *Azotobacter vinelandii*. J. Bacteriol., **68**, 555–561.

SLATER, E. C. 1949 A respiratory catalyst required for the reduction of cytochrome c by cytochrome b. Biochem. J. (London), **45**, 14–30.

STONE, R. W. AND WILSON, P. W. 1952 The effect of oxalacetate on the oxidation of succinate by azotobacter extracts. J. Bacteriol., **63**, 619–622.

TYLER, D. B. 1955 Effect of metal ions and adenosine triphosphate on an oxaloacetate-inhibited succinoxidase activity. J. Biol. Chem., **216**, 395–403.

WINTERSCHEID, L. C. AND MUDD, S. 1953 The cytology of the tubercle bacillus with reference to mitochondria and nuclei. Am. Rev. Tuberc., **67**, 59–73.

YAMAMURA, Y., KUSUNOSE, M., AND KUSUNOSE, E. 1952 Lactic oxidase of *Mycobacterium tuberculosis avium*. J. Biochem. (Japan), **39**, 227–233.

YAMAMURA, Y., KUSUNOSE, M., NAGAI, S., AND KUSUNOSE, E. 1954 Studies on the tricarboxylic acid cycle of tubercle bacillus. I. Some properties of the enzyme systems. J. Biochem. (Japan), **41**, 513–528.

YAMAMURA, Y., KUSUNOSE, M., NAGAI, S., KUSUNOSE, E., YAMAMURA, Y., JR., TANI, J., TERAI, T., AND NAGASUGA, T. 1955. Biochemical studies on the particulate fraction from *Mycobacterium tuberculosis avium*. Med. J. Osaka Univ., **6**, 489–499.