TELLURITE REDUCTASE FROM *MYCOBACTERIUM AVIUM*

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It has been reported earlier that mycobacteria (Corper, 1915) and corynebacteria (Morton and Anderson, 1941) produce black colonies on culture media containing tellurite, and this property has been used as a rapid test for the viability of tubercle bacilli (Corper, 1915).

Recently, Mudd *et al.* (1956) showed that metallic tellurium, reduced from tellurite, condensed about the reducing sites of mycobacteria in their electron micrographs, and they considered that the reduction of tellurite occurred mainly in the mitochondrial granules.

It has been previously reported from this laboratory (Yamamura and Kamahora, 1956) that the reduction of tellurite could be seen not only in mycobacteria but also in other bacteria, e. g., *Proteus vulgaris, Escherichia coli, and Staphylococcus aureus.* It has been further shown that the reduction of tellurite occurred in the cell-free extract of *Mycobacterium avium.*

The work reported here deals with the extraction, partial purification, and characterization of the tellurite-reducing enzyme from *M. avium.* This enzyme, referred to as tellurite reductase, was located in the soluble fraction of the cells, and ferrous ion was found to be one of its cofactors.

MATERIALS AND METHODS

Preparation of particulate and soluble fractions. M. avium strain Takeo was grown in glycerol-bouillon medium for 5 days, as described previously (Yamamura *et al.*, 1955). About 200 g of wet cells were ground with 200 g of sea sand in a chilled mechanical mortar for 40 min, mixed with 400 ml of distilled water, and centrifuged at 3000 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 (38,000 rpm) for 40 min. The reddish pellet formed was washed with distilled water by centrifugation at 100,000 × G for 30 min, and finally suspended in 30 ml of distilled water. This insoluble fraction was designated as R_{38}; the supernatant fluid containing soluble enzymes was designated as S_{38}.

Preparation of malic dehydrogenase. Solid ammonium sulfate was added to 100 ml of S_{38} to 30 per cent saturation, stirred for 15 min, and filtered through paper with Super-cel. Solid ammonium sulfate was added to this filtrate to 70 per cent of saturation, and it was stored in the ice box overnight. After centrifugation at 20,000 rpm for 15 min, the precipitate was dissolved in 7 ml of distilled water, and dialyzed against running water for 2 hr.

Assay methods. Malic dehydrogenase activity was measured spectrophotometrically (Kun and Aboad, 1949) by following the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) at a wave length of 480 mμ. The reaction mixture contained 1.0 ml of enzyme preparation, 0.5 ml of M/10 phosphate buffer (pH 8.0), 0.5 ml of M/2 L-malate, and 1.0 ml of 0.1 per cent TTC (freshly prepared). After 30 min incubation at 37 C, 7.0 ml of acetone was added, and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant red fluid was used for measurement. One unit is defined as the amount of enzyme which catalyzes the formation of 1 μg formazan per 20 min.

Tellurite reductase activity was measured spectrophotometrically as follows: The reaction mixture consisted of 0.5 ml of enzyme preparation, 0.3 ml of M/10 tris(hydroxymethyl)aminomethane (Tris) buffer (pH 6.5), 0.1 ml of M/200 potassium tellurite, M/2 L-malate, malic dehydrogenase preparation (described above), M/100 ferrous sulfate, and boiled extract of S_{38} in a total volume of 2.0 ml. After 30 min incubation at 37 C under aerobic conditions, 2.0 ml of 10^{-3} M mercuric chloride was added to stop the reaction. The solution was colored to blackish

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brown by the tellurite reduction, and the degree of the reaction could be measured spectrophotometrically at a wave length of 500 mμ. One unit is defined as the amount of enzyme causing an increase in optical density of 0.001 per 30 min.

Protein was determined by the colorimetric method of Lowry *et al.* (1951).

RESULTS

Fractionation of tellurite reductase. The soluble fraction (S_{38}) was heated at 50 C for 10 min, and calcium phosphate gel was added to the heated fraction in the volume ratio of 150 ml of gel (30.1 mg dry weight per ml) to 100 ml of this fraction. After being stirred for 15 min, the mixture was centrifuged, and the supernatant fluid was discarded. The gel was then stirred with 40 ml of M/10 phosphate buffer (pH 8.0), and centrifuged. This process was repeated twice. The supernatant fluids were combined, and pH was adjusted to 4.8 by the addition of 1 N acetic acid. The resulting precipitate was dissolved in 10 ml of distilled water, and pH was brought to 7.0 with dilute ammonia water. This fractionation was done for the purpose of removing the malic dehydrogenase in the soluble fraction. As shown in table 1, tellurite reductase freed of malic dehydrogenase was obtained by isoelectric precipitation.

TABLE 1

Fractionation of tellurite reductase from Mycobacterium avium

Fraction	Tellurite Reductase,* Specific Activity	Malic Dehydrogenase,† Specific Activity
	u/mg protein	*u/mg protein*
Soluble fraction (S_{38})‡	34.6	69.5
Heated at 50 C, 10 min	22.2	18.2
$Ca_3(PO_4)_2$ eluate	15.9	Trace
Ppt from acetic acid (pH 4.8)	6.3	0

* 0.5 ml of enzyme, 0.5 ml of M/10 tris(hydroxymethyl)aminomethane (Tris) buffer (pH 6.5), 0.1 ml of M/200 potassium tellurite, M/100 $FeSO_4$, boiled extract of S_{38}, M/2 L-malate, malic dehydrogenase prepared from S_{38}, and water to 2.0 ml.

† 1.0 ml of enzyme, 0.5 ml of M/10 phosphate buffer (pH 8.0), 0.5 ml of L-malate, and 1.0 ml of 0.1 per cent 2,3,5-triphenyltetrazolium chloride (TTC).

‡ Supernatant fluid centrifuged at 38,000 rpm.

Properties of tellurite reductase. As shown in table 2, neither reductase nor malic dehydrogenase preparation independently reduces tellurite, but the combination of both preparations shows a reduction.

Tellurite reductase was not found in the particulate fraction, and malic dehydrogenase in the cells exists mainly in this fraction (table 3). This particulate malic dehydrogenase, however, did not couple with tellurite reductase.

Effect of temperature. S_{38} was heated in a water bath for 10 min at various degrees of temperature, and the reductase activity was determined in the presence of excess amounts of malic dehydrogenase. The result is shown in table 4, indicating that the reductase is considerably thermostable.

Effect of pH. The effect of pH on the reduction of tellurite by the malic dehydrogenase system is seen in figure 1. The tellurite reduction proceeded maximally at pH 6.5 in Tris buffer. On the other hand, optimum pH on malic dehydrogenase was over 8.0 in phosphate buffer.

Effect of metal ions. Metal ions were added in the reaction mixture to determine their effect on the reductase. Among them, Ba^{++}, Mn^{++}, and Pb^{++} had no effect, Cd^{++}, Cu^{++}, Hg^{++}, and Ag^{+} were inhibitory over 50 per cent at the concentration of 10^{-5} M, and Zn^{++} was similar to the

TABLE 2

Malate and malic dehydrogenase of soluble fraction as electron donor for tellurite reduction

Reaction Mixture	Tellurite Reduction, $\Delta E_{600} \times 10^3$ per 30 min
Complete	270
− Malic dehydrogenase	75
− Tellurite reductase	0

Conditions identical with those in table 1, except that enzyme was calcium phosphate gel eluate.

TABLE 3

Distribution of tellurite reductase and malic dehydrogenase in Mycobacterium avium

Fraction	Tellurite Reductase	Malic Dehydrogenase
	u/mg protein	*u/mg protein*
S_{38}	34.4	59.2
R_{38}	0	996

Conditions as in table 1.

latter group at 10^{-4} M. The addition of Fe^{++} or Fe^{+++} ion to the dialyzed S_{38} caused a stimulation of tellurite reduction. Maximal activation obtained with Fe^{++} was 4-fold at the final concentration of 10^{-3} M (table 5).

Effect of metal binding agents. 8-Hydroxyquinoline, cyanide, and ethylenediaminetetraacetic acid (EDTA) were found to have a stimulatory effect, i. e., 8-hydroxyquinoline and cyanide at a concentration of 10^{-3} M activated 100 and 70 per cent, respectively (table 5). This may be due to the trapping of the reaction product, metallic tellurium.

In contrast to their effects, α,α'-dipyridyl caused an inhibition of 45 per cent at a concentration of 10^{-3} M (table 5).

Effect of boiled extract of soluble fraction. S_{38} was heated in a boiling water bath for 5 min, and centrifuged at 20,000 rpm for 20 min. The supernatant greenish-brown fluid was used as boiled extract. The effect of boiled extract is shown in table 6. Remarkable stimulation was caused by the addition of this fraction.

This stimulating effect of boiled extract was

TABLE 4

Effect of temperature on tellurite reductase

Enzyme (S_{38}) Heated at	Tellurite Reduction, $\Delta E_{500} \times 10^3$ per 30 min	Recovery
C		%
Unheated	350	100
50	334	95.4
55	250	71.4
60	185	53.0
65	57	16.3
70	0	0

S_{38}, heated with various degrees of temperature for 10 min, was used as enzyme.

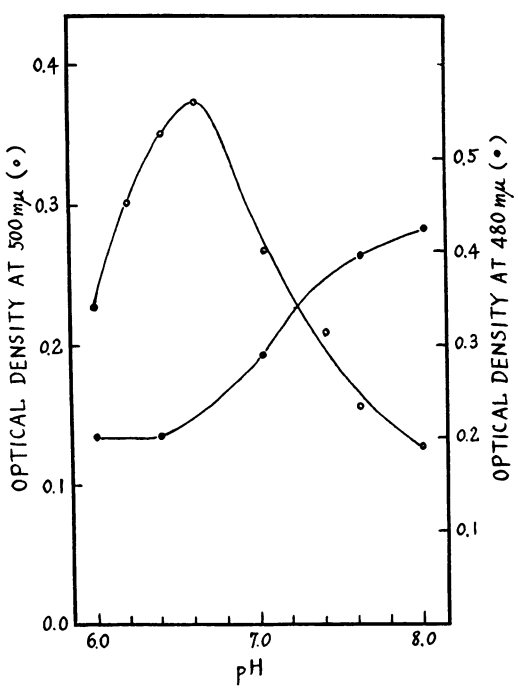

Figure 1. Effect of pH on tellurite reductase (○), and malic dehydrogenase (●) of soluble fraction. The assay conditions were as in table 1.

TABLE 5

Effect of various substances on tellurite reductase

Expt No.	Addition	Final Concentration	Tellurite Reduction, $\Delta E_{500} \times 10^3$ per 30 min
		moles	
1	None	—	245
	$CoCl_2$	10^{-3}	223
	$BaSO_4$	10^{-3}	340
	$MnCl_2$	10^{-3}	354
	$Pb(CH_3COO)_2$	10^{-3}	293
	$Fe_2(SO_4)_3$	10^{-3}	630
	$FeSO_4$	10^{-3}	1110
2*	None	—	439
	$AgNO_3$	10^{-4}	5
	$AgNO_3$	10^{-5}	215
	$CdSO_4$	10^{-4}	20
	$CdSO_4$	10^{-5}	165
	$CuSO_4$	10^{-4}	2
	$CuSO_4$	10^{-5}	175
	$HgCl_2$	10^{-4}	4
	$HgCl_2$	10^{-5}	42
	$ZnCl_2$	10^{-4}	165
3†	None	—	310
	8-Hydroxyquinoline	10^{-3}	630
	EDTA‡	10^{-3}	285
	EDTA‡	10^{-2}	698
	KCN	10^{-3}	530
	KCN	10^{-2}	1300
4†	None	—	608
	α,α'-Dipyridyl	10^{-3}	340

* Enzyme was S_{38} which dialyzed against running water for 2 hr. Conditions as in table 1 except for absence of ferrous ion.

† Conditions as in table 1.

‡ Ethylenediaminetetraacetate.

inactivated by ashing, but not by treatment with dithizone. It was dialyzable, but could not be extracted with phenol. Therefore, it seems unlikely that this active component is a flavin compound or any inorganic substance. Since ovalbumin was not effective, this effect was not attributed to protection of the surface state of the reaction system.

Electron donors. In S_{38}, containing malic dehydrogenase, L-malate was used as a hydrogen donor. By dialysis of S_{38}, the enzyme showed considerable decrease of its activity when L-malate was added. The addition of reduced diphosphopyridine nucleotide (DPNH) to this preparation resulted in marked activation of tellurite reduction (table 7).

Furthermore, it was found that leuco methylene blue and leuco diethylsaflanine were also effective as electron donors under anaerobic conditions. The reaction mixture consisted of 0.3 ml of dialyzed S_{38}, 0.1 ml of M/200 potassium tellurite, 0.3 ml of Tris buffer (pH 6.5), and 0.5 ml of 1.0 per cent leuco dye. Reaction was started by tipping the leuco dye in evacuated modified Thunberg tubes. Leuco methylene blue was reoxidized for 5 min, and leuco diethylsaflanine for 15 min. In the absence of enzyme preparation or tellurite, leuco dye was not reoxidized within 3 hr under the above conditions.

TABLE 6

Effect of boiled extract of S_{38}, untreated or treated variously, on tellurite reductase

Expt No.	Addition	Tellurite Reduction, $\Delta E_{600} \times 10^3$ per 30 min
1	None	238
	Untreated	520
2	None	270
	Untreated	406
	Dialyzable fraction	305
	Phenol extract	268
	Ashed	270
	Dithizone treated	372

Enzyme was S_{38} which dialyzed against running water for 2 hr. Each addition was equivalent to 0.2 ml of untreated boiled extract.

TABLE 7

DPNH as electron donor for tellurite reduction

Reaction Mixture	Tellurite Reduction, $\Delta E_{600} \times 10^3$ per 30 min
Complete system	264
− DPNH	68
− Tellurite reductase	0

Complete system as in table 1 except for hydrogen donor. Enzyme was S_{38}, dialyzed against running water for 2 hr. DPNH, 11 μmoles in a tube.

DISCUSSION

The existence in bacteria of particulates which in functional activity are the equivalents of the mitochondria of higher organisms has been reported by cytochemical method (Mudd, 1953, 1954; Mudd, *et al.*, 1956) and biochemical method (Yamamura *et al.*, 1955; Millman and Youmans, 1955; Nossal *et al.*, 1956).

Mudd and co-workers (1956) observed in electron micrographs that circumscribed areas of intense oxidation-reduction were revealed by reduction of tetrazolium salts or tellurite to microscopically visible deposits.

Although the activity of malic dehydrogenase concerning the reduction of tetrazolium salts exists mainly in particulate fraction, as reported previously (Yamamura *et al.*, 1955), a small amount of the activity can be seen in soluble fraction. It was shown that tellurite reductase was coupled only with malic dehydrogenase in soluble fraction, but not with the dehydrogenase in particulate fraction. This fact suggests the existence of an oxidative-reductive enzyme system in cytoplasm outside of the particulate fraction.

It is of interest that Fe^{++} or Fe^{+++} stimulates the tellurite reductase, since many of the reductases studied thus far are metallo-flavoproteins, e. g., nitrate reductase of *Neurospora crassa* requires Mo^{++} (Nicholas and Nason, 1954) and nitro reductase of *E. coli* requires Mn^{++} (Saz and Marina, 1956) as cofactors. It seems likely that this reductase is a metallo-flavoprotein, but it remains unknown whether or not any flavin compound is concerned in the reduction of tellurite. The nature of the active substance in the boiled extract has not been determined.

This reductase also has been found in the extract of animal tissues, such as pig heart or mouse liver (*unpublished data*).

SUMMARY

Cell-free extract of *Mycobacterium avium* capable of reducing tellurite was obtained, and tellurite-reducing enzyme, referred to as tellurite reductase, was separated from malic dehydrogenase in the soluble fraction. The electron donor of this reduction was malate in the presence of L-malic dehydrogenase in soluble fraction, which could be replaced by reduced diphosphopyridine nucleotide.

The optimum pH of this reductase was 6.5.

The supernatant fluid of boiled soluble fraction and ferrous or ferric ion were required for the maximal reduction of tellurite by the reductase.

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