Thiosulphate Oxidation and Cytochromes in *Thiobacillus X*

1. FRACTIONATION OF BACTERIAL EXTRACTS AND PROPERTIES OF CYTOCHROMES

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(*Received* 23 *March* 1960)

In a preliminary communication (Trudinger, 1958) the separation of some soluble cytochromes of the autotrophic sulphur bacterium *Thiobacillus X* was briefly reported. Addition of thiosulphate to intact cells results in the rapid reduction of the cytochromes and the present work was undertaken to attempt to characterize the cytochromes and to obtain some information on the electron-transport chain involved in the oxidation of thiosulphate.

This paper describes in detail the separation and some properties of the soluble cytochromes.

MATERIALS AND METHODS

Organism and growth conditions. The organism used in this study was *Thiobacillus X*, isolated and described by Parker (1947) and Parker & Prisk (1953). The bacteria were grown at 26° in the medium described by Vishniac & Santer (1957) for the culture of *Thiobacillus thioparus*, with thiosulphate as an energy source. Sufficient bromocresol purple was added to the medium to give an observable colour, and saturated Na_2CO_3 was added from time to time to keep the pH between 6 and 8. Un-neutralized cultures of *Thiobacillus X* carried out a partial oxidation of thiosulphate and growth ceased when the pH of the medium reached approx. 3 (Parker & Prisk, 1953). With neutralization, however, growth continued until all substrate was converted into sulphate.

Small amounts of bacteria were grown in penicillin flasks with mechanical shaking. Large batches of bacteria were grown in carboys containing 15 l. of medium, through which was forced air containing about 5% of CO_2. After incubation at 26° for 3–4 days the bacteria were harvested by centrifuging and washed twice with 0·1 M-phosphate, pH 7. When not used immediately the cells were freeze-dried and stored at 2°.

Mammalian cytochrome c. This was prepared from ox heart and was kindly donated by Dr C. A. Appleby.

Haemochromogens. Pyridine and cyanide haemochromogens of cytochromes were prepared according to Vernon & Kamen (1954).

Spectroscopy and spectrophotometry. Visual examinations of absorption spectra were made with a Beck–Hartridge reversion spectroscope or a Beck model 3000 microspectroscope. The latter apparatus is fitted with a comparison prism. For low-temperature spectra materials were mixed with an equal volume of glycerol, frozen in liquid air in a layer 1–2 mm. thick, and examined in the Beck spectroscope after devitrification (Keilin & Hartree, 1949, 1950).

Spectral curves were obtained with a Beckman model DU spectrophotometer with 1 cm. cells.

Terminology. The cytochromes described in this paper have been named according to their source and the locations of their α-absorption peaks in the reduced state (Scarisbrick, 1947). Different components with similar spectra have been distinguished by means of a number in parentheses (cf. Gibson & Larsen, 1955).

Chromatography on Amberlite IRC-50. Commercial Amberlite IRC-50 (XE97) was purified by washing successively with 2 N-NaOH, water, 2 N-HCl and water. Small batches (20–30 g.) of the purified resin were then stirred for 1 hr. with 1 l. of either 0·02 M-potassium phosphate, pH 7, or 0·05 M-sodium citrate, pH 5, and filtered. This procedure was repeated (generally five or six times) until the pH of the buffer changed by less than 0·1 unit. The resin was packed to a depth of 10 cm. in a 1 cm.-diameter column equipped with a jacket through which ice–water was passed. Materials applied to the column were dissolved in the buffer with which the resin was washed and were eluted with increasing buffer concentrations at the rate of 0·3–0·5 ml./min. Protein and cytochrome elution were followed by measuring the extinctions at 280 and 410 mμ respectively of 5 ml. fractions.

Paper electrophoresis. Electrophoresis of cytochromes was carried out in an EEL apparatus (Evans Electroselenium Ltd., Harlow, Essex), with 42 cm. × 1 cm. strips of Whatman no. 1 paper and 0·05 M-potassium phosphate buffers. A voltage of 200 v was applied for 16 hr. Small amounts of cytochrome were detected by spraying the papers with saturated benzidine containing 1% (v/v) of H_2O_2.

Assay of thiosulphate-oxidizing enzyme. The assay is based on results described by Trudinger (1961). The material to be analysed was mixed in a 1 cm.-diameter tube with 0·12 M-potassium phthalate, pH 5, 5 μmoles of $K_3Fe(CN)_6$ and 40 μmoles of $Na_2S_2O_3$. The final volume was 5 ml. and the temperature 23°. E at 420 mμ was recorded at 0·5 min. intervals with a Unicam SP. 600 spectrophotometer. Active enzymic fractions were diluted if necessary until the rate of reduction of ferricyanide was less than 1 μmole/2 min. Under these conditions the reduction rate was approximately linear for 2–3 min. Activities were determined from the linear portion of the curve. One unit of enzyme is defined as the amount required to reduce 1 μmole of ferricyanide/min.

Oxidation–reduction potentials of cytochromes. These experiments were carried out at 30° with the Beckman spectrophotometer and 1 cm. light-path cuvettes fitted with Thunberg side bulbs. Sufficient cytochrome to give a final $E_{\alpha max.}$ in the reduced state of 0·2–0·4 was mixed with 0·1–0·15 μmole of either 2:6-dichlorophenol-indophenol or *o*-chlorophenol-indophenol (E'_0, pH 7, 30° +0·217 v and +0·233 v respectively) in a final volume of 3 ml. of 0·1 M-phosphate, pH 7. The side bulb contained a small amount of ascorbate, sufficient to cause a partial reduction of cytochrome, and dye. This was tipped into the reaction mixture after evacuation of the system with a high-vacuum pump. Equilibrium was reached within 2–3 min. The concentrations of oxidized 2:6-dichlorophenol-indophenol and *o*-chlorophenol-indophenol were determined from the values of $E_{605\,m\mu}$ and $E_{620\,m\mu}$ respectively, after correction for the absorption due to oxidized cytochrome at these wavelengths. Although the absorption by cytochrome at 605 and 620 mμ decreased as the cytochrome was reduced the change was insufficient to cause a significant error in the estimation of dye. The relative concentrations of ferricytochrome and ferrocytochrome were calculated from the values of $E_{\alpha max.}$, after correction for the absorption due to oxidized dye, and the standard potentials of the cytochromes were derived from the normal electrode equation.

Preparation and fractionation of bacterial extracts. All operations in this and subsequent fractionations were carried out at 0–5°. Except for differences in the enzymic activity of particles, similar results were obtained with fresh and freeze-dried bacteria.

The bacteria, 5–8 g. dry wt., were suspended evenly in 30 ml. of 0·04 M-potassium phosphate buffer, pH 7, and passed twice at 15 000 lb./in.^{2} through the apparatus described by Milner, Lawrence & French (1950). This procedure disrupted more than 95% of the cells. The mixture was made up to 100 ml. with buffer, stirred overnight at 2° and centrifuged at approx. 8000 *g* for 30 min. The precipitate, fraction P_1, was usually discarded and the supernatant centrifuged again at 40 000 rev./min. (145 000 *g* at the bottom of the tube) for 60 min. in the no. 40 head of a model L Spinco centrifuge. The supernatant was collected and the particles (fraction P_2) were resuspended in 50 ml. of buffer and centrifuged again under the same conditions.

EXPERIMENTAL AND RESULTS

Spectra of intact cells

Fig. 1 shows a difference spectrum between oxidized intact cells of *Thiobacillus X* and cells in the presence of $Na_2S_2O_3$. Thiosulphate caused an instantaneous reduction of a number of cytochrome components. At room temperature the reduced α-absorption region of Fig. 1 appeared in the Beck–Hartridge spectroscope as a broad intense band from 549 to 558 mμ with a faint band at 562 mμ. At the temperature of liquid air aerated cells showed absorption bands at 510–530 mμ and at 558–562 mμ. In the presence of $Na_2S_2O_3$ a number of absorption bands appeared, the major bands being listed in Table 1. A number of very faint bands were also observed in the regions of 510 and 530 mμ, but these were too indistinct to be defined accurately. No absorption bands above 560 mμ were detected.

All cytochrome absorption bands of thiosulphate-reduced cell suspensions were slightly intensified by the addition of $Na_2S_2O_4$ (e.g. the height of the α peak of the spectrum shown in Fig. 1 was increased by about 10%). No additional bands were observed in the presence of $Na_2S_2O_4$.

Separation of cytochromes and thiosulphate-oxidizing enzyme

Extracts of bacterial cells after high-speed centrifuging showed spectra qualitatively similar to those of intact cells with the exception that the band in the reduced state at 562 mμ was missing. The extracts were fractionated as shown in Fig. 2. At each stage fractions were examined with the hand spectroscope in the presence of $Na_2S_2O_4$ and tested for thiosulphate-oxidizing activity by the standard test described above.

Fractionation with ammonium sulphate. Although only a partial separation of cytochromes and thiosulphate-oxidizing enzyme was achieved by this treatment, subsequent fractionation on Amberlite IRC-50 was much facilitated. Fraction AS 40–70 contained all the recovered enzyme activity together with a mixture of cytochrome components. Fractions AS 70–95 was enriched with respect to those cytochromes absorbing between 552 and 558 mμ.

Chromatography on IRC-50 at pH 7. At pH 7, the thiosulphate-oxidizing activity of fraction AS 40–70 was adsorbed on to Amberlite IRC-50 from 0·02 M-

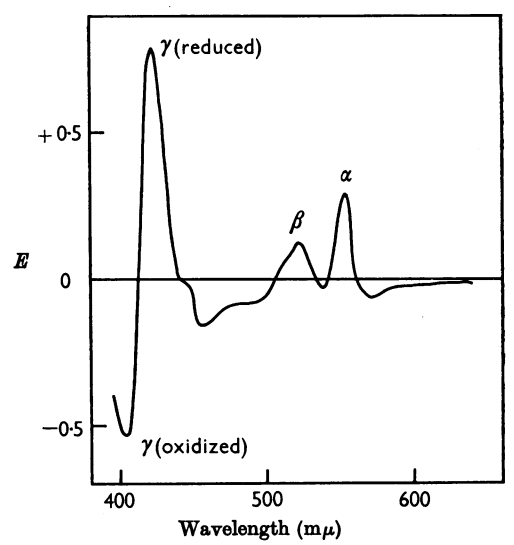

Fig. 1. Difference spectrum of intact *Thiobacillus X* (5 mg. dry wt./ml.) reduced with thiosulphate against oxidized cells. The positions of the absorption peaks are at α, 553 mμ; β, 522 mμ; γ (reduced), 422 mμ and γ (oxidized), 403 mμ.

Table 1. *Low-temperature spectra of intact cells and soluble cytochromes*

The reduced materials in 50% glycerol were examined with the hand spectroscope at liquid-air temperature. Wavelengths of the absorption bands (mμ) and their approximate relative intensities (in parentheses) are given.

Region	Intact cells	Cytochrome 550 (1)	Cytochrome 553·5	Cytochrome 557 (1)	Acidic fraction
α	547–549 (+ + + + +)	547–549 (+ + + + + +)	550–552 (+ + + + +)	553–556 (+ + + + + +)	547–549 (+ + + + + +)
	550–556 (+ + + + + +)	—	—	—	—
	560 (+)	—	—	—	—
	—	537 (+)*	532 (+)*	532–533 (+)*	—
β	525 (+ +)	525 (+ + +)	522–523 (+ + +)	524 (+ + +)	524–525 (+ + +)
	519–521 (+ + +)	519 (+ + +)	520 (+)	519 (+)	519 (+)
	—	508 (+)	510 (+)	510–511 (+)	—

* These bands were tentatively assigned to the α region by analogy with mammalian cytochrome *c* (Estabrook, 1956).

phosphate and eluted by 0·1 M-phosphate. The cytochromes (fraction AMB 1) passed through the resin column. Provided that only small amounts (5–10 mg. dry wt.) of fraction AS 40–70 were applied to the column, a complete separation of oxidizing enzyme from cytochromes was achieved at this stage. However, it was more rapid and convenient as a routine procedure to process larger amounts (up to 100 mg. dry wt.) of fraction AS 40–70. Under these conditions the fraction containing enzyme (AMB 2) was still contaminated with small amounts of cytochrome, which was removed by a second treatment with IRC-50. The yield of enzyme in this case was about 20% less than in the single-step separation.

Two cytochrome components of fraction AS 70–95 were adsorbed from 0·02 M-phosphate on IRC-50 at pH 7. These were eluted by 0·1 M- and 0·2 M-phosphate and have been named *Thiobacillus X* cytochromes 557 (1) and 550 (1) respectively. The unadsorbed material had similar spectroscopic properties to fraction AMB 1, with which it was combined. It was sometimes necessary to re-treat the cytochrome 550 (1) and 557 (1) fractions with IRC-50 to remove traces of contaminating cytochrome components.

Resolution of fraction AMB 1. Fraction AMB 1 had considerable absorption at 260 mμ. This was almost entirely removed by treating the solution dropwise with 1% (w/v) protamine sulphate until no further precipitation occurred, centrifuging and passing the supernatant through a column of Dowex-2 (X8) (formate). In a typical case the extinction ratio E_{260}/E_{280} was reduced from 1·7 to 0·68 by this treatment with little loss of cytochrome. The cytochrome components still failed to adsorb on IRC-50 at pH 7, but as the subsequent fractionation at pH 5 was more precise after the treatment described, the latter was retained in the separation procedure. Treatment of the original bacterial extract with protamine sulphate and Dowex-2 decreased the yield of enzyme without affecting the overall separation of enzyme and cytochromes.

A cytochrome component of the Dowex-treated fraction AMB 1 was adsorbed on to Amberlite IRC-50 at pH 5 from 0·05 M-citrate. This component (*Thiobacillus X* cytochrome 553·5) was eluted with 0·1 M-phosphate, pH 7. The unadsorbed material (acidic fraction) contained two cytochrome components with bands in the presence of $Na_2S_2O_4$ at 550 and 557 mμ respectively. Attempts to separate these components by fractional precipitation with acetone or ethanol, by paper electrophoresis and by chromatography on calcium phosphate gel were unsuccessful.

Recovery and purity of thiosulphate-oxidizing enzyme and cytochromes. Table 2 shows the activities and recoveries of enzyme at various stages of the

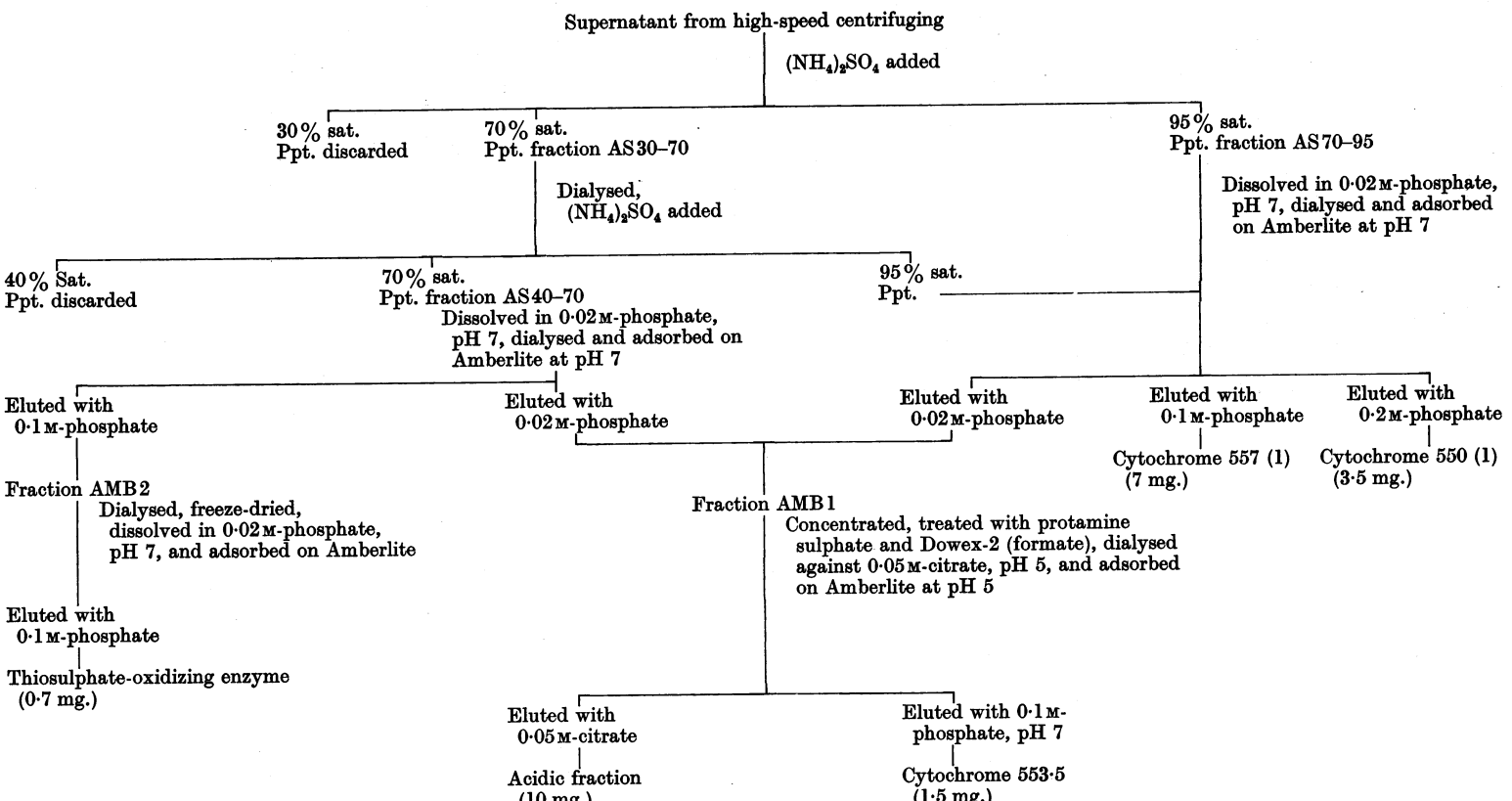

Fig. 2. Flow sheet of procedure for the separation of soluble cytochromes and thiosulphate-oxidizing enzyme. The figures in parentheses refer to the average yield/g. dry wt. of bacteria extracted.

separation procedure. On the basis of extinction at 280 mμ a considerable purification of the crude extract was achieved with a recovery of about 40 %. The final preparation showed no cytochrome bands at −190° and did not fluoresce under ultraviolet light in the presence or absence of thiosulphate.

An assessment of the concentrations of the cytochromes in crude extracts could not be made owing to considerable overlapping of their spectra. This prevented a determination of the recoveries of the individual cytochromes. It was apparent, however, that considerable losses occurred, particularly of cytochrome 553·5, of which only small amounts were obtained. At several stages in the separation procedure insoluble pink precipitates were produced, whereas IRC-50 columns invariably retained pigmented material which resisted elution. No attempts were made in the present study to achieve high degrees of purity for the soluble cytochromes, nor were the various fractions examined by physical tests. However, assuming their molecular weights and α-extinctions in the reduced state to be similar to those of mammalian cytochrome *c*, the best preparations of cytochrome 550 (1) were about 40 % pure and those of cytochromes 553·5 and 557 (1) about 10 % pure.

Properties of the soluble cytochromes

Spectra. At room temperature the reduced cytochromes 550 (1), 553·5 and 557 (1) exhibited typical three-banded spectra between 400 and 600 mμ (Figs. 3–5). All three preparations absorbed strongly in the region of 280 mμ. At the temperature of liquid air the spectra of the reduced soluble cytochromes were much more complex (Table 1) and showed evidence of splitting of the α and β peaks similar to that found with other cytochromes (Keilin & Hartree, 1949; Estabrook, 1956). The main α peaks were at wavelengths 1–4 mμ shorter than those at room temperatures and covered the range visible in intact cells under similar conditions. Indeed, a mixture of cytochromes 550 (1), 553·5 and 557 (1) of approximately equal concentration (based on extinction) showed a spectrum virtually indistinguishable from that of intact cells with the exception of the band at 560 mμ in the latter.

Table 2. *Purification and recovery of thiosulphate oxidase*

For purification procedure and definition of the enzyme unit see text.

Stage of purification	Ratio: Units of enzyme/ml. / $E_{1\,cm.}$ at 280 mμ	Total units
Crude extract	0·5	3900
Fraction AS 40–70	0·85	3470
First IRC-50 treatment	2·1	2150
Enzyme fraction	10·0	1650

Haemochromogens. The main absorption bands of the reduced pyridine and cyanide haemochromogens of the soluble cytochromes are shown in Table 3. Apart from minor variations in the position of absorption maxima the spectra were very similar to those observed when mammalian cytochrome *c* was treated similarly.

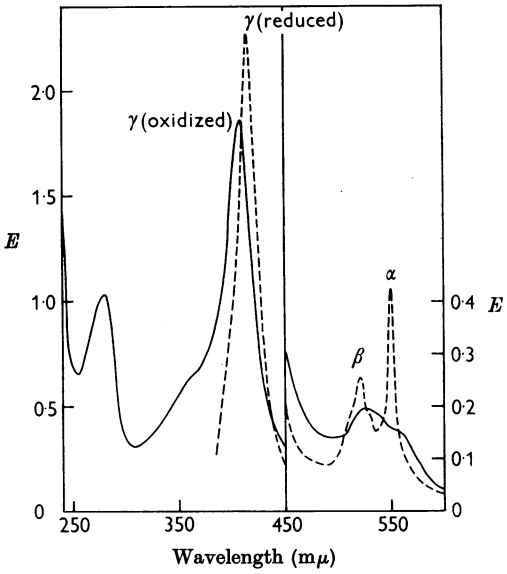

Fig. 3. Absorption spectra of cytochrome 550 (1) (0·55 mg./ml.), oxidized (continuous line) and reduced (broken line). Absorption maxima are: α, 550 mμ; β, 521 mμ; γ (reduced), 416 mμ and γ (oxidized), 409·5 mμ.

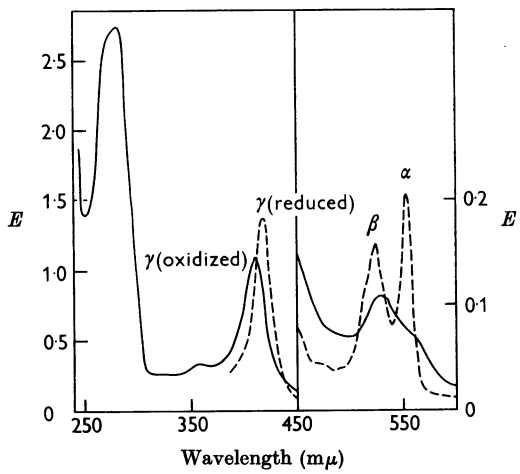

Fig. 4. Absorption spectra of cytochrome 553·5 (2·4 mg./ml.), oxidized (continuous line) and reduced (broken line). Absorption maxima are: α, 553·5 mμ; β, 524 mμ; γ (reduced), 418 mμ and γ (oxidized), 410·5 mμ.

Oxidation–reduction potentials. The standard potentials, E_0' at pH 7 and 30°, were found to be +0·200v for cytochrome 550 (1), +0·210v for cytochrome 553·5 and +0·155v for cytochrome 557 (1).

Effect of carbon monoxide. Carbon monoxide had no effect on the spectra of the reduced soluble cytochromes when freshly prepared or stored at −20° for 2 months. After treatment with acid (pH 3), 2N-NaOH or heating for 3 min. at 60° at pH 7 the α band of all the reduced cytochromes was at 550 mμ. They then reacted with CO, when absorption bands in the reduced state appeared at 560–565 mμ. These changes were also visible after storing the cytochromes for 4–5 days at 2°.

Auto-oxidation. Solutions of reduced cytochrome 557 (1) became oxidized after 1–2 days' storage at 0°. When air was bubbled (30 ml./min.) through a solution of reduced cytochrome 550 (1) it was oxidized at the rate of about 1%/min. Reduced cytochrome 553·5 was completely oxidized within 5 min. under these conditions. The cytochromes of the acidic fraction were also slowly re-oxidized on shaking or aeration. The addition of cytochrome 553·5 accelerated the auto-oxidation of the other soluble cytochromes. Auto-oxidation of the soluble cytochromes was not inhibited by 0·01M-KCN and was independent of pH within the range 5·5–8·5. After denaturation by acid, alkali or heat all the cytochromes were rapidly auto-oxidized. Addition of denatured cytochrome to untreated samples did not accelerate auto-oxidation of the latter.

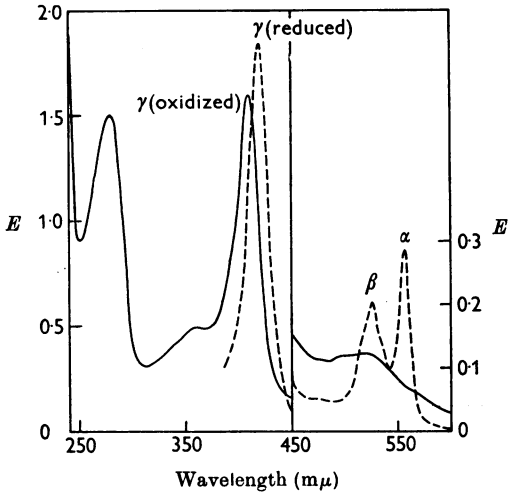

Fig. 5. Absorption spectra of cytochrome 557 (1) (1·4 mg./ml.), oxidized (continuous line) and reduced (broken line). The absorption maxima are: α, 557 mμ; β, 525 mμ; γ (reduced), 419 mμ and γ (oxidized), 409 mμ.

Paper electrophoresis. Detailed electrophoretic studies were not undertaken. However, at pH 7 cytochromes 550 (1) and 557 (1) moved towards the cathode whereas cytochrome 553·5 and the components of the acidic fraction moved to the anode, in agreement with their behaviour on Amberlite IRC-50.

Nature of the cytochrome components of the acidic fraction. Apart from their behaviour on Amberlite IRC-50 the components of the acidic fraction had similar properties to those of the separated basic cytochromes 550 (1) and 557 (1). The spectrum of this fraction in the presence of $Na_2S_2O_4$ and at liquid-air temperature showed absorption peaks at 546–549 mμ and at 552–555 mμ (Table 1). Both components were auto-oxidizable, and were denatured by heat, acid and alkali. They did not combine with CO. The main absorption maxima of the cyanide and pyridine haemochromogens of the components of the acidic fraction are listed in Table 3. An approximate estimation of oxidation–reduction potentials was made by the technique described above, except that the extent of reduction was determined with the hand spectroscope by comparison of the bands with those of cytochromes 550 (1) and 557 (1) at different stages of reduction. This indicated potentials, at pH 7, of the order of 0·20v and 0·15v for the 550 and 557 mμ components respectively. No basic components were released

Table 3. *Haemochromogens of soluble cytochromes*

Spectra of the reduced haemochromogens, prepared as described under Materials and Methods, were determined spectrophotometrically.

Cytochrome	Haemochromogen	Absorption maxima (mμ)		
550 (1)	Cyanide	555	525	420·5
550 (1)	Pyridine	550	520	413
553·5	Cyanide	555	524·5	420
553·5	Pyridine	550	521	414·5
557 (1)	Cyanide	556·5	525	420
557 (1)	Pyridine	550	520	413
Acidic fraction	Cyanide	555–557	525	420
Acidic fraction	Pyridine	550	520	413
Mammalian *c*	Cyanide	554	525	420
Mammalian *c*	Pyridine	550	520	414

when the acidic fraction was subjected to the complete separation procedure.

Cytochromes in the particle fractions

Fraction P_1 was almost devoid of cytochrome and thiosulphate-oxidizing enzyme. After reduction with $Na_2S_2O_4$ faint bands at 548 mμ and at 551–555 mμ were observed at liquid-air temperature and were probably due to the small number of intact cells present in this fraction. Fraction P_2 contained no intact cells and in the presence of $Na_2S_2O_4$ absorption bands at 550, 553–558 and 562 mμ were visible at room temperature. A rough comparison with intact cells indicated that about 30–40 % of the total cell cytochrome remained in the particles. At liquid-air temperature the particles showed intense absorption at 553–555 mμ and at 547–549 mμ together with faint bands at 560 mμ and between 550 and 553 mμ. The particulate cytochromes were not brought into solution by passage through the Hughes press (Hughes, 1951) nor by treatment with butan-1-ol or detergents.

DISCUSSION

On the basis of the spectra of their haemochromogens the soluble cytochromes described in this paper may be classified tentatively as *c* type. It is now evident that different micro-organisms possess characteristic haem–proteins of the *c* type which differ in various properties from each other and from mammalian cytochrome *c* (cf. Kamen, 1955; Mahler, 1957). Although the cytochrome 553·5 of *Thiobacillus X* closely resembles, in all properties studied, cytochrome c_1 of *Pseudomonas* (Vernon, 1956), both cytochromes 550 (1) and 557 (1) exhibit properties which distinguish them from other *c* cytochromes described in the literature (see review by Morton, 1958).

In Table 4 some properties of the soluble cytochromes of *Thiobacillus X* are summarized and compared with the properties of cytochromes isolated from other thiobacilli. All the cytochromes of *Thiobacillus X* differ in a number of respects from the *c* cytochrome of *T. denitrificans* and cytochrome *s* of *T. thioparus*. So far only a single cytochrome of the *c* type has been isolated from each of the last-named two organisms. Indeed, *T. thioparus* has been reported to contain only one *c* cytochrome (Szczepkowski & Skarzynski, 1952); preliminary observations in this laboratory suggest a similar situation in *T. denitrificans*.

The nature of the two cytochrome components in the acidic fraction is obscure although such properties as have been studied indicate that they are very similar to cytochromes 550 (1) and 557 (1) except for their electrophoretic and ion-exchange behaviour. At first it was thought that cytochromes 550 and 557 may be bound in the cell to acidic molecules and became partly dissociated during separation. However, the stability of the components of the acidic fraction towards treatment with Dowex-2, protamine sulphate and to a further complete fractionation does not support this view.

Alternative views appear to be that either the components of the acidic fraction are two quite distinct cytochromes or that both cytochromes 550 and 557 exist in two forms: one free, the other firmly bound to an acidic molecule. A similar situation exists in *Chlorobium thiosulphatophilum*, from which both a basic and an acidic cytochrome component [*Chlorobium* cytochromes 554 (1) and 554 (2)], with very similar properties, have been isolated (Gibson & Larsen, 1955).

The relationship between the acidic and basic forms will probably be resolved when some knowledge of their function is obtained.

SUMMARY

1. Three soluble cytochromes, *Thiobacillus X* cytochromes 550 (1), 553·5 and 557 (1), and an enzyme system oxidizing thiosulphate, have been separated from extracts of the aerobic sulphur

Table 4. *Properties of* c *cytochromes from different thiobacilli*

Source	α Band (reduced) (mμ)	E'_0	Absorption on IRC-50 at pH 7	Auto-oxidation at pH 7	Stability to heat	References*
T. denitrificans	552	+0·270	+	−	+	(1)
T. thioparus (cytochrome *s*)	551	+0·140–0·150	−	+		(2)
Thiobacillus X	550	+0·200	+	+	−	
	553·5	+0·210	−	+	−	
	557	+0·155	+	V. slow	−	
Thiobacillus X Acidic fraction	550/557	+0·2 +0·15	− −	+	−	

* (1) Klimek, Skarzynski & Szczepkowski (1956); (2) Aubert, Milhaud, Moncel & Millet (1958).

bacterium *Thiobacillus X*. A fifth fraction (acidic fraction) contained two additional cytochrome components with α bands at 550 and 557 mμ respectively in the reduced state.

2. All the cytochromes appear to be of the *c* type, are labile to heat and acid and do not combine with carbon monoxide. They are auto-oxidizable to varying degrees.

3. Cytochromes 550 (1) and 557 (1) are basic, and are adsorbed on to Amberlite IRC-50 at pH 7. Their E'_0 values at pH 7 are +0·20 and +0·15 v respectively. Cytochrome 553·5 is acidic but is adsorbed on to Amberlite at pH 5. Its E'_0 value is +0·21 v.

4. The cytochromes of the acidic fraction are not adsorbed on IRC-50. Otherwise their properties are very similar to those of cytochromes 550 (1) and 557 (1) respectively.

5. Addition of thiosulphate to whole cells causes a rapid reduction of cytochrome components with α peaks at 549–558 mμ and 562 mμ.

The author thanks Mr P. Davies for skilled technical assistance.

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Thiosulphate Oxidation and Cytochromes in *Thiobacillus X*

2. THIOSULPHATE-OXIDIZING ENZYME

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(*Received* 23 *March* 1960)

The aerobic sulphur autotroph *Thiobacillus X* oxidizes thiosulphate to sulphate with the intermediate formation of tetrathionate (Trudinger, 1959). In the preceding paper (Trudinger, 1961) the separation from extracts of this bacterium of some cytochrome components and an enzyme oxidizing thiosulphate was reported. In this paper some properties of the enzyme system are described and the role of cytochromes in the oxidation of thiosulphate is discussed.

MATERIALS AND METHODS

Thiosulphate-oxidizing enzyme and cytochromes. The preparation of these is described in the preceding paper (Trudinger, 1961).

Enzymic reaction procedures. All reactions were carried out at 23° unless otherwise stated. Ferricyanide reduction was followed with a Unicam SP. 600 spectrophotometer as described by Trudinger (1961). Cytochrome reduction was studied in 5 cm. × 0·4 cm. tubes which were viewed vertically through a Beck microspectroscope, model 3000, mounted on a microscope stage; the condensing lens was used as a source of illumination. Complete reduction of cytochrome was gauged by comparison with a second sample reduced with $Na_2S_2O_4$.

Gas exchanges were measured by the conventional Warburg manometric technique (Umbreit, Burris & Stauffer, 1949).

Radioactive thiosulphate. $Na_2(S{\cdot}SO_3)$, labelled with ^{35}S in either the inner ($-SO_3$) or outer ($-S$) positions, was obtained from The Radiochemical Centre, Amersham, Bucks. Doubly labelled thiosulphate refers to a mixture of equal activities of inner- and outer-labelled thiosulphate.

Potassium tetrathionate. $Na_2S_2O_3,5H_2O$ (50 g.) was dissolved in 100 ml. of water and oxidized to completion with iodine. An excess of potassium acetate was added and $K_2S_4O_6$ precipitated by the addition of 4 vol. of absolute ethanol. The material was recrystallized from 0·5 N-HCl.