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ELECTRON TRANSFER DURING SULPHIDE AND SULPHITE OXIDATION BY *THIOBACILLUS CONCRETIVORUS*

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SUMMARY

1. Cytochromes of the *b*, *c*, *a* and *d* types have been detected in *Thiobacillus concretivorus*. The relative amounts of each component, however, were found to vary with growth conditions. These cytochromes, reduced immediately on adding S^{2-} or SO_3^{2-}, were reoxidised by O_2. Flavin and ubiquinone may also be components of the electron transfer chain.

2. At least two distinct electron transfer pathways are present. The one operating during S^{2-} oxidation is not sensitive to CO, N_3^-, NH_2OH or piericidin A, whereas the other, which is associated with SO_3^{2-} oxidation, is inhibited by these compounds.

3. Phosphorylation of ADP to ATP occurred concomitantly with S^{2-} or SO_3^{2-} oxidation.

INTRODUCTION

Several cytochromes of the *c* type have been partially purified and characterised in the Thiobacilli. Thus MILHAUD *et al.*[1] extracted a cytochrome *c* (552) from *Thiobacillus denitrificans* which was reduced by $S_2O_3^{2-}$ and SO_3^{2-} and reoxidised by cytochrome a_3 in the presence of NO_3^- (ref. 2). TRUDINGER[3,4] separated five types of cytochrome *c* from *T. neapolitanus*, none of which combine with CO. A cytochrome of the *c* type which binds CO, has, however, been isolated from *T. concretivorus*[5].

Cytochromes of the *b* type have also been found in *T. neapolitanus*[6,4] and *T. denitrificans*[7]. ALEEM[8] reported that electron transfer in *T. novellus* from $S_2O_3^{2-}$ to O_2 was mediated by cytochromes of the *c* and *a* types, but flavins and cytochrome *b* were not involved.

The inhibition of sulphur and SO_3^{2-} oxidation by CO has been reported in *T. thiooxidans*[9,10]; light reversed the latter but not the former effect. In extracts of *T. novellus*[8] the inhibition of $S_2O_3^{2-}$ oxidation by CO was reversed by light. Reduced bands between 600 and 610 nm associated with cytochromes of the *a* type were found in *T. novellus*[8]. On the basis of absorption spectra with and without CO, cytochrome a_3 has been proposed as a terminal oxidase in *T. denitrificans*[1], whereas in *T. neapolitanus*, cytochrome *o* was implicated (cited in ref. 7).

Cytochromes of the *b*, *c* and *a* types are involved in S^{2-} oxidation in *T. concre-*

tivorus[5]. S^{2-} oxidation proceeds in two stages; the first is rapid and its concomitant O_2 consumption is not inhibited by CO, whereas the second is slow and its associated O_2 uptake which is inhibited by CO is reversed by light[5].

Ubiquinone-8, tentatively identified in *T. thiooxidans*[13] and *T. thioparus*[14], is thought to function during sulphur oxidation. The involvement of ubiquinones in S^{2-} oxidation by *T. concretivorus* has also been reported[5].

Although cytochromes mediate electron transfer during oxidation of inorganic sulphur compounds by the thiobacilli, the precise mechanisms involved are not known. In this paper data on the respiratory pathways for the oxidation of S_2^- and SO_3^{2-} in *T. concretivorus* are presented.

METHODS

Cultures of organism

T. concretivorus (NCIB 9514) was grown and harvested as described previously[5]. Cells comparable to those described in the previous paper will be referred to as Batch A, and those grown in continuous cultures over a period of 3 years, resulting in a changed cytochrome pattern, will be referred to as Batch B.

Preparation of extracts

Cells suspended in 50 mM phosphate buffer (pH 7.0) containing 0.2 mM EDTA (sodium salt) (25 %, w/v) were passed twice through a French pressure cell at 20000 lb/inch^2 at 4°. The crude homogenate was centrifuged at 20000 × *g* for 40 min, and the supernatant fraction, dialyzed for 12 h against 200 vol. of the same buffer, was used as the crude extract (S_{20}). This extract, centrifuged at 144000 × *g* for 1 h, yielded a supernatant fraction (S_{144}) and a pellet (P_{144}). The pellet was resuspended in 50 mM phosphate buffer (pH 7.0) containing 0.2 mM EDTA (sodium salt), and this will be referred to as the membrane fraction.

Protein was determined by the method of ITZHAKI AND GILL[11], using bovine serum albumin as a standard.

Acetone extraction of the membrane fraction was carried out as described previously[5]. The acetone-soluble fractions were used as a source of ubiquinone.

O_2 uptake

O_2 uptake was measured polarographically as described previously[5].

Spectrophotometry

A Unicam SP800 recording spectrophotometer was used in conjunction with a scale expander and Goerz Electro Servoscribe recorder to measure absorption spectra. A Cary-14 recording spectrophotometer (with and without a low-temperature attachment) was also used for some of the spectra.

Phosphorylation

The crude extract used for phosphorylation studies was prepared by passing the cell suspension once through a French pressure cell at 15000 lb/inch^2 at 4°. The homogenate was centrifuged at 10000 × *g* for 20 min and the supernatant fraction (S_{10}), dialyzed for 3 h at 4° against the phosphate buffer, was used immediately. Crude extracts after storage overnight at 4° did not phosphorylate ADP.

ATP formation during S^{2-} and SO_3^{2-} oxidation by crude extracts (S_{20}) was determined by the firefly method using a Packard Tricarb scintillation spectrometer (Model 3375)[12]. The reaction mixture, containing 1 ml of 10 mM phosphate buffer (pH 7.3), 1 ml 50 mM arsenate (pH 7.3), 1 ml water, 50 μl of firefly extract (4 lanterns) and 0.2 ml of 0.1 M $MgCl_2$, was pipetted into the scintillation vial, and the background counts were determined. Then 10 nmoles ADP were added, followed by 0.1 ml S_{10} extract containing 15 mg protein/ml. Background counts were again determined. The reaction was started by adding 10 μl of 50 mM Na_2S solution, or 10 μl of 100 mM Na_2SO_3 and assayed over a period of 5 min at 0.1-min intervals.

Chemicals

Standard A.R. chemicals were dispensed in double glass-distilled water. Na_2S solution was freshly prepared each day from washed crystals of $Na_2S \cdot 9H_2O$.

ATP, ADP and bovine serum albumin were purchased from Sigma Chemical, St. Louis, U.S.A.; sodium diethyldithiocarbamate from Merck, Darmstadt, Germany; bathocuproin and bathophenanthroline (disodium sulphonate salts) from Fluka AG, Buchs, Switzerland; ubiquinones from Calbiochem, Los Angeles, U.S.A.; piericidin A was a gift from Professor S. Tamura, Department of Agricultural Chemistry, University of Tokyo. Other chemicals were obtained either from the British Drug Houses, Poole, Great Britain, or from May and Baker, Dagenham, Great Britain.

RESULTS

Spectra of cytochromes

Difference spectra of S_{20} extracts treated with S^{2-} *minus* oxidised, indicate that cytochromes of the *b*, *c* and *a* types were reduced (Fig. 1(a)). Since similar difference spectra were obtained with $S_2O_4^{2-}$ instead of S^{2-}, it is clear that S^{2-} fully reduced these cytochromes. When SO_3^{2-} was used as a reductant in crude extracts (S_{20}), however, the absorption spectrum for reduced cytochrome *b* was not evident (Fig. 1(b)).

The difference spectra of the cytochromes in the membrane fraction (P_{144}), reduced with either S^{2-} or SO_3^{2-} *minus* oxidised, are illustrated in Fig. 1(c,d). Cytochrome *b* is only partially reduced by SO_3^{2-} (430 nm), whereas with S^{2-} it is completely reduced. In crude extracts (S_{20}) the absorption spectrum of cytochrome *b* reduced by SO_3^{2-} was masked by the more intense bands of cytochrome *c*.

At liquid N_2 temperatures, the α band of the $Na_2S_2O_4$ reduced *minus* oxidised spectrum of cytochrome *a* in the S_{20} extract was observed at 612 nm, with shoulders at 590 and 570 nm (Fig. 2). This suggests that cytochromes of *a* and possibly *d* types contribute to the broad band centred around 607 nm of room temperature spectra.

Components of Batch A and B cells

The ratio of cytochrome *b* (563) to cytochrome *c* (551) was much less in cells of A than in those of B. Thus when S_{20} extracts of Batch A were reduced with S^{2-} the absorbance of the α band of cytochrome *b* was about 12 % of that of the α band of cytochrome *c* (Figs. 1(a) and 3(a)).

A large maximum at 415 nm and minima at 436, 521, 551 nm were observed in

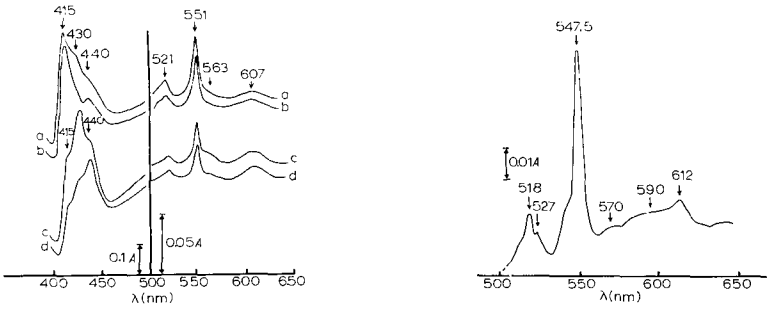


Fig. 1. Reduced *minus* oxidised difference spectra of crude extracts or P_{144-1h} fractions (Batch A cells). Conditions: 1-cm cuvettes in "close-up" position of the SP800 spectrophotometer, contained 2.5 ml extract (20 mg protein) in 50 mM phosphate buffer (pH 7.0). A few small crystals of reductant were added to the sample cuvette. a, crude extract reduced with Na_2S or $Na_2S_2O_4$; b, crude extract reduced with Na_2SO_3; c, P_{144-1h} fraction reduced with Na_2S and $Na_2S_2O_4$; d, P_{144-1h} fraction reduced with Na_2SO_3.

Fig. 2. Low-temperature difference spectrum of crude extract S_{20} (Batch A cells) reduced with $Na_2S_2O_4$ *minus* oxidised. 0.2-cm cuvettes containing 0.5 ml crude extract (5 mg protein) in 50 mM phosphate buffer (pH 7.0) were immersed in liquid N_2. Spectra recorded in a Cary-14 spectrophotometer, fitted with a low-temperature attachment. The 0–0.1 slidewire was used.

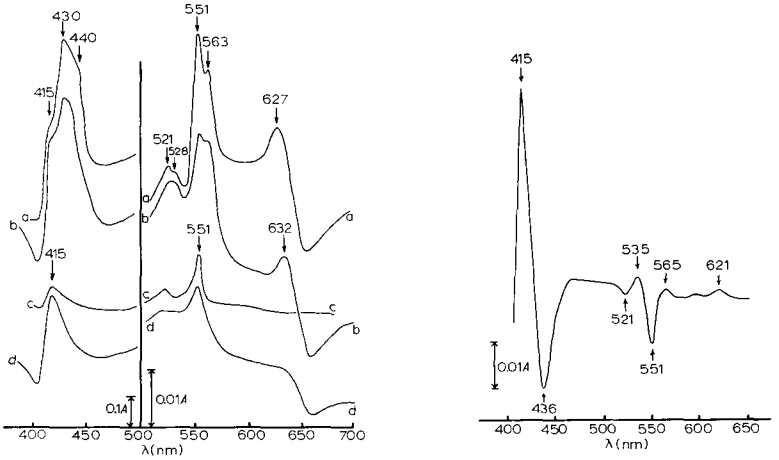


Fig. 3. Difference spectra of crude extracts from Batch B cells. Conditions: 1-cm cuvettes in close-up position of the SP800 spectrophotometer, contained 2.5 ml crude extract (20 mg protein) in 50 mM phosphate buffer (pH 7.0). a, reduced with 40 μmoles S^{2-} *minus* oxidised; b, 30 μmoles S^{2-} were added to sample cuvette, then CO was bubbled through it for 2 min. Another 30 μmoles S^{2-} were added immediately prior to recording the spectrum; c, a (above) reoxidised by shaking briefly in air; d, b (above) reoxidised by shaking briefly in air.

Fig. 4. CO reduced with $Na_2S_2O_4$ *minus* reduced with $Na_2S_2O_4$ difference spectrum of crude extract (Batch A cells). 0.2-cm cuvettes in the Cary-14 spectrophotometer contained 0.5 ml crude extract (5 mg protein) in 50 mM phosphate buffer (pH 7.0), reduced first with a few crystals of $Na_2S_2O_4$. CO was flushed through the sample cuvette for 2 min. The 0–0.1 slidewire was used.

the CO–$Na_2S_2O_4$ reduced *versus* $Na_2S_2O_4$ reduced difference spectra of S_{20} extracts of the A cells (Fig. 4).

The maximum at 415 nm and minima at 521 and 551 nm are due, at least in part, to the binding of CO to cytochrome *c* (ref. 5).

The α band at 621 nm of S_{20} extracts (Fig. 4) may be associated with a cytochrome of the *d* type.

In S_{20} extracts of the B cells, a shoulder at 440 and an α band at 627 nm were detected in the S^{2-} reduced *minus* oxidised difference spectrum (Fig. 3(a)). The α maximum shifted from 627 to 632 nm in the CO–S^{2-} reduced *minus* oxidised spectrum (Fig. 3(b)). A maximum α band at 640 nm was observed in the CO–S^{2-} reduced *minus* S^{2-} reduced difference spectrum for the same extract (Fig. 5). Thus it is likely that a cytochrome of the *d* (627) type was present in the B cells.

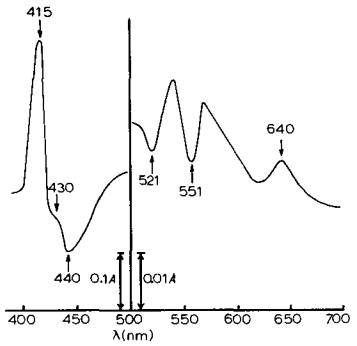


Fig. 5. CO–S^{2-} reduced *minus* S^{2-} reduced difference spectrum of crude extract (S_{20}) (Batch B cells). 1-cm cuvettes in the close-up position of the SP800 spectrophotometer, contained 2.5 ml crude extract (20 mg protein) in 50 mM phosphate buffer (pH 7.0) reduced first with a few crystals of Na_2S. CO was bubbled through the sample cuvette for 2 min.

Pyridine haemochromogens

Particulate fractions (P_{144}) of the B cells were treated with pyridine and alkali by the method of FALK[15]. The reduced *minus* oxidised difference spectrum had a broad maximum between 550 and 557 nm (cytochromes *b* and *c*, respectively) and also a maximum at 585 nm (cytochrome *a*). A broad shoulder between 600 and 650 nm was observed, which is probably associated with haem *d*.

S^{2-} and SO_3^{2-} oxidation

The cytochromes, reduced immediately on adding S^{2-} or SO_3^{2-} were reoxidised in the presence of O_2 when all the S^{2-} was utilised (Fig. 3(c)), but the reoxidation of cytochrome *c*, however, was not complete.

Cytochromes *b*, *c* and *d* reduced by S^{2-}, were partly reoxidised by O_2 even in the presence of CO (Fig. 3 (b and d)). The shift in the α maximum of the cytochrome *d* from 627 to 632 indicates that it had combined with CO. Similarly the increase in intensity of the Soret band at 415 nm and the decrease in those at 521 and 551 nm suggest that some of the cytochrome *c* had combined with CO (Fig. 3 (b)) (see ref. 5). The CO complexes of cytochrome *d* and possibly of an *a* type were not fully reoxidised by O_2 since the minimum at 655 nm (*d*) and the broad shoulder between 580 (*a*) and 640 nm (*d*) persisted.

Ubiquinone (Q)

Acetone-soluble fractions of Cells A and B were examined for ubiquinone components by means of a reversed phase thin-layer chromatography[16]. The following R_F values were recorded in this system for ubiquinone-10 (Q_{10}) 0.2; ubiquinone-6 (Q_6) 0.6; ubiquinone-8 (Q_8) (prepared from *Azotobacter vinelandii*)[17] 0.32; ubiquinone (from the acetone-soluble fractions of *T. concretivorus*) 0.32. Thus the P_{144} fraction of *T. concretivorus* contains Q_8.

O_2 uptake did not occur in crude extracts (S_{20}) which had been extracted with cold acetone when either S^{2-} or SO_3^{2-} was added. The addition of pure Q_6 or the acetone-soluble lipid fraction of the bacterium (which contained Q_8) to these extracts initiated O_2 uptake in the presence of S^{2-} but not of SO_3^{2-}. The rate of S^{2-} oxidation, however, was considerably less than for normal extracts, and was not increased by further additions of either Q_6 or the acetone-soluble lipid fraction of the cells. These effects may be non-specific.

Inhibitors

The reduction of the cytochromes by S^{2-} or SO_3^{2-} was inhibited by sodium diethyldithiocarbamate, NaN_3 and Tris·HCl at 5 mM. In addition, NH_2OH inhibited the reduction of the cytochromes by SO_3^{2-} only. Thus the site of action of these inhibitors is prior to the cytochrome components of the respiratory chain.

The effects of various inhibitors on O_2 uptake during S^{2-} and SO_3^{2-} oxidation are shown in Table I. Thus CO inhibits O_2 uptake associated with SO_3^{2-} oxidation but not that for the utilization of S^{2-}. The reversal by light of the CO inhibition of SO_3^{2-} oxidation suggests the involvement of a terminal oxidase of the haem *a* type.

Phosphorylation

ATP was generated during the oxidation of either S^{2-} or SO_3^{2-} by S_{10} extracts

TABLE I

EFFECTS OF INHIBITORS ON O_2 UPTAKE DURING S^{2-} AND SO_3^{2-} OXIDATION BY P_{144} FRACTION

O_2 uptake was determined by the oxygen electrode. P_{144} (2 mg protein) in 50 mM phosphate buffer (pH 7.0) with 0.2 mM EDTA (sodium salt) in a final volume of 3 ml incubated with each compound (except CO) for 20 min prior to reaction. CO was flushed through P_{144} for 2 min prior to adding 100 μmoles S^{2-} or 30 μmoles SO_3^{2-} to start the reaction. Where indicated, Q_6 (25 μg in 50 μl acetone) was added during the reaction; acetone alone had no effect on the reaction.

Inhibitor	*Inhibition of SO_3^{2-} oxidation* (%)	*Inhibition of S^{2-} oxidation* (%)
p-Chloromercuribenzoate (5 mM)	90	—
Mepacrine (0.5 mM)	40	70
CO (light reversible)	95	0
NaN_3 (5 mM)	60	0
Tris·HCl (5 mM)	50	65
Sodium diethyldithiocarbamate (5 mM)	60	85
Bathocuproin (10 mM)	—	70
Bathophenanthroline (30 mM)	—	50
Piericidin A (40 μM)	95	0
Piericidin A (40 μM) + 25 μg Q_6	80	0

(Fig. 6); ATP was formed during the initial fast reaction of S^{2-} oxidation (Stage 1) (see ref. 5).

An active ATPase was found in these extracts so that the decrease in the amount of ATP recorded at this stage when all the S^{2-} had been completely oxidised (Fig. 6(a)) was probably due to ATPase activity. On adding further amounts of S^{2-} however, ATP was again produced.

Adenylate kinase activity was negligible over the time period of the experiments (5 min) and ATP was formed only when either S^{2-} or SO_3^{2-} was added.

Crude extracts (S_{10}) were more effective in phosphorylating ADP to ATP than those extracts prepared by centrifuging at 20000 × *g* for 40 min (S_{20}).

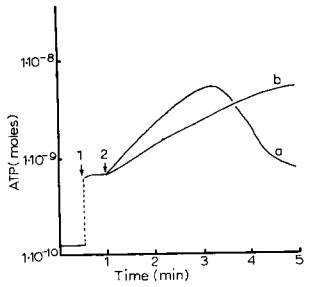


Fig. 6. Phosphorylation during S^{2-} and SO_3^{2-} oxidation. Reaction mixtures contained 1 ml 10 mM phosphate buffer (pH 7.3), 1 ml 50 mM arsenate (pH 7.3), 1 ml water, 50 μl firefly extract, 0.25 ml 0.1 M $MgCl_2$, 10 μl 1 mM ADP. At point 1, 0.1 ml S_{10} extract (10000 × *g* during 20 min) (1.5 mg protein) was added. For details of method: a, 0.5 μmole Na_2S added at Point 2; b, 5 μmoles Na_2SO_3 added at Point 2.

DISCUSSION

Components of the respiratory chain

It is likely that the terminal oxidases of *T. concretivorus* contain cytochromes of the a_1 and *d* types.

The CO–sulphide reduced α maximum of the *d* haem at 640 nm is similar to that reported for cytochrome *d* in other bacteria[17]. The a–a_3 cytochromes are probably absent from the B cells because bands at 432 or 590 nm in the CO-reduced spectra were not detected.

CASTOR AND CHANCE[18] reported that cytochrome *d* usually occurs when cytochromes a_1 and *o* are also present. YAMANAKA AND OKUNUKI[19] showed that the Soret band of cytochrome *d* (a_2) in *Pseudomonas aeruginosa* was of relatively low intensity compared to other cytochromes, and was therefore difficult to detect in crude extracts. This may explain why the γ band of haem *d* was not observed in preparations of *T. concretivorus*. The Soret band at 415 nm in the CO–S^{2-} reduced *minus* S^{2-} reduced difference spectra of S_{20} extracts of *T. concretivorus* may well correspond in part with the 413-nm band reported for similar spectra for the *d* haem of *Ps. aeruginosa*[19]. The CO–cytochrome a_1 absorbs at 428 nm in *Proteus vulgaris*[17], thus in the S_{20} extracts of *T. concretivorus* the CO–S^{2-} reduced *versus* S^{2-} reduced difference spectrum (Fig. 5), the shoulder at 430 nm may be due to cytochrome a_1. There is no conclusive evidence for cytochrome *o*, but its spectrum would be masked in crude preparations by those of cytochromes *c* and a_1.

Inhibitor studies suggest that flavin is required for S^{2-} and SO_3^{2-} oxidation and that ubiquinone is essential for the SO_3^{2-} system only.

SO_3^{2-} oxidation

The partial reduction of cytochrome *b* (563) by SO_3^{2-} suggests that electrons enter the respiratory chain prior to cytochrome *c*. This is supported by the probable participation of Q_8 in electron transfer during SO_3^{2-} oxidation as shown by the piericidin A inhibition of O_2 uptake, and its partial reversal by Q_6. Diethyldithiocarbamate, Tris·HCl, and N_3^- inhibit the reduction of the cytochromes by SO_3^{2-}, suggesting the involvement of a metal at a site prior to the cytochromes.

The CO inhibition reversed by light implicates a cytochrome oxidase of the *a* type as a terminal acceptor during SO_3^{2-} oxidation. Oxidative phosphorylation occurred during electron transfer *via* the cytochrome chain.

S^{2-} oxidation

All the cytochromes are readily reduced by S^{2-}, with concomitant reoxidation by O_2. The accompanying phosphorylation of ADP to ATP indicates that this electron transfer *via* the cytochromes during the first stage of S^{2-} oxidation[5] is part of the enzymic mechanism and not simply a non-specific effect. Some features of this process distinguish it from the electron transfer system associated with SO_3^{2-} oxidation: (1) only a small fraction of the cytochrome *b* was reduced by SO_3^{2-}, whereas it was completely reduced by S^{2-}; (2) piericidin A strongly inhibited SO_3^{2-} oxidation, but had no effect on S^{2-} oxidation; (3) mepacrine inhibited SO_3^{2-} oxidation by 40% and S^{2-} oxidation by 70%; (4) CO did not restrict O_2 uptake during the first rapid oxidation stage of SO_3^{2-} oxidation[5] whereas it inhibited S^{2-} oxidation.

Since CO–cytochrome *d* is readily reoxidised by O_2 in the dark it is likely that this is the terminal oxidase for S^{2-} oxidation. Because the inhibition of SO_3^{2-} oxidation by CO is only reversed in the light it is likely that it utilizes cytochrome a_1 as a terminal oxidase. The data presented herein suggest that the sulphite oxidase is coupled to a terminal electron transfer system which is not utilized by the S^{2-} system.

Thus the initial oxidation of S^{2-}, which probably involves the loss of two electrons to give atoms at the oxidation level of elemental sulphur[20], is probably associated with an electron transfer system with cytochrome *d* as the terminal acceptor. A detailed study of this respiratory sequence will have to await a satisfactory method for separating the membrane bound sulphide oxidase.

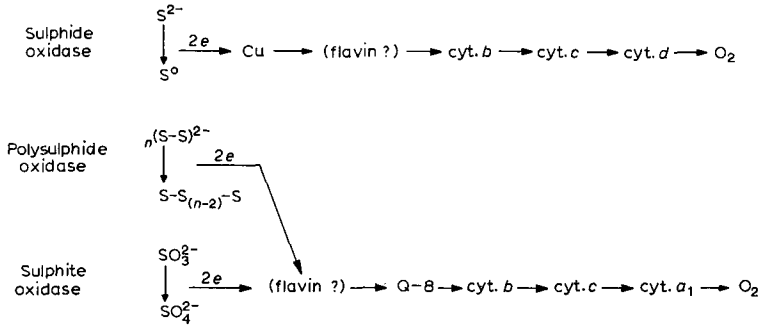
The data obtained previously on particles extracted with acetone[5], and those reported in this paper, suggest that ubiquinone is a component of the electron transfer system which operates during the oxidation of S^{2-}. This conclusion, however, is not supported by experiments with piericidin A, because although this compound inhibited SO_3^{2-} oxidation, it was without effect on the S^{2-} oxidising system. It was noted that the rate of oxidation of S^{2-} in acetone-extracted particles, reactivated with ubiquinone, was considerably less than that in untreated particles; thus it might be a non-specific effect. Without further evidence, it is not certain whether ubiquinone is a component of the electron transfer system for the oxidation of S^{2-}.

A copper protein has been implicated in sulphide oxidase as a binding site for S^{2-} (ref. 5). The inhibition by bathocuproin, which is more marked than that of

bathophenanthroline, further supports this hypothesis. Its involvement at the beginning of the electron transfer sequence is inferred, because sodium diethyldithiocarbamate inhibited the reduction of the cytochromes by S^{2-}.

The cytochromes were not fully reoxidised by O_2 immediately after the completion of the initial rapid oxidation of S^{2-}. Thus electron transfer continued during the slower second stage, and its concomitant O_2 uptake was inhibited by CO (ref. 5). O_2 uptake during the second stage may be accounted for by the oxidation of short chain polysulphides to polymeric compounds with a longer chain length[20].

A tentative scheme to account for these results is as follows:



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