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A COMPARISON OF THE NADH OXIDASE ELECTRON TRANSPORT SYSTEMS OF TWO OBLIGATELY CHEMOLITHOTROPHIC BACTERIA

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SUMMARY

1. The NADH oxidase electron transport systems of two obligate autotrophs were investigated. Cytochromes c_{547}, c_{550}, c_{552}, b or c_{554}, b_{558} and a were found in *Thiobacillus neapolitanus*, and cytochromes $c_{549,\ 551}$, c or b_{555}, a_1, and a or a_3 in *Thiobacillus thioparus*. A soluble cytochrome c_{552} not present in the particulate fractions was detected in *T. neopolitanus*. Low potential *c*-type cytochromes were found in both organisms. NADH reduced both cytochromes c_{547} and c_{550} in the large particle fraction of *T. neapolitanus*, but only c_{550} in the small particle fraction.

2. Both organisms contained the ubiquinone, Q-8. The levels of flavin, quinone, and cytochrome *c* were comparable to those of heterotrophic bacteria. No naphthoquinone was detected.

3. The levels of NADH and ascorbate oxidases were similar to those of heterotrophic bacteria, while NADH dehydrogenase and ascorbate: *N,N,N',N'*-tetramethyl-*p*-phenylenediamine·2HCl (TMPD) oxidase levels were higher. In *T. Thioparus*, NADH oxidase activity was located exclusively in the large-particle fraction, and in *T. neapolitanus* in both the large- and small-particle fractions.

4. The NADH oxidase activities of both organisms were sensitive to inhibitors usually employed in studies of electron transport. NADH oxidase of *T. thioparus* was completely inhibited by KCN, while that of *T. neapolitanus* was never inhibited by more than 80%. Ascorbate and ascorbate:TMPD oxidases were sensitive to KCN but insensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide.

5. Electron transport pathways are proposed for both organisms.

INTRODUCTION

The obligately autotrophic bacteria, *Thiobacillus neapolitanus* and *Thiobacillus thioparus*, oxidize reduced sulfur compounds as their energy source and fix CO_2 *via* the Calvin cycle for their carbon source. NADH is required for the reduction of 3-phosphoglyceric acid, the product of the carboxylation of ribulose 1,5-diphosphate, to 3-phosphoglyceraldehyde. As high levels of NADH are needed for CO_2 reduction,

Abbreviations: HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; CN^-, KCN; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine·2 HCl.

it is difficult to assign a role to NADH oxidase. Uncontrolled, it would deprive the cell of indispensable reducing power unless: (1) intracellular compartmentalization exists, whereby only excess NADH is oxidized to regenerate NAD^+; (2) fine control by metabolic intermediates is exerted on the oxidase, or (3) ATP is produced by oxidative phosphorylation. Thus far phosphorylation linked to NADH oxidation has not been demonstrated in these organisms[1].

The electron transport chains of both of these autotrophs are sensitive to the usual inhibitors of electron transport, and the levels of activity are similar to those found in other bacteria, both heterotrophic and facultatively autotrophic[1,2]. The only suggestion of a function for NADH oxidase in autotrophs has been to operate in the reverse direction to generate NADH by energy-dependent reversed electron flow. Reversed electron transport has been demonstrated in extracts of *T. neapolitanus*[1], but only under anaerobic conditions, and the rate of NADH production is low compared to that of mitochondria[3]. These organisms are highly aerobic, and under physiological conditions reversed electron flow may not exist.

In order to shed further light on the matter, it has been considered important that the qualitative and quantitative aspects of the electron transport chains of these two obligate autotrophs be examined in detail. The present report provides information on the levels and subcellular localization of activities, of components of the chains, and the effect of inhibitors on NADH and ascorbate oxidases.

MATERIALS AND METHODS

Cell culture

T. neapolitanus (obtained from W. Vishniac) and *T. thioparus* (ATCC 8158) were grown as previously described.[4]

Cell fractionation

A 25 % (w/v) suspension of cells in 0.05 M Tris–HCl, pH 7.2, was ruptured by passage through a chilled French pressure cell under 20000 lb/inch^2. The viscosity of the cell extract was reduced by the addition of a few crystals of deoxyribonuclease and incubation at room temperature for 15 min. A crude extract was obtained by centrifuging this homogenate at 2000 $\times$ *g* for 10 min to remove whole cells. The crude extract was centrifuged at 20000 $\times$ *g* for 30 min to obtain the large particle fraction. The supernatant fluid from the 20000 $\times$ *g* centrifugation was further centrifuged at 144000 $\times$ *g* for 120 min to obtain the small particle fraction. The supernatant fluid from the 144000 $\times$ *g* centrifugation was designated as the soluble fraction.

Measurement of enzyme activity

Oxidase activities were determined by measuring the initial oxygen uptake at 30 °C with a GME oxygraph using a Clark oxygen electrode. The rate of absorbance change at 340 nm was used to determine NADH:$Fe(CN)_6^{3-}$ reductase activity in the presence of 1 μmole $Fe(CN)_6^{3-}$, since a large change in absorbance at 410 nm was observed, due to endogenous reduction of $Fe(CN)_6^{3-}$. The reaction mixture contained 100 μmoles Tris–HCl (pH 7.2 for *T. thioparus* and pH 8.0 for *T. neapolitanus*), extract, and 0.4 μmole NADH, 10 μmoles ascorbate, or 10 μmoles ascorbate *plus*

0.1 μmole *N,N,N'N'*-tetramethyl-*p*-phenylenediamine·2HCl (TMPD), in a total volume of 1.8 ml.

All inhibitor effects were determined on oxygen uptake. Atabrine and KCN(CN^-) were added as aqueous solutions. The remainder of the inhibitors were added as ethanolic solutions in volumes of 25 μl or less; 25 μl of abs. ethanol had no effect on NADH or ascorbate oxidases. All inhibitors were neutralized before use.

The optimal pH for oxidase activity was determined as described for the NADH oxidase activity, substituting 100 μmoles of the following buffers for the Tris–HCl (pH 7.2 or pH 8.0): Tris–maleate, pH 5.0–7.2; Tris–HCl, pH 7.0–9.2; glycine–NaOH, pH 9.0–10.5.

Determination of quinone

The quinones were extracted from whole cells by the method of Kashket and Brodie[5] and purified by chromatography on a Decalso (Ionac C-101, Folin Decalso, Ionac Chemical Co., Birmingham, N.J.) column by the method of Lester and Crane[6]. For use as standards, Q-6 and Q-10 were extracted and purified from *Candida utilis* (St. Regis, Lakes State Division, Rhinelander, Wisc.) and Q-8 from *Escherichia coli* by the methods described above. The thiobacillary quinones were identified by thin-layer chromatography using the method of Gloor[7]. The quinones were detected by spraying with 0.5 % Rhodamine B in ethanol and visualized by ultraviolet light. The concentration of quinones was determined by the method of Redfearn[8]. The absorption spectra of the purified quinones were recorded with a Beckman DB spectrophotometer. The levels of total flavin were measured by the method of Rao *et al.*[9].

Difference spectra

Room temperature and liquid nitrogen difference spectra were determined with a Phoenix PDM-1010 Dual Beam spectrophotometer as described by Lanyi[10] with the fraction diluted in 0.05 M Tris–HCl buffer (pH 7.2). The cytochrome *c* concentrations were approximated from the room temperature spectra using the reading at 550 nm *minus* 540 nm, and 19.6 $mM^{-1} \cdot cm^{-1}$ as the extinction coefficient[11].

All chemicals were purchased from Sigma Chemical Co., unless otherwise stated.

RESULTS

Quinones

The quinones from both *T. neapolitanus* and *T. thioparus* migrated as a single yellow band on the Decalso column and were eluted with 5 % ethyl ether in iso-octane. On thin-layer plates the thiobacillary quinones (*T. thioparus* No. 6 and *T. neapolitanus* No. 5, Fig. 1) migrated as single spots and at the same distance from the origin as Q-8. Using coincidence chromatography with the two thiobacillary quinones and Q-8, a single spot was observed (not shown). The thiobacillary quinones dissolved in abs. ethanol gave spectra typical of ubiquinones with an absorbance maximum at 275 nm in the oxidized state and at 290 nm in the reduced state. There was no evidence of naphthoquinones.

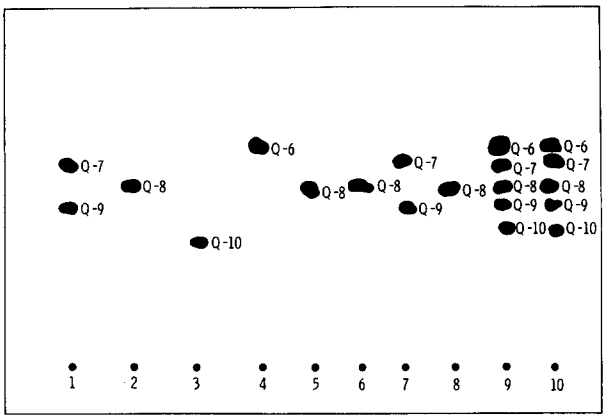

Fig. 1. Thin-layer chromatography of thiobacillary quinones. Thin-layer plates of silica gel impregnated with paraffin oil were developed with acetone–water (19:1, v/v) and visualized with 0.5% Rhodamine B and ultraviolet light. Quinones: 1 and 7, Q-7 and Q-9 from *Candida utilis*; 2 and 8, Q-8 from *E. coli*; 3, Q-10; 4, Q-6; 5, Q-8 from *T. neapolitanus*; 6, Q-8 from *T. thioparus*; 9 and 10, a mixture of 1, 2, 3, and 4.

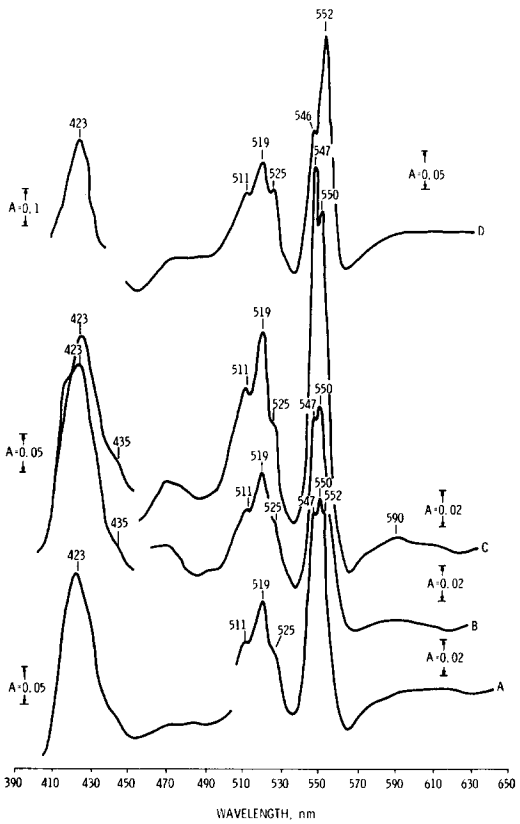

Fig. 2. Dithionite reduced *minus* oxidized difference spectra of subcellular fractions of *T. neapolitanus*. A, crude extract, 27.7 mg/ml protein; B, large particle, 18.8 mg/ml protein; C, small particle, 16.2 mg/ml protein; D, soluble fraction, 14.5 mg/ml protein.

Difference spectra

The cytochrome profile was not resolvable at room temperature. All cytochrome spectra shown were therefore obtained at the temperature of liquid nitrogen. Under these conditions there should be negligible splitting of the α bands[12]. The total cytochrome complement of *T. neapolitanus* is illustrated in Fig. 2 which shows dithionite reduced *vs* oxidized difference spectra of the crude extract (A), the large particle (B), the small particle (C), and the soluble fraction (D). In the α region of the crude extract, there were three bands at 547, 550, and 552 nm, respectively. In both the large-particle and the small-particle fractions, there were bands in the α region at 547 and 550 nm, but the ratio of 547 nm:550 nm absorption was different, the large particle fraction having relatively more absorption of 550 nm and the small particle more at 547 nm, assuming that the extinction coefficients were the same. In the α region of the small-particle fraction there was an absorption band at 590 nm and a shoulder in the Soret region at 435 nm suggesting a cytochrome of the *a* type. The soluble fraction contained absorption bands in the α region at 546 and 552 nm which were not seen in the particulate fractions.

The dithionite reduced *vs* oxidized difference spectra of *T. thioparus* (Fig. 3)

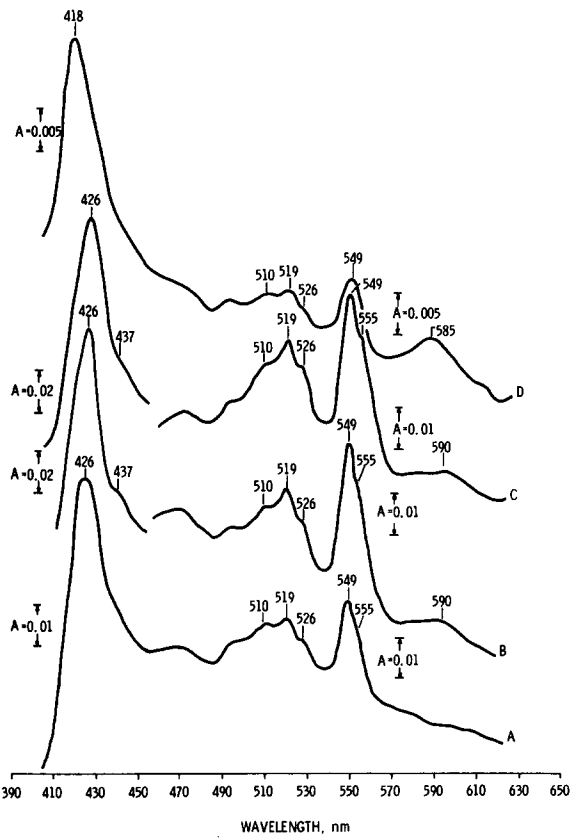

Fig. 3. Dithionite reduced *minus* oxidized difference spectra of subcellular fractions of *T. thioparus*. A, crude extract, 18.0 mg/ml protein; B, large particle, 21.9 mg/ml protein; C, small particle, 8.9 mg/ml protein; D, soluble fraction, 5.5 mg/ml protein.

were quite different from those of *T. neapolitanus*. The crude extract and large and small particle fractions showed an absorption band in the α region at 549 nm with a shoulder at 555 nm. Also, in the large and small particle fractions there were absorption bands at 590 nm in the α region, and shoulders at 437 nm in the Soret region, which are typical of cytochromes of the *a* type. The soluble fraction displayed a broad band at 549 nm and also a band at 585 in the α region.

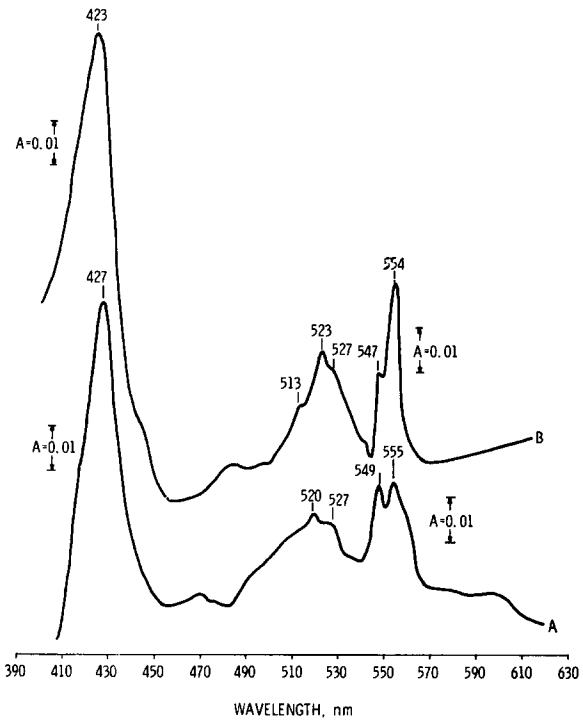

Fig. 4. Dithionite reduced *minus* ascorbate reduced difference spectra of the small particle fractions of *T. thioparus* (A), and *T. neapolitanus* (B). A, 8.9 mg/ml protein; B, 16.2 mg/ml protein.

The dithionite reduced *vs* ascorbate reduced difference spectra of the small particle fractions of both organisms are shown in Fig. 4. The small particle fraction of *T. thioparus* (A) had α bands at 549 and 555 nm, while that of *T. neapolitanus* (B) had α bands at 547 and 554 nm. The cytochromes represented by α bands at 549 in *T. thioparus*, and 547 nm in *T. neapolitanus* were probably cytochromes of the *c*-type with low redox potentials, since they were not reduced by ascorbate. The α bands at 555 nm in *T. thioparus* and 554 nm in *T. neapolitanus* probably indicate cytochromes of the *b*-type, although it is possible that they may also be low potential *c*-type cytochromes.

In the NADH reduced *vs* oxidized spectra of fractions of *T. neapolitanus* (Fig. 5), three bands in the α region were resolved in both the crude extract (A) and large particle fraction (B), at 547, 550, and 558 nm. The cytochrome represented by an α band at 558 nm was probably a cytochrome of the *b* type. In the small particle fraction, only one cytochrome band was seen in the α region at 550 nm.

The NADH reduced *vs* oxidized difference spectra of the cytochromes of *T. thioparus* are shown in Fig. 6. In both the crude extract and large-particle fraction there were α bands at 549 and 551 nm. Also in the crude extract there was a broad absorption band in the α region at 586 nm and in the large particle fraction at 596 nm, which corresponds to cytochromes of the *a* type. Little cytochrome reduction by NADH was seen in the small-particle fraction, but there was a minor peak at 551 nm.

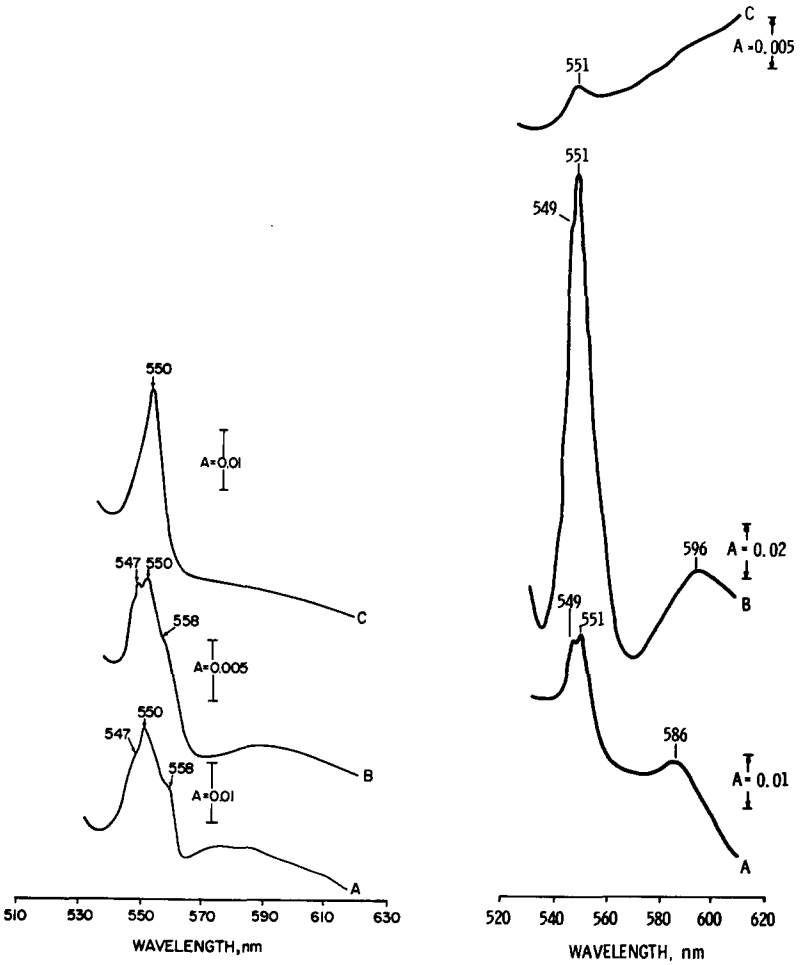

Fig. 5. NADH reduced *minus* oxidized difference spectra of subcellular fractions of *T. neapolitanus*. A, crude extract; B, large particle and C, small particle fraction. Protein concentrations are the same as those for Fig. 2. The conditions of reduction were as described by Lanyi[10].

Fig. 6. NADH reduced *minus* oxidized difference spectra of subcellular fractions of *T. thioparus*. A, crude extract, 18.0 mg/ml protein; B, large particle, 33.8 mg/ml protein; and C, small particle fraction, 18.5 mg/ml protein.

Concentration of quinone, flavin, and cytochrome c

The approximate molar content of cytochrome *c*, flavin, and quinone in *T. neapolitanus* and *T. thioparus* is shown in Table I. Most of the flavin was located in

TABLE I

FLAVIN, QUINONE, AND CYTOCHROME *c* CONTENT (μmoles/g PROTEIN) OF *T. neapolitanus* AND *T. thioparus*

Concentrations were determined as described in the text.

Fraction	*Flavin*		*Quinone*		*Cytochrome c*	
	Concn	% *Recovery*	*Concn*	% *Recovery*	*Concn*	% *Recovery*
T. neapolitanus						
Crude extract	0.56	100	2.12	100	2.10	100
Large particle	0.38	51	3.52	63	2.23	40
Small particle	0.10	3	3.05	26	2.33	20
Soluble	0.77	51	0.29	5	1.92	34
T. thioparus						
Crude extract	0.45	100	17.04	100	0.83	100
Large particle	0.43	51	27.71	86	1.05	67
Small particle	0.44	6	2.34	1	0.54	4
Soluble	1.00	38	0.57	1	0.24	5

the large particle and soluble fractions with very little in the small particle fractions. The level of quinone was higher in *T. thioparus* than in *T. neapolitanus*. The greatest concentration of quinone was found in the large particle fraction of both organisms. Cytochrome *c* levels were higher in *T. neapolitanus* than in *T. thioparus*. Cytochrome *c* was distributed among all of the fractions of *T. neapolitanus*, but in *T. thioparus* it was concentrated in the large particle fraction.

Distribution of activities

The NADH oxidase activity (Table II) of *T. neapolitanus* was located in both the large and small particle fractions, with about 3 times as much in the large particle fraction while that of *T. thioparus* was located exclusively in the large-particle fraction.

TABLE II

DISTRIBUTION OF SEVERAL ELECTRON TRANSPORT ACTIVITIES IN VARIOUS FRACTIONS OF *T. neapolitanus* AND *T. thioparus*

Activities were measured as described in the text.

Fraction	*NADH oxidase (natoms O_2/min per mg protein)*	*NADH:$Fe(CN)_6^{3-}$ reductase (nmoles NADH/min per mg protein)*	*Ascorbate oxidase (natoms O_2/min per mg protein)*	*Ascorbate:TMPD oxidase (natoms O_2/min per mg protein)*
T. neapolitanus				
Crude extract	19	98	11	808
Large particle	68	289	11	1064
Small particle	21	258	13	1370
Soluble	0	212	14	78
T. thioparus				
Crude extract	26	479	12	850
Large particle	29	664	38	1180
Small particle	0	288	37	2620
Soluble	0	2838	0	0

In both organisms NADH:$Fe(CN)_6^{3-}$ reductase activity was present in all fractions and was found at much higher levels than the NADH oxidase activity. Ascorbate oxidase was present in each fraction of both organisms, except the soluble fraction of *T. thioparus*. Ascorbate:TMPD oxidase levels were much higher than ascorbate oxidase levels, and the ascorbate:TMPD activity was located in all fractions of both organisms, except the soluble fraction of *T. thioparus*.

The effect of inhibitors

The NADH oxidase activities of *T. neapolitanus* (Table III) were sensitive to all of the inhibitors used, except in the activity located in the small particle fraction, which was not sensitive to atabrine. The activity located in the large particle fraction showed a greater sensitivity to all inhibitors tested than did the activity located in the small particle fraction. The NADH oxidase activity in *T. thioparus* (Table IV) was also sensitive to these inhibitors, with the exception of the insensitivity of the activity in the large particle fraction to atabrine. The major difference in the sensitivities of these activities of the two organisms was seen in the case of CN^-. The NADH oxidase activity of *T. thioparus* was always 100% inhibited by CN^-, while

TABLE III

EFFECT OF INHIBITORS ON NADH OXIDASE ACTIVITY OF *T. neapolitanus*

Inhibition of oxygen uptake was measured as described in the text.

Inhibitor	*Concn* (*M*)	% *Inhibition*		
		Crude extract	*Large particle*	*Small particle*
Amytal	$5.6 \cdot 10^{-4}$	87	87	66
Atabrine	$5.6 \cdot 10^{-4}$	29	48	0
Rotenone	$1.8 \cdot 10^{-4}$	88	97	78
o-Phenanthroline	$7.7 \cdot 10^{-5}$ *	57	84	9
Antimycin A	$2.5 \cdot 10^{-5}$ *	75	70	28
HQNO	$5.4 \cdot 10^{-5}$ *	80	86	51
CN^-	$5.6 \cdot 10^{-3}$	73	80	67

* Approximately 1 μg of inhibitor/mg protein.

TABLE IV

EFFECT OF INHIBITORS ON NADH OXIDASE OF *T. thioparus*

Inhibition of oxygen uptake was measured as described in the text.

Inhibitor	*Concn* (*M*)	% *Inhibition*	
		Crude extract	*Large particle*
Amytal	$5.6 \cdot 10^{-4}$	76	88
Atabrine	$5.6 \cdot 10^{-4}$	55	0
Rotenone	$1.8 \cdot 10^{-4}$	74	94
o-Phenanthroline	$7.7 \cdot 10^{-5}$ *	40	44
Antimycin A	$2.5 \cdot 10^{-5}$ *	72	55
HQNO	$5.4 \cdot 10^{-5}$ *	51	48
CN^-	$5.6 \cdot 10^{-3}$	100	100

* Approximately 1 μg of inhibitor/mg protein.

TABLE V

EFFECT OF CN^- ON ASCORBATE AND ASCORBATE:TMPD OXIDASES OF *T. neapolitanus*

Inhibition was measured on oxygen uptake as described in the text.

Fraction	% *Inhibition*	
	Ascorbate oxidase	*Ascorbate:TMPD oxidase*
Crude extract	43	91
Large particle	41	89
Small particle	66	85
Soluble	0	0

that of *T. neapolitanus* was never inhibited by more than 80 %, even at levels up to 0.01 M. The ascorbate oxidase and ascorbate:TMPD oxidase activities of *T. neapolitanus* and *T. thioparus* were insensitive to 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) but were sensitive to CN^-. The percent inhibition by CN^- of NADH oxidase of *T. neapolitanus* was intermediate between that of the ascorbate and TNPD oxidase (Table V), while the NADH oxidase activity of *T. thioparus* was completely inhibited.

DISCUSSION

The electron transport systems of these two obligate autotrophs were shown to contain components common to heterotrophic electron chains. The levels of quinone, flavin, and cytochrome *c* were comparable to those found in heterotrophic bacteria. Both organisms contained the same quinone, Q-8, which is also found in several other Gram negative bacteria[6]. The presence of Q-8 in *T. thioparus* has been reported previously[13], but adequate verification, as presented here, was not included.

The cytochrome complements of *T. neapolitanus* and *T. thioparus* appear to be distinct based upon difference spectrophotometry analyses. This conclusion, however, must be considered tentative until purification of the cytochromes, since in some instances there were only 1 nm differences. *T. neapolitanus* was shown to contain cytochromes c_{547}, c_{550}, c_{552}, *b* or c_{554}, b_{558}, and *a*. The α bands at 590 nm and the Soret bands at 435 nm indicate that the *a* cytochrome is probably of the a_1 type. Aleem[1] reported cytochromes c_{550}, c_{553}, c_{557}, and *b* using tetrathionite reduction at room temperature, but in our experience these bands are unresolvable at room temperature. Those bands he alludes to in his text would, however, correspond to those which we can clearly resolve at liquid nitrogen temperature, since there would be a 1–3-nm shift to the shorter wavelengths due to liquid nitrogen temperature. Ross *et al.*[14] also suggested the presence of *o*- and a_3-type cytochromes from CO difference spectra. From our data cytochrome *a* seemed to be present, and the b_{558} may be a cytochrome of the *o* type. Trudinger[15,16] demonstrated three soluble *c*-type cytochromes in *T. neapolitanus* (c_{550}, $c_{553.5}$, and c_{557}), while we found two (c_{546} and c_{552}). Those differences might be due to differences in growth conditions, since we know now that the metal mix originally recommended for growth of the sulfur oxidizing chemolithotrophs[16] did not provide an optimal medium for their growth.

T. thioparus contained cytochromes c_{549}, c_{551}, *c* or b_{555}, and *a*. The α band at

596 nm in the NADH reduced *minus* oxidized large-particle fraction may represent a cytochrome oxidase of the $a + a_3$-type similar to that of *Prototheca zopfii* at 598 nm[17]. Only one cytochrome has been reported previously in *T. thioparus*, namely cytochrome s_{552}[18,19], under growth conditions quite different from ours; since the yields were only 3–6 g/300 l of medium in 6–8 days, whereas in our hands, the usual growth yield is 0.5–1 g/l in 36–48 h, it is difficult to assess that report.

The large and small particle fractions of *T. neapolitanus* did not oxidize NADH in the same manner since in the large particle fraction cytochromes c_{547} and c_{550} were reduced, while in the small particle fraction only c_{550} was reduced.

The presence of low-potential *c*-type cytochromes in both organisms may offer a possibility for direct reduction of NAD^+ by an inorganic substrate, if the redox potentials of these cytochromes are low enough. The thiobacilli must provide reducing power in the form of NADH for CO_2 fixation. The only suggestion which has been made to date to provide the needed NADH, is reversed electron transport from the inorganic substrate to NAD^+, but this would prove energetically unfavorable to the organism, since it requires as musch as 5 moles of ATP per mole of NAD^+ reduced. A mechanism for direct reduction would be energetically far more beneficial to these organisms, and a low potential cytochrome *c* provides a possibility for direct reduction of NAD^+. These may be c_3-type cytochromes such as have been reported in the sulfate-reducing bacteria *Desulfovibrio* and some photosynthetic bacteria[20].

The presence in *T. neapolitanus* of a considerable amount of a soluble cytochrome not found in the particulate fractions may be similar to the situation with soluble type cytochromes of photosynthetic bacteria[21]. Along with other evidence — light stimulation of growth[22], AMP inhibition pattern[22], presence of methylated phospholipids[23] in *T. neapolitanus* but not *T. thioparus* — this reinforces the concept of an evolutionary link[23] between photosynthetic bacteria and *T. neapolitanus*.

The levels of NADH and ascorbate oxidases in these two thiobacilli were similar to levels reported in both facultative autotrophic and heterotrophic bacteria[24,25]. The levels of the NADH:$Fe(CN)_6^{3-}$ reductases and ascorbate:TMPD oxidases were higher than those reported in other organisms[25,26]. The NADH dehydrogenases of these two organisms were not the limiting factor in the oxidase activity, as reported for *Hemophilis*[26], since the NADH:$Fe(CN)_6^{3-}$ reductase levels were much higher than the NADH oxidase levels.

A major difference in the electron transport chains of these two thiobacilli was the subcellular localization of the NADH oxidase activity. The presence of NADH dehydrogenase, ascorbate oxidase, and ascorbate:TMPD oxidase activities in each fraction of *T. thioparus* suggests that the localization of activity of NADH oxidase in the large particle fraction could be due to: (1) the inability of the activities to function as a unit because of the loss, or absence of a necessary component, or (2) improper orientation of the activities in the absence of binding to the large particles. The atabrine insensitivity of the large-particle fraction perhaps reflected a loss of a soluble component which interacted with the large-particle fraction and was sensitive to atabrine in the crude extract. Also, there was a loss of activity, presumably due to inactivation during fractionation, since the activity of the crude extract was approximately the same as that of the large-particle fraction. If all of the activity had been preserved in the large particles, the observed activity should have been higher.

Although NADH oxidase activity of *T. neapolitanus* was found in both the large and small-particle fractions, the activity was always greater (approximately 3-fold) in the large-particle fraction. The lower activity of the small-particle fraction was probably due to the inability of NADH to reduce the cytochrome c_{547} in this fraction. The absence or loss of this ability might be due to: (1) an inherent difference in the interaction of the components of these two fractions; (2) the loss of a component from the small particle fraction during cell breakage; (3) inaccessibility of c_{547} to NADH resulting from its spatial orientation in the small particle.

The absence of sensitivity to atabrine observed with the small-particle fraction of *T. neapolitanus* indicates that the flavoprotein that reduced c_{550} was insensitive to atabrine, although it was sensitive to amytal and rotenone. This suggests the presence in *T. neopolitanus* of at least two flavoprotein dehydrogenases: one insensitive to atabrine and which reduces cytochrome c_{550}, one sensitive to atabrine and which reduces cytochrome c_{547}. The NADH oxidase system located in the large particle fraction must either be more accessible to, or inherently more sensitive to, the inhibitors tested than the small-particle fraction since the degree of observed inhibition was always greater with the large particle fraction.

In the electron transport chains of both organisms, ascorbate and ascorbate:TMPD must enter after cytochrome *b* since these two oxidases were insensitive to amytal and HQNO, but were sensitive to CN^-.

In *T. neapolitanus*, electrons from ascorbate and ascorbate:TMPD must enter the chain at different locations, since the ascorbate:TMPD activity was so much greater than the ascorbate oxidase. Electrons from ascorbate:TMPD probably enter at the low potential cytochrome c_{547} as well as the high potential cytochrome c_{550} (although this has not been shown spectrophotometrically), while ascorbate electrons enter at the high potential cytochrome c_{550}. Also ascorbate and ascorbate:TMPD utilized different terminal oxidases as shown by the difference in CN^- inhibition. Electrons from ascorbate:TMPD may also enter at cytochrome c_{554}, which was not involved in NADH oxidation, but may be involved in sulfur oxidation.

In *T. thioparus* electrons from ascorbate:TMPD entered the chain at a different location and probably also use a different terminal oxidase from ascorbate, since the ascorbate:TMPD oxidase levels were much higher than ascorbate oxidase levels.

The following electron transport chains are proposed

T. neapolitanus

$$\text{NADH} \rightarrow F_{p_1}(\text{NHI}) \rightarrow \text{cytochrome } b\ (?) \rightarrow \text{cytochrome } c_{547} \rightarrow \text{cytochrome } o\ (b_{558}) \rightarrow O_2$$

$$\text{NADH} \rightarrow F_{p_2}(\text{NHI}) \rightarrow \text{cytochrome } b\ (?) \rightarrow \text{cytochrome } c_{550} \rightarrow \text{cytochrome } a \rightarrow O_2$$

Cytochrome b_{558} is included as a terminal oxidase since it was reduced by ascorbate, indicating a high potential. Also cytochrome b_{558} is probably reduced by cytochrome c_{547}, since no reduction of b_{558} was seen with the reduced cytochrome c_{550} in the small particle fraction.

T. thioparus

$$\text{NADH} \rightarrow F_p(\text{NHI}) \rightarrow \text{cytochrome } b\ (?) \rightarrow \text{cytochrome } c_{549} \rightarrow \text{cytochrome } a \rightarrow O_2$$

$$\text{NADH} \rightarrow F_p(\text{NHI}) \rightarrow \text{cytochrome } b\ (?) \rightarrow \text{cytochrome } c_{551} \rightarrow \text{cytochrome } a \rightarrow O_2$$

It is possible that the electron transport chain of *T. thioparus* is branched at cytochrome *b*, using the same dehydrogenase and the same cytochrome *b*, although the

atabrine sensitivity of the crude extract and the insensitivity of the large particle fraction suggests the presence of two different dehydrogenases.

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REFERENCES

1 M. I. H. Aleem, *Antonie van Leeuwenhoek*, 35 (1969) 379.
2 M. H. Sadler and E. J. Johnson, *Bacteriol. Proc.*, (1970) p. 78.
3 H. Low and I. Vallin, *Biochem. Biophys. Res. Commun.*, 9 (1962) 307.
4 A. S. Cornish and E. J. Johnson, *Arch. Biochem. Biophys.*, 142 (1971) 584.
5 E. R. Kashket and A. F. Brodie, *Biochim. Biophys. Acta*, 40 (1960) 550.
6 R. L. Lester and F. L. Crane, *J. Biol. Chem.*, 234 (1959) 2169.
7 U. Gloor, in E. Stahl, *Thin-Layer Chromatography*, Academic Press, New York, 1965, p. 233.
8 E. R. Redfearn, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. X, Academic Press, New York, 1967, p. 381.
9 N. A. Rao, S. P. Felton and F. M. Huennekens, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. X, Academic Press, New York, 1967, p. 494.
10 J. K. Lanyi, *Arch. Biochem. Biophys.*, 128 (1968) 716.
11 T. Yonetani, *J. Biol. Chem.*, 240 (1965) 4509.
12 R. W. Estabrook, in J. E. Falk, R. Lemberg and R. K. Morton, *Haematin Enzymes*, Pergamon Press, New York, 1961, p. 442.
13 T. M. Cook and W. W. Umbreit, *Biochemistry*, 2 (1963) 194.
14 A. J. Ross, R. L. Schoenhoft and M. I. H. Aleem, *Biochem. Biophys. Res. Commun.*, 32 (1968) 301.
15 P. A. Trudinger, *Biochim. Biophys. Acta*, 30 (1958) 211.
16 P. A. Trudinger, *Biochem. J.*, 78 (1961) 673.
17 B. Epel and W. L. Butler, *Science*, 166 (1969) 621.
18 T. W. Szczepkowski and B. Skarzynski, *Acta Microbiol. Pol.*, 1 (1952) 93.
19 R. Klimek, B. Skarzynski and T. W. Szczepkowski, *Acta Biochim., Pol.*, 3 (1956) 261.
20 T. E. Meyer, R. G. Bartch and M. D. Kamen, *Biochim. Biophys. Acta*, 245 (1971) 453.
21 M. D. Kamen and T. Horio, in E. E. Snell, *Annu. Rev. Biochem.*, 39 (1970) 690.
22 J. V. Mayeux and E. J. Johnson, *J. Bacteriol.*, 94 (1967) 409.
23 J. M. Shively and A. A. Benson, *J. Bacteriol.*, 94 (1967) 1679.
24 A. J. Smith, J. London and R. Y. Stanier, *J. Bacteriol.*, 94 (1967) 972.
25 J. Biggins and U. E. Dietrich, Jr, *Arch. Biochem. Biophys.*, 128 (1968) 40.
26 D. C. White, *J. Biol. Chem.*, 239 (1964) 2055.