ENZYMOLOGY AND ENERGETICS OF THIOSULPHATE OXIDATION BY *THIOBACILLUS* A2

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Warwick

by

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DECLARATION

The work described in this thesis is the original work of the author. It was carried out in the Department of Environmental Sciences, University of Warwick, between December 1980 and August 1983 and has not been submitted previously for a degree at any Institution.

SUMMARY

The mechanism of thiosulphate oxidation by Thiobacillus versutus (A2) was investigated in an attempt to elucidate the enzyme system and the energetic processes involved. The theoretical maximum growth yield of the organism on thiosulphate was 7 (g dry wt/mol). Thiosulphate oxidation was coupled to the electron transport chain through c-type cytochrome(s). Cell-free extracts catalyzed the complete oxidation of thiosulphate and linked the associated electron transport to ATP synthesis with a P/O ratio of about one. On the basis of these data and a knowledge of the biochemistry of the organism, a requirement reduction of one mole of approximately three ATP for NAD^+ was deduced and the involvement of an oxygenase in thiosulphate oxidation ruled out. Enzymes for conversion of thiosulphate to sulphate were located in the cytoplasm, but oxygen uptake required the membrane fraction, which provided cytochrome c and cytochrome oxidase and could be replaced by its counterparts from mammalian sources.

The crude extract was fractionated by ammonium sulphate, then resolved into three major fractions involved in thiosulphate oxidation by chromatography on DEAE-Sepharose-CL 6B. Four essential components, enzyme A, enzyme B, cytochrome c_{551} and cytochrome $c_{552.5}$ and sulphite:cytochrome c oxidoreductase were finally highly purified from the three fractions by means of gel filtration, hydrophobic interaction chromatography, isoelectric focusing, chromatofocusing and gel electrophoresis.

Enzyme A (Mr 16,000) and Enzyme B (Mr 64,000) were colourless proteins both necessary for the thiosulphate-oxidizing reaction. Cytochrome c_{551} (Mr 260,000) and cytochrome $c_{552.5}$ (Mr 56,000) were multihaem c-type cytochromes, each with a dual Em, 7 of -115, +240 and about -50 and +220 mV respectively. The four components, each comprising 0.6 to 1.5% of the cellular protein, catalyzed complete oxidation of thiosulphate in the presence of an electron transport system. The reaction proceeded in an integral way as neither cleavage of thiosulphate nor any intermediates (e.g. sulphite) were detected and the individual or specific functions of the four components were not identified. The two cytochromes may serve not only as electron carriers but could also have other enzyme functions. Sulphite:cytochrome c oxidoreductase was an important component in the enzyme system, but further study of its function was hampered by its low concentration (less than 0.5% of the cellular proteins) and its intimate association with cytochrome c_{551}.

Rhodanese was proven not to be a part of the thiosulphate-oxidizing system.

A generalized mechanism and a model system are proposed for the path of thiosulphate oxidation to sulphate, the involvement of the four essential components and the role of dual Em 7 of the cytochromes in feeding electrons into ATP and NADH-generating electron transport. The problems remaining to be resolved and possible routes to their solution, are outlined.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Biochemical background to the oxidation of inorganic sulphur compounds in thiobacilli

Thiobacilli are chemolithotrophic bacteria capable of obtaining all the energy required for growth from oxidizing inorganic forms of sulphur, such as thiosulphate, elemental sulphur, sulphide and polythionate and cell carbon from fixing carbon dioxide using the Calvin cycle (Kelly 1971). Thiobacilli occur in diverse aquatic and terrestrial environments and are believed to be responsible for a major part of the biological oxidation of inorganic sulphur in nature.

Progress in elucidating the mechanisms of sulphur oxidation and biochemical energy-coupling in thiobacilli has been slow, mainly because of technical difficulties in handling the organisms (e.g. low growth yield) and the chemistry of their substrates. During the last fifteen years, however, there have been some achievements, mainly on the bioenergetics of the chemolithotrophic organisms, of which I shall give a brief summary in this Chapter. As the title of my thesis has indicated, the Introduction will stress the biochemistry of thiosulphate oxidation and related subjects.

1.1.2 Growth yields

Chemostat culture techniques are generally used to estimate the energy efficiency (growth yield, Y_g) of thiobacilli grown on sulphur compounds, which is very difficult to obtain by using batch culture methods. Theoretical maximum (or true) growth yield, Y_g^{max}, corrected for energy expended in cell maintenance, is calculated graphically using the following equations (Pirt 1965, 1975):

$$1/Y_g = 1/Y_g^{max} + m/\mu \qquad (1.1)$$

or

$$q = 1/Y_g^{max} + m \qquad (1.2)$$

where m = maintenance coefficient, μ = specific growth rate (= D, dilution rate, in steady state of continuous culture), q = specific rate of substrate consumption. From equation 1.1 it follows that if m is constant the graph of $1/Y_g$ against $1/\mu$ will be a straight line with slope m and intercept, $1/Y_g^{max}$ on the ordinate. Alternatively Y_g^{max} and m may be estimated from the plot of q against μ (equation 1.2).

As shown on Table 1.1, the theoretical maximum (or true) growth yields of thiobacilli growing on thiosulphate examined so far fall into more or less the same category, around 6.8 g dry wt cells per mol thiosulphate, except T. denitrificans which gives a doubled value in aerobic growth condition and about 11 in anaerobic growth conditions. The results suggest that the efficiency of energy conservation by these aerobic thiobacilli on thiosulphate is similar. Does this mean that the same biochemical mechanism of thiosulphate oxidation is also operative in these organisms (and thereby extend the concept of "unity in biochemistry", (Kluyver and Donker, 1926)? The present answer is "possible", but direct evidence, such as highly purified enzymes, isolated intermediates (if any) etc., is necessary for a proper comparison before any conclusions can be reached. Nevertheless these values can provide a rough but useful guide to their metabolic patterns.

Based on the Y_g^{max} of 6.8 and P/O ratio about 1 (therefore 4 ATP produced per thiosulphate metabolised) (see 1.1.2 and 2.3.5) a Y_{ATP} value of 1.7 could be obtained. The value for T. denitrificans is 1.93 (Kelly 1982). The values of Y_{ATP}^{max} for T. versutus and T. denitrificans are 2.2 (see 2.3) and 3.07 (Timmer-ten-Hoor 1976) respectively. These figures are very much lower than those found in heterotrophic bacteria from 8.4 to 14 (Jones 1977). That is because a vast amount of energy (ATP) is consumed for the reduction of NAD^+ through reversed electron transport by the thiobacilli mainly for the purpose of CO_2 fixation. (See 2.4 and 2.5 for further discussion).

TABLE 1.1 Theoretical maximum growth yields and observed yields of thiobacilli and other bacteria growing in continuous chemostat culture

Organism	Substrate	Y_g^{max}	Y_g	(D)
T. neapolitanus	$S_2O_3^{2-}$	7.7,6.3,6.3, 5.5		
T. versutus (A2)	$S_2O_3^{2-}$	7.0,6.4		
T. ferrooxidans	$S_2O_3^{2-}$	7.5		
	$S_4O_6^{2-}$	12.2		
T. novellus [a]	$S_2O_3^{2-}$	7.6	5.7	(0.055)
T. denitrificans	$S_2O_3^{2-}(O_2)$	14.7		
	$S_2O_3^{2-}(NO_3)$	11.4,10.0		
	S^{2-} (NO_3)		9.4	(0.03)
Thiomicrospira denitrificans	$S_2O_3^{2-}(NO_3^-)$		5.2	(0.03)
	$S_2O_3^{2-}(O_2)$		7.7	(0.03)
	S^{2-} (NO_3^-)		5.9	(0.03)
Thiomicrospira pelophila	$S_2O_3^{2-}(O_2)$	7.0		
Paracoccus denitrificans [b]	$S_2O_3^{2-}$ (O_2)		4.5	(0.14)

Data are taken from Kelly (1982) except (a) the figure of T. novellus which is calculated from Fig. 1 of Leefeldt and Matin (1980) for $D = 0.02 - 0.05h^{-1}$ after a plot of Y^{-1} to D^{-1} and (b) from Friedrich and Mitrenga (1980).

1.1.3 Electron transport systems and energy coupling

Basically the respiratory chain in thiobacilli is similar to that in mitochondria and some heterotrophic bacteria such as Paracoccus denitrificans (Kelly 1978; Suzuki 1979). The components found so far are cytochrome a, a3, b, c, and O, ubiquinone Q8 and flavoprotein. The organisms normally also contain succinate dehydrogenase and NADH dehydrogenase. However, the exact compositions of the electron transport system vary in different organisms. A few c-type cytochromes have been partially purified and characterized from thiobacilli, which will be discussed in Chapter 6.

The oxidation of inorganic sulphur compounds is ultimately coupled by the electron transport system to oxygen or to nitrate (in the case of T. denitrificans) as a terminal electron acceptor (Kelly and Syrett, 1963; Aleem 1975, 1978; Peck 1968; Suzuki 1979). It is generally accepted that oxidative phosphorylation is a major or only (in some organisms) way for energy conservation, and substrate level phosphorylation (APS pathway) is also involved in some organisms.

It has been established by considerable work that electrons from thiosulphate (and probably other reduced sulphur compounds) enter the electron transport chain at the level of cytochrome c in most thiobacilli, such as T. novellus, T. neapolitanus, T. versutus, T. thiooxidans (Aleem 1975; Kelly 1978, 1982), but at the level of flavoprotein or cytochrome b in T. denitrificans. These results suggest that only one ATP could be produced per atom of oxygen consumed in the aerobic thiobacilli and two in T. denitrificans which is in harmony with the observations growth yield of the aerobic thiobacilli. Much of this evidence has been obtained from inhibition studies with intact cells and cell-free extracts of thiobacilli. In such study some note should be taken that the mechanism of action of many inhibitors is not yet fully understood, and a selective blocking of electron transport at certain sites by certain inhibitors may not be universal among

all electron transport systems (Suzuki 1979). This view may account for some controversial results such as the partial inhibition of thiosulphate oxidation by rotenone amytal (flavoprotein inhibitors), antimycin A and 2-n-heptyl 4-hydroxyquinoline N-oxide (blocking electron transfer between the Ubiquinone-cytochrome b and cytochrome c segments) in some experiments with T. neapolitanus (Saxena and Aleem 1972) and T. novellus (Cole and Aleem 1973). Nevertheless, this evidence is clearly supported by the other means of approaches to the problem as follows.

ATP formation coupled to thiosulphate oxidation has been successfully demonstrated in extracts of T. neapolitanus with a P/O ratio of 0.8 for T. neapolitanus and 0.9 for T. novellus (Aleem 1975), although rather low P/O ratios were obtained by others (Davis and Johnson 1967, Moriarty and Nicholas 1970) with sulphite or sulphide as substrates. Unfortunately the data for T. denitrificans are not available. Drozd (1974) demonstrated by measurement of the outward translocation of protons coupled to thiosulphate oxidation a H^+/O value of 2 in intact cells of T. neapolitanus consistent with the presence of only one energy coupling site. The same value was shown with sulphide, sulphite and ascorbate-TMPD as electron donor (Drozd 1978) in the same organism. A proton-induced ATP formation was also demonstrated in whole cells or cell-free extracts of T. novellus by using an artificially established pH gradient (Cole and Aleem 1973). Using the quinacrine fluorescence technique, Tuovinen et al. (1977) also showed a proton translocation in intact cells of T. denitrificans during thiosulphate oxidation. These observations suggest that the chemiosmotic energy-conserving mechanism is also involved in the oxidation of sulphur compounds by Thiobacillus.

1.1.3 Reduction of nicotinamide adenine dinucleotides

Since the redox values of the probable oxidation steps in the metabolism of sulphur compounds are not sufficiently electronegative to couple directly to NAD^+ or $NADP^+$ reduction (see 1.3), the pyridine nucleotides reduction must occur by an energy-dependent reversed electron transport. This thermodynamic consideration is confirmed by the direct evidence that (a) electrons from thiosulphate, sulphide or sulphite enter

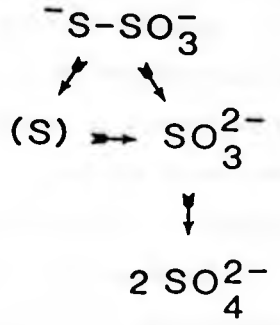
the respiratory chain at the levels of cytochrome c, flavoprotein or cytochrome b (see 1.1.3) and (b) ATP-dependent reduction of pyridine nucleotides by thiosulphate was demonstrated in the cell-free extracts of T. novellus (Aleem 1966) and T. neapolitanus (Saxena and Aleem 1972); both systems were inhibited by amytal, antimycin A and uncouplers of oxiditive phosphorylation. Roth et al. (1970) also observed that the pathway of pyridine nucleotide reduction in intact T. neapolitanus proceeded through an energy-dependent and amytal sensitive step when either thiosulphate or sulphide was used as the substrate.

The amount of energy required for NAD^+ reduction by thiosulphate is still uncertain. A value of 2.5 or 1 ATP per mol of NAD^+ in T. neapolitanus and T. novellus respectively was reported by Aleem and his co-workers (Aleem 1966; Saxena and Aleem 1972). These values, however, are probably underestimated due to the complexity of conducting and interpreting this kind of experiment. For example, the presence of an active adenylate kinase in T. neapolitanus would re-synthesize a substantial amount of ATP from ADP produced from the reaction of NAD^+ by the ATP-driven reversal of electron transport. If this extra amount of ATP was considered in the calculation, the actual value could reach about five ATP per NADH (Kelly 1982). On the basis of Y_g^{max} the P/O ratio of one and the complete oxidation thiosulphate to sulphate (thus ATP/$S_2O_3^{2-}$ ratio of about four) the value was estimated to be more than three for T. versutus (A2) (for details see 2.4, 2.5 and Kelly 1982). A value of five was found in Rhodopseudomonas palustris, a photosynthetic purple bacterium, with thiosulphate as an electron donor (Knobloch et al. 1971). Nevertheless from the present knowledge of the biochemistry of aerobic thiobacilli, such as growth yield, CO_2 fixation by the Calvin cycle, P/O ratio about 1 and similar biosynthetic pathway for cell components, one could expect that the major energy (or ATP) sink would be the reduction of pyridine nucleotides, which, together with the CO_2 fixation probably account for the low Y_{ATP} of this type of living organisms

(see 1.1.1).

1.1.4 Enzymes reported to be involved in thiosulphate oxidation

From a chemical consideration, the basic process of the complete oxidation of thiosulphate can be given by



This simple concept was put forward about 15 years ago (Kelly 1968; Suzuki 1974), before and since then a few enzymes involved have been detected and some of them partially purified.

Peck (1960, 1962) demonstrated that the following pathway (APS pathway) is present in T. thioparus for sulphite oxidation.

$$SO_3^{2-} + AMP \xrightarrow{\text{APS-reductase}} APS + 2e$$

$$APS + P \xrightarrow{\text{ADP-sulphurylase}} ADP + SO_4^{2-}$$

The APS-reductase was also shown and partially purified from T. denitrificans (Bowen et al. 1966). Later, another AMP-independent sulphite oxidizing enzyme (sulphite:cytochrome c oxidoreductase or sulphite oxidase) was found and partially purified from T. novellus by Charles and Suzuki (1965). This enzyme catalyzes the reduction of horse heart (or other sources) cytochrome c or ferricyanide by sulphite. The enzyme activity was shown in several thiobacilli, including T. thiooxidans, T. versutus, T. ferrooxidans and T. neapolitanus (see 8.4) as well as T. thioparus and T. denitrificans (Roy and Trudinger 1970). Both enzymes were claimed to play an important part in thiosulphate oxidation by thiobacilli. These two enzymes account, however, only for the terminal step of thiosulphate oxidation, and do not account for oxidation of the sulphane-sulphur.

It is not clear why some thiobacilli contain both types of sulphite

oxidizing enzymes and how these two enzymes operate during the oxidation of thiosulphate. Although the possible advantage for organisms in having the APS pathway is to enable them to obtain 0.5 or 1 extra ATP through the oxidation of 1 mol sulphite (Kelly 1982), there is certainly no thermodynamic reason for this pathway to occur in the sulphur-oxidizing bacteria as there is in the sulphate-reducing bacteria, due to the fact that the E'_o of $SO_3^=/SO_4^=$ pair is too low to accept electrons from NADH. High purification of both enzymes from T. denitrificans or T. thioparus is necessary to prove the co-existence of them in one organism.

Trudinger (1961) demonstrated the existence of a thiosulphate-oxidizing enzyme, catalyzing:

$$2\ S_2O_3^{2-} + 2\ Fe(CN)_6^{3-} \rightarrow S_4O_6^{2-} + 2\ Fe(CN)_6^{4-}$$

Cytochrome c can be used to replace ferricyanide as the electron acceptor but with very low activity. The enzyme was subsequently found in numerous thiobacilli and other bacteria (Roy and Trudinger 1970), but not in T. versutus and T. denitrificans (Justin and Kelly 1978). In any case this enzyme cannot play an essential role in the complete oxidation of thiosulphate to sulphate.

The initial "activation" or scission of thiosulphate is uncertain. Rhodanese (thiosulphate:cyanide sulphur transpherase), catalyzing the reaction:

$$S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-}$$

is present in all the strains of thiobacilli growing chemoautotrophically or heterotrophically and was speculated upon as a candidate for this role (Charles and Suzuki 1966), but direct evidence has never appeared (for further discussion of this subject see Chapter 4).

Peck (1960, 1962) proposed a thiosulphate reductase in T. thioparus with reduced glutathione (GSH) as electron donor to produce sulphide and sulphite

$$^{-}SSO_3^{-} + 2H^{+} + 2\,e^{-} \rightarrow SO_3^{2-} + H_2S$$

This enzyme seems unlikely to be operative in the aerobic thiobacilli because of the heavy burden of regeneration of GSH and the insensitivity of the oxidation of thiosulphate by the aerobic thiobacilli to flavoprotein inhibitors (see 1.1.2 and below).

The central uncertainty is the mechanism of oxidation of sulphur (including sulphane-sulphur from thiosulphate) to sulphite. A sulphur-oxidizing oxygenase enzyme capable of oxidation of elemental sulphur requiring reduced glutathione (GSH) and producing sulphite or thiosulphate was found in T. thiooxidans (Suzuki 1965). This enzyme was subsequently shown in several other thiobacilli, including T. thioparus, T. novellus and T. ferrooxidans (Suzuki 1979), although generally with rather low activities (see also Justin and Kelly 1978). It is noted that all of the organisms studied except T. thiooxidans were cultivated in thiosulphate medium. Suzuki (1966, 1979) proposed that the enzyme actually catalyzed the oxidation of sulphane-sulphur of thiosulphate to sulphite in the oxidation of thiosulphate by thiobacilli. However little progress in purification of these enzymes has been achieved since then (Professor Suzuki's group is still trying to do this, personal communication). So, we know virtually nothing about its properties. Moreover, the existence of such an oxygenase was challenged by the following arguments:
(a) in Suzuki's original $^{18}O_2$ experiment (Suzuki 1965b), the whole cells crude extract and the two ethanol-concentrated fractions did not incorporate any ^{18}O from $^{18}O_2$ into sulphate during sulphuroxidation. Only one of the ethanol-concentrated fractions showed a small amount of incorporation of ^{18}O into sulphate, which could arise from a number of exchange reactions occurring during the experiment (Aleem 1975). In this experiment the extract of elemental sulphur-grown T. thiooxidans was used, so the result may not be applicable to other thiobacilli systems growing on thiosulphate; (b) if reduced glutathione is required for the oxidation of sulphur and NADPH is utilized to regenerate GSH as postulated by Suzuki (1965b), then

sulphur oxidation to sulphite should be inhibited by uncoupling agents simply due to the involvement of an ATP-dependent reversal of electron transport for reduction of NAD^+ (Aleem 1975). This is certainly not the case in the oxidation of thiosulphate by thiobacilli; (c) if an oxygenase reaction was involved, the P/O ratio would be 0.5 rather than 1 as expected from previous evidence (see 1.1.2) for the aerobic thiobacilli, so only 2 ATP could be produced per thiosulphate oxidized to sulphate, which would then be insufficient to support the observed yields, as we have calculated that the minimum requirement for ATP production from thiosulphate is 2.5, 3.1 or 3.7, depending on whether two, three or four ATP are required to reduce NAD^+ or $NADP^+$ (see Kelly 1982 and 2.4 for detailed calculations). If the generation of reduced glutathione is taken into account an extra amount of ATP has to be added to these figures; (d) the high concentration of c-type cytochromes in thiosulphate-grown T. novellus, T. versutus and T. neapolitanus (see Chapter 6, Kula et al. 1975, and Sadler and Johnson 1972) indicates their essential role in thiosulphate oxidation. Our recent results (see Chapter 7) showed that none of four soluble c-type cytochromes purified from T. versutus combined with CO, suggesting that they are not available for direct oxidation by oxygen.

It is noted that the elemental sulphur-oxidizing system from T. thiooxidans were resolved into four components, a particulate fraction sensitive to oxygen, two soluble components (A and B) and a pyridine nucleotide as a cofactor (Takakuwa 1975). All of the four were necessary for sulphur oxidation. Components A and B were partially purified, but the specific activities of the crude extract and the reconstituted system were not mentioned throughout the paper.

Schedel and Trüper (1979, 1980) demonstrated a sirohaem sulphite reductase in T. denitrificans, comprising about 2% of the total cellular protein, and purified it to homogeneity. The enzyme might provide the missing link in the organism for the conversion of sulphane-sulphur to sulphite if it is operative in the oxidative direction in vivo as proposed

by the authors, but they failed to show the oxidation of thiosulphate with the purified enzyme. As the mechanism of thiosulphate oxidation in T. denitrificans might be different from other thiobacilli according to the results of growth yields (see 1.1.1) and ^{35}S-thiosulphate oxidation and the present data (see 1.1.5) are insufficient for proper comments, I shall not go further into the detail of this subject.

Finally a few words about metabolism of polythionates. As clearly pointed out by Kelly (1982), polythionates have no central role as free intermediates of inorganic sulphur oxidation in thiobacilli. Their degradation seems always to require reductive or hydrolytic scission to simpler molecules such as thiosulphate and sulphite, with which energy conservation processes definitely involved. Moreover the only enzyme in polythionate metabolism that has ever been isolated is the tetrathionate-producing thiosulphate-oxidizing enzyme mentioned above.

1.1.5 Oxidation of ^{35}S-thiosulphate by whole cells: test for the initial reaction

The work by Kelly and Syrett (1965) revealed that washed intact cells of T. neapolitanus oxidized both sulphur atoms of thiosulphate to sulphate at a fairly rapid and more or less equal rate, the formation of ^{35}S from $(S\ ^{35}SO_3)^{2-}$ being slightly faster (about 1 min) than that from $(^{35}S\ SO_3)^{2-}$. A similar result was observed by Aleem (1975) with intact cells from T. novellus and T. neapolitanus. He also found that the cell-free extracts from both organisms catalyzed the oxidation of both the sulphur atoms at approximately the same rate. Those findings suggest that in these two organisms there is no discrimination for oxidation of either sulphur atom of thiosulphate.

In contrast, Schedel and Trüper (1980) found that elemental sulphur was transiently formed from sulphane-sulphur and deposited within the cells of T. denitrificans. Its oxidation to sulphate was delayed (about half hour in their experimental conditions) as long as thiosulphate was present. So, they concluded that thiosulphate was split (by rhodanese)

to sulphite and elemental sulphur (an intracellular hydrophilic sulphur sol). Similarly, Smith and Lascelles (1966) showed that the oxidation of thiosulphate by Chromatium strain D, a photosynthetic purple sulphur bacterium is bisphasic in character. In the initial fast phase about half the sulphur of thiosulphate is converted to sulphate which arose largely from the sulphonate group. The sulphane-sulphur accumulated within the cells during the initial phase and was subsequently oxidized to sulphate at a slower rate. A similar observation was made by Trüper and Pfening (1966) on Chromatium and Thiocapsa floridana. From those observations it follows that there may be two different types of initial mechanisms of thiosulphate oxidation, one involves a distinguishable cleavage reaction and discriminative oxidation of two atoms of thiosulphate, another oxidizes thiosulphate in a more or less integral way.

1.1.6 Cell-free extracts catalyzing oxidation of thiosulphate and the sub-cellular location of the enzyme systems

Crude, cell-free preparations of T. thiooxidans (London and Rittenberg, 1964) and T. novellus (Aleem 1965; Charles and Suzuki 1966; Oh and Suzuki 1977) were described which catalyzed the oxidation of thiosulphate to sulphate without added cofactors or special treatment. The cell-free systems from all other thiobacilli carried out incomplete thiosulphate oxidation with sulphate and tetrathionate as the major products (Roy and Trudinger 1970). This is one of the major problems to complicate the study of thiosulphate oxidation pathway and to hinder the investigation of the enzyme systems involved in the oxidation.

Aleem (1965) reported a soluble enzyme system from T. novellus which catalyzed the reduction of horse heart cytochrome c by thiosulphate with an appreciable activity. This so called soluble fraction, a supernatant fraction after centrifugation of the crude extract produced by ultrasonication at 140,000 xg for 2 h probably still contained a certain amount of small membrane fragments (as we found in the work with T. versutus , see 2.3.4), thereby it could also effect the complete oxidation of thiosulphate and even oxidative

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phosphorylation as reported (Cole and Aleem 1970, 1973). (For further comments, see 2.4). However a converse observation was made later (Oh and Suzuki 1977 a,b), showing that a membrane-bound thiosulphate oxidation system could be obtained from the same organism, this might be explained by the enzyme system being associated with the electron transport chain in the intact cell, but the tightness and stability of the association could be affected by the growth conditions method of preparation of the crude extract and subsequent purification procedures. Saxena and Aleem (1972) reported that a thiosulphate:cytochrome c oxidoreductase also resided mainly in the soluble fraction from T. neapolitanus.

It is generally accepted that oxidation of sulphur compounds by thiobacilli must involve oxidative phosphorylation as a major or only energy-conserving process (Aleem 1977, see 1.1.2), therefore the membrane system is always necessary for the whole process. But a membrane-dissociated enzyme system (like a soluble thiosulphate:cytochrome c oxidoreductase) will be very useful in the further elucidation of the enzyme(s) involved. It is not surprising that most sulphur or sulphide oxidation systems reported were also membrane-associated, some of these required both soluble and particulate fractions (for a general review, see Suzuki 1974). However, none of them was further resolved and studied, except the work done by Takauwa (1975) mentioned above (1.1.4). Although Suzuki stated in his review (1974) "it would be interesting if these membrane systems were further fractionated and the specific enzymes involved were identified. It might be possible then to reconstitute a sulphur-oxidizing system capable of oxidizing sulphur to sulphate with purified enzymes and membranes."

1.1.7 Thiosulphate as a model substrate to study the biochemical mechanisms of oxidation of sulphur compounds

Thiosulphate is the most commonly used substrate for growth of the sulphur oxidizer to investigate the metabolism of sulphur compounds simply because it is easily soluble and reasonably stable at 120°C in the autoclave and at the pH range suitable for thiobacilli and good methods for

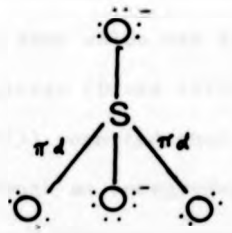
its qualitative and quantitative determination are available. All species and strains of thiobacilli can oxidize thiosulphate and grow on it very well as an energy source (Tuovinen and Kuenen 1981).

There is some evidence showing that in the natural environment (especially in seawater) sulphide produced by sulphate-reducing bacteria and microbial breakdown of organic matter could be chemically re-oxidized to thiosulphate in the presence of oxygen (Sorokin 1971, Almgren and Hagström 1974, and Kuenen 1975), probably by the following reactions (Nedwell 1982):

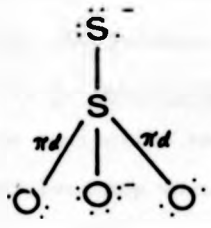
$$H_2S + O_2 \rightarrow 2\,S^o + 2H_2O$$
$$H_2S + 2O_2 \rightarrow SO_3^{2-} + H_2O$$
$$SO_3^{2-} + S^o \rightarrow S_2O_3^{2-}$$

Biological oxidation was subsequently responsible for the further oxidation of thiosulphate to sulphate (Sorokin 1972, Tuttle and Jannasch 1973). Suzuki and Silver (1966) also reported a non-enzymatic condensation of sulphur and sulphite to thiosulphate in their reaction mixture. It is likely that thiosulphate is one of the major and widespread forms of sulphur combined in the natural environment, and the activity of its oxidation by sulphur-oxidizing bacteria plays an important part in the cycle of sulphur compounds.

Chemically, thiosulphate can be regarded as sulphate in which one of the oxygen atoms has been replaced by sulphur (i.e. the sulphane group)



sulphate



thiosulphate

The two sulphur atoms are thus unequal, the outer one having an oxidation number of -2, whereas the central sulphonate group sulphur atom has an oxidation number of +6 (Roy and Trudinger 1970).

From these considerations, several postulations can be made regarding the

nature of the cleavage of the bond between the sulphane and sulphonate sulphurs in thiosulphate. Hydrolytic cleavage could give rise to sulphur and sulphite or to sulphide and sulphate:

$$^{-}SSO_3^{-} + H_2O \rightarrow HSO_3^{-} + S^{o} + OH^{-}$$

$$^{-}SSO_3^{-} + H_2O \rightarrow HSO_4^{-} + S^{2-} + H^{+}$$

Thus, the mechanism of thiosulphate oxidation probably involves and shares the mechanisms necessary for sulphide and sulphur oxidations, though we do not know which of the two reactions is operating in thiobacilli. It is likely that the S^{o} or S^{2-} formed after the cleavage reaction are associated or bound to an enzyme or acceptors for further oxidation, and thus would not be exactly the same as the free sulphide and sulphur. In this sense, oxidation of elemental sulphur in the presence of GSH or not with cell-free extracts from thiosulphate-grown cells could not reflect the real reactions and enzyme systems of thiosulphate oxidation *in vivo*. Nevertheless, I guess that a similar enzyme or acceptor-bound S^{o} or S^{2-} might be formed after elemental sulphur or sulphide was transported (by what mechanisms?) into the cells.

There is some preliminary experimental evidence to support the suggestion that mechanisms of oxidation of thiosulphate and sulphide might be similar, at least in the aspects of energetics. Drozd (1977) demonstrated that in *T. neapolitanus*, sulphide (and also sulphite) oxidation was associated with a maximal H^{+}/O ratio slightly under 2, i.e. a P/O ratio of about 1. The same value was found for the oxidation of thiosulphate in the same organism (Drozd 1974). Using intact cells of *T. neapolitanus*, Roth *et al.* (1973) reported that the pathway of pyridine nucleotide reduction proceeded through an energy-dependent and amytal-sensitive step when either thiosulphate or sulphide was used as the substrate, and the ATP formation with either substrate was not interfered with by amytal. These observations were supported by the results that the anaerobic growth yields of *T. denitrificans* and *Thiomicrospira denitrificans* on sulphide were comparable to those on

thiosulphate (Timmer-ten-Hoor 1981), even though sulphide is generally thought to be a stronger biological reductant than thiosulphate and some inhibitor studies indicated that the electrons from sulphide entered the respiratory chain at the level of cytochrome b or even flavoprotein in the aerobic thiobacilli such as T. neapolitanus and T. novellus (Aleem 1975).

Sulphide and elemental sulphur are less useful experimental substrates owing to the low aqueous solubility of the latter making quantitative study difficult and the instability of the former: sulphide in neutral solution is largely present as H_2S, which is volatile (the first ionization constant is 1.15×10^{-7}), auto-oxidizable (oxidation being chemically catalyzed by some metal ions and haem compounds) and toxic at other than low concentrations.

1.2 Thiobacillus versutus (A2)

1.2.1 Description of the organism

Thiobacillus versutus (former name Thiobacillus A2, (Harrison 1983)), isolated by Taylor and Hoare (1969), is an aerobic, mesophilic, gram-negative, non-sporulating, non-mobile, rod-shaped, non-acidophilic bacterium. Thiosulphate is the only sulphur compound known to support the autotrophic growth (Taylor and Hoare 1964). It can grow autotrophically in a formate minerals medium (Wood et al. 1981). The organism grows anaerobically respiring nitrate, which is coupled to the oxidation of organic substrates (e.g. glucose, acetate, formate) but not inorganic sulphur compounds. T. versutus can be differentiated from other non-acidophilic mixotrophic or facultative thiobacilli by its versatility in using various organic compounds as sole sources of energy with rapid rate of growth and good cell yield, by its ready transition from chemolithotrophy to organotrophy and vice versa and by its denitrifying activity with organic substrates. Like the other thiobacilli, T. versutus uses the Calvin-Benson cycle for its autotrophic CO_2 fixation.

1.2.2 *T. versutus* as a model organism for studying thiosulphate oxidation

Thiobacillus versutus has proved to be the organism of choice in attempting to elucidate sulfur oxidation mechanisms, in that it does not exhibit many of the complicating properties found with other species, such as unpredictable accumulations of elemental sulfur or polythionates and does not lose all its thiosulfate-oxidizing activity on being disrupted. Its main advantages are thus (a) both whole organisms and cell-free extracts oxidize thiosulfate completely to sulfate; (b) no tetrathionate or sulfur are accumulated; (c) no tetrathionate-producing thiosulfate-oxidizing enzyme is present; (d) there is no APS pathway involved in the oxidation of sulfite by the organism; (e) large quantities of bacteria in good yield can be obtained, completely free of inorganic precipitates, by continuous chemostat cultivation (Kelly and Tuovinen 1975; Smith *et al.* 1980; Kelly 1982 and also see Chapter 2).

1.3 Some thermodynamic calculations for the oxidation of sulphur compounds

The standard electrode potential of a redox pair can be calculated from the following relationship:

$$\Delta G'_o = nF\Delta E'_o \qquad (1)$$

where $\Delta G'_o$ is the standard free energy change, n the number of electrons transferred per mol, F Faraday's constant (23,063 cal x volt^{-1} x equivalents) and $\Delta E'_o$ difference between the redox potentials of the two half reactions. $\Delta E'_o$ = (E'_o of the half-reaction containing the oxidizing agent) - (E'_o of the half-reaction containing the reducing agent) (Segel 1976).

To calculate E'_o we need to know $\Delta G'_o$, which can be estimated from the equation below (Lehniger 1975):

$$\Delta G'_o = \Sigma \Delta G^o_{f\,product} - \Sigma \Delta G^o_{f\,reactor} \qquad (2)$$

where ΔG^o_f is the standard free energy of formation. It should be

recognised that the error in such calculations for $\Delta G'_o$ can be rather large because the volume for $\Delta G'_o$ usually involves a small difference between two large numbers. Nevertheless this approach represents the only convenient way of obtaining the $\Delta G'_o$ of the redox reactions of sulphur compounds.

The standard free energy of formation for compounds related with sulphur metabolism are listed in Table 1.2. As an example, the redox potential of SO_3^{2-}/SO_4^{2-} pair is calculated as follows:

The overall reaction: $SO_3^= + \frac{1}{2}O_2 \rightarrow SO_4^=$

According to equation (2) and Table 1.2, we have $\Delta G'_o = -178-(-116) = -62$ Kcal/mol

The overall reaction consists of two half reactions:

$SO_3^= + 1\frac{1}{2}O \rightarrow SO_4^= + 2H^+ + 2e^-$ $E'_o = ?$

$\frac{1}{2}O_2 + 2H^+ + 2e \rightarrow H_2O$ $E'_o = +0.82$volt

Therefore, according to equation (1):

$-62,000 = -2 \times 23,063\ (0.82 - E'_o)$

$E'_o = -0.51$ volt

In the same way, I calculated the $\Delta G'_o$ and $\Delta E'_o$ for other sulphur reactions which appear on Table 1.3 and Table 1.4 respectively. The data of $\Delta G'_o$ are similar to those published by Aleem (1975). The calculation procedure for thiosulphate oxidation in this thesis is as follows:

Overall reaction: $S_2O_3^= + 2O_2 + H_2O \rightarrow 2SO_4^= + 2H^+$ $\Delta G'_o = 212$ Kcal/mol

Two half reactions: $S_2O_3^= + 5H_2O \rightarrow 2SO_4^= + 8e^- + 10H^+$

$2O_2 + 8H^+ + 8e^- \rightarrow 4H_2O$

Therefore: $-212,000 = -8 \times 23,063\ (0.82 - E'_o)$

$E'_o = -0.31$ volt

In this calculation, it is assumed that the electrons from the substrate are transferred to oxygen through the electron transport chain, as it would be impossible to calculate E'_o by this way if there were any oxygenases involved.

In Table 1.4 I list the calculated and published values of electrode potentials of various redox half reactions involved in oxidation of sulphur

TABLE 1.2 Gibbs free energies of formation from the elements for compounds related with sulphur metabolism

Compound	ΔG_f^o (Kcal/mol)
$SO_3^=$	-116
HSO_3^-	-126
H_2SO_3	-129
SO_2	- 72
$SO_4^=$	-178
HSO_4^-	-181
$S_2O_3^=$	-123
$S_2O_4^=$	-144
$S^=$	20.5
HS^-	- 2.9
H_2S	- 6.7
S (rhombic)	0
H_2O (liq)	- 56.7
H^+ (pH 7)	- 9.5
O_2 (gas)	0

The data are taken from Wagman et al. (1968). All of the compounds are in aqueous solution except where specified.

TABLE 1.3 Standard free energy changes of important reactions of sulphur metabolism

	Reaction	$\Delta G'^{\circ}$ (Kcal/mol) (published)[a]	(calculated)[b]
1.	$S_2O_3^{=} + 2O_2 + H_2O \rightarrow 2SO_4^{=} + 2H^+$	-211	-212
2.	$SO_3^{=} + \frac{1}{2}O_2 \rightarrow SO_4^{=}$	- 60	- 62
3.	$SO_3^{=} + H_2O \rightarrow SO_4^{=} + 2H^+$		- 25
4.	$SO_4^{=} + H_2 \rightarrow SO_3^{=} + H_2O$		- 5
5.	$H_2S + 2O_2 \rightarrow H_2SO_4$	-160	-165
6.	$S^{=} + 2O_2 \rightarrow SO_4^{=}$		-199
7.	$S^{o} + 1\frac{1}{2}O_2 + 2H^+ \rightarrow H_2SO_4$	-118	-158
8.	$5S_2O_3^{=} + 8NO_3^{-} + H_2O \rightarrow 10SO_4^{=} + 4N_2 + 2H^+$	-893	

[a] Taken from Aleem (1975)

[b] See the text for detailed calculations

TABLE 1.4 Electrode potentials of some redox half reactions involved in oxidation of sulphur compounds

	System	E'_o (a) (pH 7)	E (volt) E_o (b)	E'_o (c) (pH 7)
1.	$S_2O_3^= + 5H_2O/2SO_4^= + 10H^+ + 8e$	-0.31		
2.	$S_2O_3^= + 3H_2O/2H_2SO_3 + 2H^+ + 4e$	-0.02*	-0.4	+0.013
3.	$SO_3^= + H_2O/SO_4^= + 2H^+ + 2e$	-0.51		
4.	$H_2SO_3 + H_2O/SO_4^= + 4H^+ + 2e$		-017	+0.26
5.	$H_2SO_3 + H_2O/H_2SO_4 + 2H^+ + 2e$	-0.28*		
6.	$HSO_3^- / SO_4^=$	-0.516+		
7.	$H_2S + 4H_2O/H_2SO_4 + 8H^+ + 8e$	-0.07		
8.	$S^= + 4H_2O/SO_4^= + 8H^+ + 8e$	-0.26		
9.	$S^o + 3H_2O/SO_4^= + 6H^+ + 6e$	-0.03 (d) or -0.32		
10.	$S + 3H_2O/H_2SO_4 + 4H^+ + 4e$		-0.45	-0.037

(a) See the text for detailed calculations, except that + taken from Thauer et al. (1977) and * from Kelly (1982).

(b) Taken from Latimer (1952), who calculated the values by the following method, for example, eq. (2), $\Delta F^o = F^o_{H_2SO_3}\times 2 - F^o_{S_2O_3^=} - F^o_{H_2O}\times 3 = 128{,}600 - -124{,}000 - 3\times(-56{,}700) = 37{,}000$; then, $E^o = -\frac{\Delta F^o}{n \times 23{,}060} = -\frac{37{,}000}{4 \times 23{,}060} = -0.4$. But, using the same method of calculation for eq.(1), the E^o value will be + 0.33. Also the value of + 0.26 for eq. (4) is obviously too high.

(c) Calculated from E_o (b) by using the equation $E'_o = E_o - 0.059 \times pH$.

(d) Calculated from $\Delta G'_o$ of -118 and -153 Kcal of Table 1.3 respectively.

compounds. One can see that the values vary considerably depending on the value of $\Delta G'_o$ and the method of calculation. Therefore these figures can only provide a rough guide to the metabolic patterns of those reactions, such as that they are all too high to reduce NAD^+ or $NADP^+$ directly and the E'_o of the sulphide/sulphate pair is not lower than that of the thiosulphate/sulphate pair. However one should bear in mind that by these calculations any value of half reactions containing more than one pair of electrons probably represents an average value of the pairs of electrons which may actually have different electrode potentials.

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CHAPTER 2

CHEMOSTAT GROWTH KINETICS, THIOSULPHATE OXIDATION, ELECTRON TRANSPORT AND PHOSPHORYLATION IN WHOLE CELLS AND CELL-FREE SYSTEM

2.1 INTRODUCTION

There have been relatively few reports of the preparation of cell-free systems from thiobacilli capable of the complete oxidation of thiosulphate to sulphate. Examples are extracts of *Thiobacillus novellus* (Aleem, 1965; Charles & Suzuki, 1966; Oh & Suzuki, 1977a,b) and *T. thiooxidans* (London & Rittenberg, 1964). Recently a membrane-associated thiosulphate-oxidizing system was obtained from *T. novellus*, but was not active on reconstitution from its constituent enzymes (Oh & Suzuki, 1977b). Few studies have been reported of efficient electron transport phosphorylation dependent on cell-free thiosulphate oxidation. In cases where $P/_O$ ratios of about 1·0 have been obtained it has not always been clear whether complete oxidation of thiosulphate was occurring (involving oxidation of both the reduced sulphane and oxidized sulphone atoms of the ion) or whether phosphorylation was accompanying the oxidation of sulphite derived from thiosulphate. The occurrence in some thiobacilli of substrate-level phosphorylation, dependent on APS reductase and ADP- or ATP-sulphurylase, has probably also been an interfering factor in attempts to demonstrate oxidative phosphorylation (Ross *et al.*, 1968; Peck, 1968; Kelly, 1978). There have in fact been no published reports of phosphorylation by cell-free extracts of thiobacilli in which the complete oxidation of thiosulphate to sulphate was simultaneously and unequivocally known to be occurring. Earlier work with *T. novellus* (Aleem, 1965) showed thiosulphate oxidation by crude extracts and a 144,000 xg soluble fraction with a ratio of oxygen consumed/thiosulphate oxidized of 1·6 - 1·8. Oxidation was stimulated by added cytochrome *c* and recent work with *Thiobacillus* A2 indicated electrons from thiosulphate oxidation to enter the electron transport chain at the level of cytochrome *c* (Kuła *et al.*, 1982). We have undertaken to prepare cell-free systems from *Thiobacillus* A2 that would simultaneously effect complete oxidation of thiosulphate and ATP

synthesis by electron transport phosphorylation. We have sought to establish the electron transport pathway involved in thiosulphate oxidation and the subcellular location of the thiosulphate-oxidizing system.

2.2 MATERIALS AND METHODS

2.2.1

Organism and culture conditions. Thiosulphate-agar media were used for culture maintenance as previously described for *Thiobacillus* A2 (Wood & Kelly, 1977). Continuous chemostat culture was used both for estimating maximum theoretical growth yield and as a procedure for the generation of large quantities of the organisms in a medium containing (g ℓ^{-1}): $Na_2S_2O_3.5H_2O$, 12.5 or 25; $MgSO_4.7H_2O$, 0.1; NH_4Cl, 0.8; KH_2PO_4, 1.5; $Na_2HPO_4.2H_2O$, 7.9; H_2SO_4 as required; trace metal solution (Tuovinen & Kelly, 1973), 10 ml. The culture was established in an LH Engineering (Slough, Bucks) modular type series 500 fermenter with a culture volume of 750 ml, stirred (500 rpm) and aerated (55 ml min^{-1}) with air containing 1.4% (v/v) CO_2. Temperature was maintained at 30°C and pH 7.7 by automatic addition of 2M NaOH.

2.2.2

Preparation of crude cell-free extract. The effluent from the chemostat culture was collected in an ice-cooled vessel and kept at 4°C until used. The bacteria were washed once with 55 mM phosphate buffer (pH 7.5) and resuspended (20 to 40 mg dry wt ml^{-1}) in the same buffer. The suspension was passed twice through a French Pressure cell at 140 MPa. The disintegrated material was treated at 4°C for 10 min with deoxyribonuclease (2 µg ml^{-1}) and 10 mM $MgCl_2$, then centrifuged at 10,000 xg for 15 min at 4°C to remove any unbroken cells. The supernatant fraction was used as the crude extract.

2.2.3

Alternatively a crude extract was prepared with a lysozyme treatment based on the method described by Kaback (1971). Washed cells were suspended to a final 15 mg dry wt ml^{-1} in 0.5 M sucrose containing 100 mM EDTA and 10 mM phosphate buffer, pH 7.0. Lysozyme was added to the suspension at a concentration of 1 mg ml^{-1}, then incubated at 30°C for 20 - 30 min until the absorbance at 540 nm of a diluted aliquot of the suspension had decreased 5 to

6-fold compared with that before addition of lysozyme. The lysozyme-treated cells were sedimented by centrifugation at 12,000 xg for 15 min and resuspended to one-fifth of the original volume in 55 mM phosphate buffer (pH 7.5). The suspension was then diluted 5 - 10-fold with water to disrupt the cells. The resulting suspension had a high viscosity which was rapidly lowered by adding deoxyribonuclease (10 μg ml^{-1}) and $MgCl_2$ (2 mM). The suspension was then centrifuged at 5,000 xg for 30 min to remove any unbroken cells. The supernatant was also termed the crude cell-free extract.

2.2.4 *Preparation of "membrane fraction".* The extract from either French Pressure cell or lysozyme treatment was centrifuged at 48,000 xg or 130,000 xg for 45 min or 90 min at 4°C. The pellet fraction was washed with a 20-fold volume of 55 mM phosphate buffer (pH 7.5) and recentrifuged. The particulate fraction, containing cell membrane particles, was resuspended in the same buffer to give 8 - 15 mg protein ml^{-1}.

2.2.5 *Preparation of "soluble fraction".* The extract from lysozyme treatment was centrifuged at 48,000 xg for 30 min and the supernatant further spun at 130,000 xg for 90 min. The resultant supernatant was carefully removed and designated "soluble fraction". The supernatant (130,000 xg) from French Pressure cell treatment was not exclusively a "soluble fraction" as it contained membrane material (see Results).

2.2.6 *Enzyme assays.* The thiosulphate-oxidizing activity was routinely assayed at 30°C by measuring oxygen consumption in a Teflon-covered Clark oxygen electrode cell (Rank Brothers, Bottisham, England) with a chart recorder. Oxygen concentration in the experimental system was calibrated using the method described by Robinson & Cooper (1970). The reaction mixture (final volume, 1 ml) contained, unless otherwise specified, 2 μmol thiosulphate; 50 μmol phosphate buffer (pH 7.5); 0.2 or 0.5 mg dry wt cells or 5 mg enzyme protein. The reaction was started by injecting thiosulphate solution with a Hamilton microsyringe. Activity was expressed as nmol or μmol oxygen uptake min^{-1} $(mg\ protein)^{-1}$ or $(dry\ wt)^{-1}$.

The NADH-, succinate- or sulphite-oxidizing activities were measured polarographically in the same way as above except that 0.2 µmol NADH, 0.5 µmol succinate or 1 µmol Na_2SO_3 in 5 mM EDTA was used instead of thiosulphate.

Rhodanese activity was measured by a method based on that of Silver & Kelly, (1976). The reaction mixture contained 50 µmol $Na_2S_2O_3$, 125 µmol Tris (2-amino-2-hydroxymethyl-1,3-propanediol), pH 10.6; 100 µmol KCN; enzyme and water to give 2.1 ml. After incubation for 10 min at 30°C, thiocyanate was determined as described previously (Wood & Kelly, 1981). For the assay of small quantities of cells, the organisms were lysed by the modified EDTA-lysozyme method of Vandenbergh *et al.* (1979). Lysozyme and EDTA (final concentrations 0.5 mg ml^{-1} and 10 mM, respectively) were added to the cell suspension (final concentration about 1 mg dry wt ml^{-1}), which was then incubated at 30°C for 20 - 30 min until the turbid cell mixtures became clear and some precipitation occurred. The lysed cell suspension was used to assay rhodanese activity as described above. Alternatively the cells were incubated with 5% (v/v) Triton X-100 for 20 min at room temperature and then the rhodanese activity was measured. The rhodanese activities measured by either method were similar.

Thiosulphate:cytochrome *c* oxidoreductase activity was measured by following the reduction of cytochrome *c* (horse heart III) at 550 nm in 1 cm cuvettes with a Pye-Unicam SP1700 spectrophotometer.

The assay mixture contained, unless otherwise stated, 2 µmol $Na_2S_2O_3$, 50 µmol phosphate buffer (pH 7.5); 35 or 70 nmol cytochrome *c*; 3 mg protein and water to give a final volume of 1 ml. The same procedure was employed to assay sulphite: cytochrome *c* oxidoreductase activity, except that 2 µmol sulphite in 5 mM EDTA, 100 µmol Tris-HCl (pH 7.5) and 0.5 mg protein were used instead of thiosulphate and phosphate. Activity was expressed as nmol cytochrome *c* reduced min^{-1} $(mg\ protein)^{-1}$; a millimolar extinction coefficient at 550 nm of 28.0 was used.

2.2.7 Spectrophotometry. Difference spectra were obtained with Pye-Unicam SP1700 or SP1800 recording spectrophotometers. Experimental details are provided in the legends to figures.

2.2.8
Oxidative phosphorylation. Reaction mixtures were incubated at 30°C in the oxygen electrode cell as described above. The assay mixture contained in a total volume of 1.0 ml (µmol): 40 phosphate buffer (pH 7.5); 1 AMP; 1 ADP; 20 NaF (to inhibit endogenous ATPase activity); 6 $MgCl_2$; 10 $Na_2S_2O_3$; and cell-free extract (3 mg protein). NADH (0.2 µmol) was added instead of thiosulphate when NADH was used as substrate. The reaction was started by adding substrate with a microsyringe. The rate of oxygen uptake was recorded throughout the reaction. To terminate the reaction, 0.5 ml of assay mixture was removed quickly into a tube containing 0.4 ml of 3% (w/v) perchloric acid, to which 0.1 ml of 2.6 M NaOH was added shortly (3 min) afterwards. The amount of ATP in the neutralized reaction mixture was determined by a luciferin-luciferase method using a Lumac celltester M1030 (Boro Labs Ltd, England). Endogenous respiration and ATP formation by controls were determined by the same procedure except that the substrate was omitted.

2.2.9
Analytical procedures. Thiosulphate was estimated colorimetrically as described by Kelly *et al.* (1969) or by Sörbo (1957). Biomass was estimated from absorbance at 440 nm (1 cm) using appropriate dry weight-absorbance calibration curves. Direct determination of dry weight of organisms in culture confirmed that this was a reliable method for monitoring biomass concentration. Protein was estimated according to the procedure of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard. Carbon content of dried bacteria and cultures was determined with a Beckman Total Organic Carbon Analyzer.

Chemicals. Lysozyme (egg white), cytochrome *c* (horse heart III), cytochrome oxidase (bovine heart), rhodanese (bovine liver), catalase (bovine liver), bovine serum albumin, NADH, antimycin A, HQNO (2-heptyl-4-hydroxquinoline-N-oxide) and rotenone were from Sigma (London) Ltd. Luciferin-luciferase and ATP were purchased from Boro Labs Ltd, England; ADP from Boehringer Mannheim GmbH; and deoxyribonuclease (bovine) from Seravac Lab. England

2.3 RESULTS

2.3.1
Aerobic thiosulphate-limited chemostat culture. A continuous culture was established using a medium containing approximately 30 mM thiosulphate. Steady states were maintained at eight dilution rate (D) between 0.024 and 0.084 h^{-1} (Fig.2.1 a during which no thiosulphate was detectable in the cultures.

Complete oxidation of thiosulphate occurred in all steady

Fig.2.1(a) Steady state yield, thiosulphate oxidation rate, protein content and rhodanese activity of a thiosulphate-limited chemostat of *Thiobacillus* A2. Yield (g dry wt (mol $Na_2S_2O_3$ consumed)$^{-1}$; ●); Rate of oxidation of thiosulphate by organisms removed from the chemostat and assayed polarographically (nmol O_2 uptake min^{-1} (mg protein)$^{-1}$; ○); protein content of the organisms (g protein (g dry wt)$^{-1}$; △); rhodanese activity in lysozyme-treated organisms (nmol thiocyanate formed min^{-1} (mg dry wt)$^{-1}$; ▲).

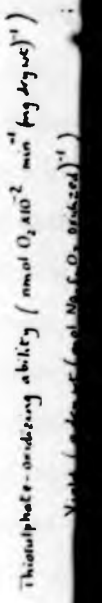

Fig. 2.1(a)

Fig. 2.1b Plot of the specific rate of thiosulphate oxidation ($q_{S_2O_3^{=}}$) against the dilution rate. For details see the text and 1.1.1.

2.1.(b)

oxidation rate, protein content and
ited chemostat of *Thiobacillus* A2.
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$(mg\ dry\ wt)^{-1}$; ▲).

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FIG 1

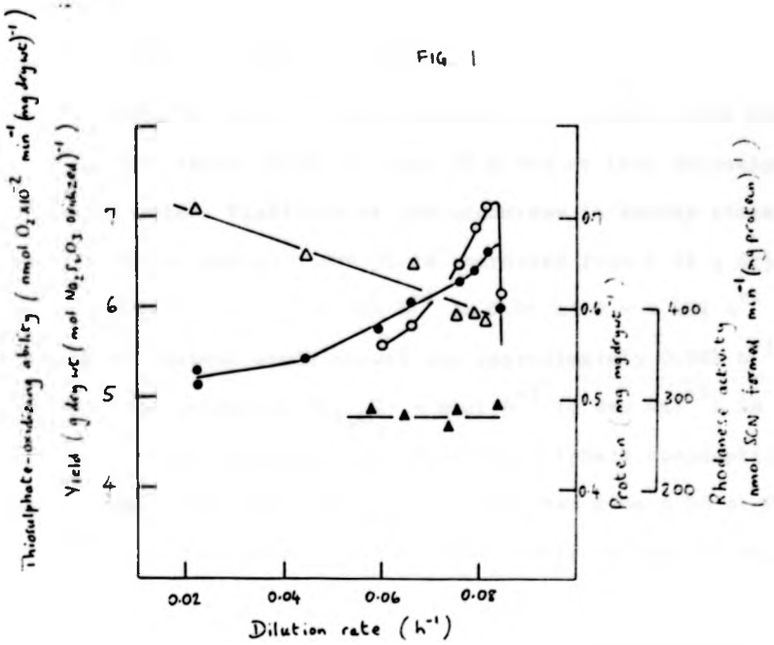
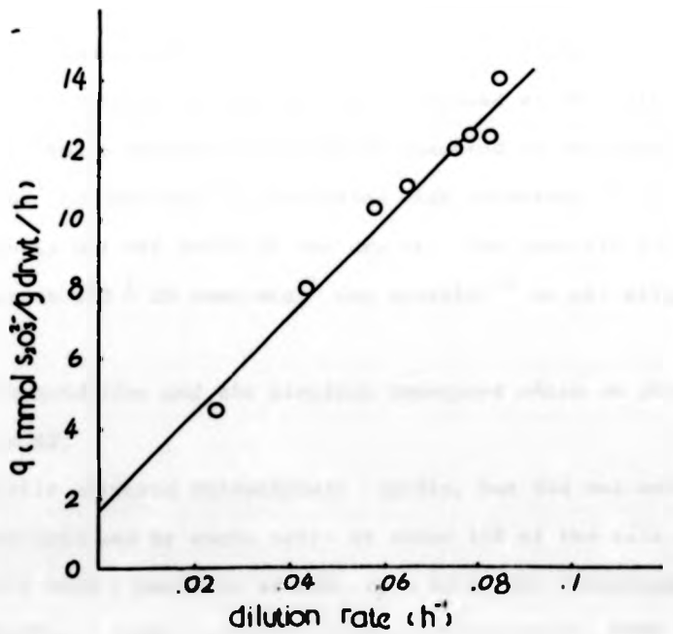


Fig. 2.1(a)



2.1(b)

states in agreement with the following equation:

$$S_2O_3^{2-} + 2O_2 \rightarrow 2SO_4^{2-} + 2H^+ \quad \ldots\ldots\ldots\ldots\ldots\ldots(1)$$

Precise determination of input concentration of thiosulphate and biomass enabled estimation of steady state yield in terms of g dry wt (mol thiosulphate consumed)$^{-1}$ for each dilution rate. Viability of the organisms in steady states was high (>90%) at all D values tested. The yield increased from 5.35 g dry wt (mol thiosulphate consumed)$^{-1}$ at D = 0.024 h^{-1} to 6.66 at D = 0.081 h^{-1} (Fig.2.1a). D_{crit} (above which washout would occur) was approximately 0.085 h^{-1}. The specific rate of thiosulphate oxidation ($q_{S_2O_3^{2-}}$ = mmol h^{-1} (g dry wt)$^{-1}$) in the steady state was calculated for each dilution rate from thiosulphate consumption rate and the steady state biomass. The value of $q_{S_2O_3^{2-}}$ increased from 4.58 at D, 0.025 h^{-1} to 14.01 at D, 0.084 h^{-1} and gave a linear relationship (fitted by regression analysis) when plotted against D. The correlation coefficient for fit of the eight data points to a straight line was 1.003. The reciprocal of the slope of this plot indicated a maximum theoretical growth yield (Y^{max}) of 7.04 g dry wt (mol thiosulphate)$^{-1}$. The q intercept of the plot gave a maintenance coefficient (m) of 1.44 mmol thiosulphate h^{-1} (g dry wt)$^{-1}$, (Fig. 2.1b)

The protein content of the bacteria decreased as the dilution rate increased, but the capacity to oxidize thiosulphate measured in the oxygen electrode cell (nmol O_2 min^{-1} (mg dry wt)$^{-1}$), increased with increased D. The carbon content of the bacteria was 46% (w/w) of the dry wt. The specific activity of rhodanese was constant at 280 ± 10 nmol min^{-1} (mg protein)$^{-1}$ at all dilution rates tested (Fig.2.1c)

2.3.2
Thiosulphate oxidation and the electron transport chain in thiosulphate-grown Thiobacillus A2.

Whole cells oxidized thiosulphate rapidly, but did not oxidize succinate. Sulphite was oxidized by whole cells at about 15% of the rate of thiosulphate in 0.1M Tris-HCl buffer but only at less than 5% of the thiosulphate rate in phosphate buffer. Crude extracts oxidized thiosulphate, NADH and succinate but showed only very slight ability to oxidize sulphite or formate when assayed in the oxygen electrode. Thiosulphate was oxidized at similar rates by crude extracts (10 mg protein ml^{-1}) in Tris (0.01 - 0.1M) or phosphate (0.03-0.01M) buffers.

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The reduced *minus* oxidized spectra of the crude extract, membrane fraction and soluble fraction, using NADH, succinate, $S_2O_3^{2-}$ or SO_3^{2-} as reductants, indicated that cytochromes *c*, *b* and *a* were present in *Thiobacillus* A2 with α bands at 552, 560 and 605-620 nm respectively (Figs.2.2, 2.3) A trough at 450 nm suggested that flavin-protein was also present.

The cytochromes were reduced immediately on addition of NADH or succinate to the crude extract or membrane fraction. The cytochrome *b* band at 560 nm was visible in the membrane fraction (Fig.2.3) but was masked by the very high concentration of cytochrome *c* in the crude extract (approximately 1.4 nmol (mg protein)$^{-1}$). With thiosulphate or sulphite as reductants, the spectra of the crude extract appeared in 5 to 15 min during which the reaction mixture in the cuvette apparently became anaerobic since the peaks would disappear if oxygen was introduced simply by inverting the cuvettes, indicating high activity of cytochrome oxidase in the crude extract. Reoxidation did not occur with NADH or succinate as reductants because the oxidation rates of NADH or succinate in the crude extract were about 15 and 5 times higher respectively than that of thiosulphate. Cytochrome c_{522} reduced in the soluble fraction by thiosulphate could not be oxidized by adding mammalian cytochrome oxidase. Horse heart cytochrome *c* reduced in the soluble fraction by thiosulphate could easily be oxidized by the membrane fraction.

The difference spectra in the soluble fraction were formed as soon as thiosulphate or sulphite was added (Fig.2.4). This fraction probably contained only *c*-type cytochrome (α band at 552 nm). Thiosulphate or sulphite failed to reduce the cytochromes in the membrane fraction (Fig.2.3). Similarly NADH or succinate did not reduce cytochromes in the soluble fraction (Fig.2.4).

Antimycin A or HQNO inhibited the reduction of cytochrome *c* with NADH or succinate as reductants but had no effect with thiosulphate or sulphite as substrates (Fig.2.2 and2.3b).

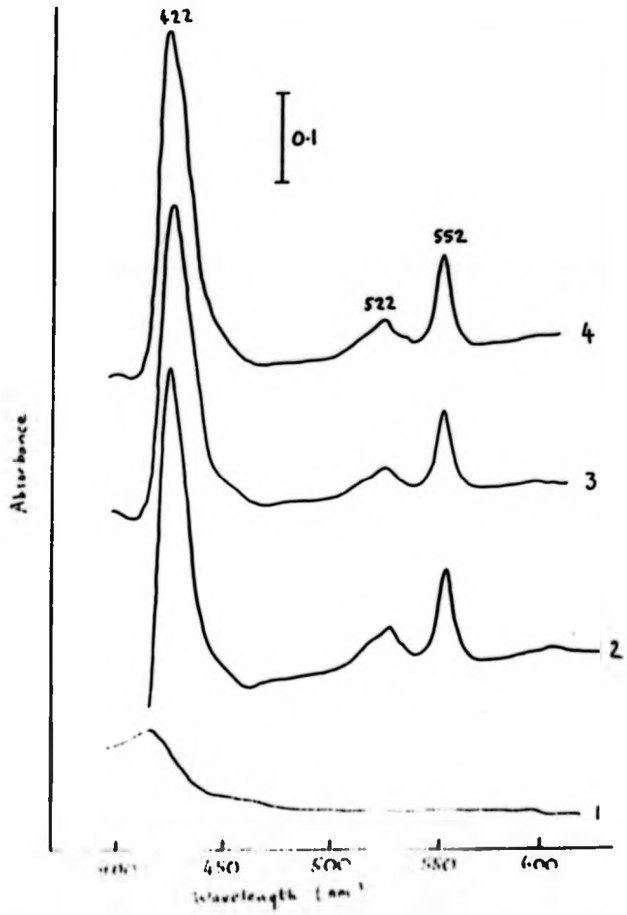

Fig. 2.2 Difference spectra of *Thiobacillus* A2 crude extract (6 mg protein ml^{-1}) incubated without further additions (trace 1); with 1 mM NADH or 5 mM succinate (trace 2); 10 mM thiosulphate or sulphite (trace 3); 10 mM thiosulphate with either rotenone, antimycin A (50 µg ml^{-1}) or HQNO (25 µg ml^{-1}) (trace 4).

Fig. 2.3

Fig. 2.3 Cytochrome reduction in *Thiobacillus* A2 membrane fraction and the effect of electron transport inhibitors. (a) Difference spectra immediately after addition to membrane fraction (7.5 mg protein) of 10 mM thiosulphate or sulphite (trace 1) or 1 mM NADH or 5 mM succinate (trace 2). Prolonged incubation did not alter trace 1. (b) Cytochrome reduction using membrane fraction (5 mg protein) with NADH or succinate after 2 min pre-incubation with either antimycin A (50 μg ml^{-1}) or HQNO (25 μg ml^{-1}), measured after 0 min (trace 1), 2 min (trace 2), 8 min (trace 3), 9 min (trace 4), 10.5 min (trace 5).

Fig. 2.3

Zlus A2 membrane fraction and the

(a) Difference spectra immediately

mg protein) of 10 mM thiosulphate

mM succinate (trace 2). Prolonged

Cytochrome reduction using membrane

ccinate after 2 min pre-incubation

HQNO (25 μg ml^{-1}), measured after

(trace 3), 9 min (trace 4), 10.5

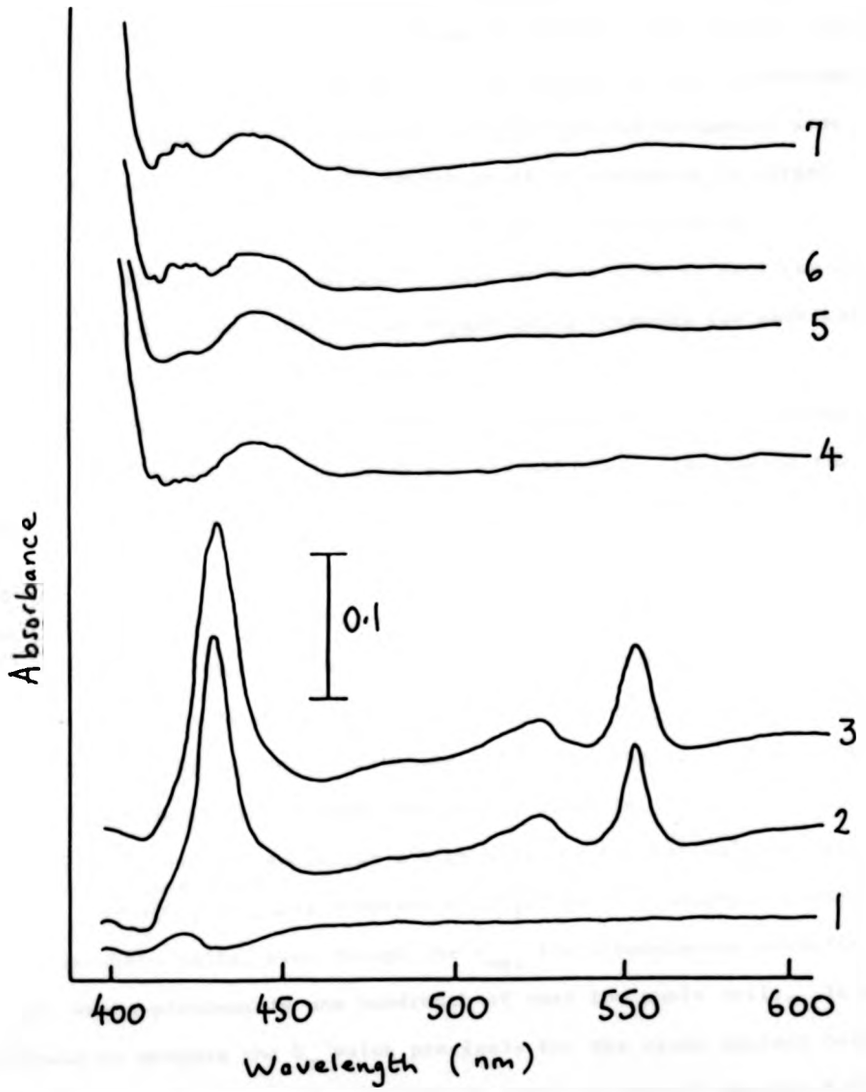

Fig. 2.4 Difference spectra of the 'soluble fraction' (5 mg protein) from *Thiobacillus* A2 incubated with no further addition (trace 1), 10 mM thiosulphate or sulphite for 1 and 4 min (traces 2 and 3), 1 mM NADH or 5 mM succinate for 2, 5, 8 and 25 min (traces 4, 5, 6 and 7).

The effects of several inhibitors on NADH, succinate or thiosulphate oxidation by the crude extract are shown in Table 2.1. The results confirmed the finding from the difference spectra that neither *b*-type cytochrome nor any electron transport chain component on the NADH dehydrogenase side of cytochrome *b* was involved in the oxidation of thiosulphate by oxygen.

2.3.3
Stoichiometry of thiosulphate oxidation by cell-free extracts.

Thiosulphate oxidation by whole cells or cell-free extract was consistent with equation (1), two molecules of oxygen being consumed for each thiosulphate oxidized (Table 2.2).

Neither the soluble nor the membrane fraction (from centrifuging at 130,000 x g) could separately carry out the oxidation, indicating that factors present in both fractions were necessary (Table 2.2). NADH- or succinate-oxidizing activities were, however, mainly membrane bound, hardly any activity being found with the soluble fraction alone.

Cytochrome *c* (horse heart III) caused little stimulation of the activity of thiosulphate oxidation by the crude extract which was, however, slightly inhibited (30%) by reduced glutathione (5 mM) and strongly (80%) by sulphite (2 mM) if the sulphite was added before thiosulphate.

The crude extract exhibited a high affinity for thiosulphate with a K_m value between 33 μM and 90 μM (e.g. Fig. 2.5) compared to 37 μM for thiosulphate-dependent oxygen uptake by whole cells, even though the V_{max} for thiosulphate oxidation by the crude extract was approximately one hundredth of that for whole cells. It was very difficult to measure the K_m value precisely for the crude extract because of (a) the occurrence of a lag and acceleration phase of several minutes before the maximum rate of oxidation was reached; and (b) the specific activity of thiosulphate oxidation was related to the concentration of extract (Fig. 2.6a), thus the specific activity was increased with increase of protein concentration up to 12 mg ml^{-1}, indicating that a complex enzyme system was involved in thiosulphate oxidation. There was also an interdependence of Tris buffer concentration and protein concentration in determing the specific activity

Table 2.1 *Effect of electron transport inhibitors on the oxidation of NADH, succinate and thiosulphate by a crude extract of Thiobacillus A2*

Substrate	Inhibitor	Inhibition of oxidation rate (%)
NADH	Antimycin A (10 µg)	96
	HQNO (5 µg)	97
	Rotenone (10 µg)	93
Succinate	Antimycin A (10 µg)	75
	HQNO (5 µg)	80
$Na_2S_2O_3$	Antimycin A (10 µg)	13
	HQNO (5 µg)	11
	Rotenone (10 µg)	10
	Cyanide (0.1 mM)	95

Initial oxidation rates were determined using the oxygen electrode cell (1 ml final volume) as described in the Methods. Inhibitors were added after the initial rates of substrate oxidation had been established.

Table 2.2 *Thiosulphate oxidation by whole cells and extracts of Thiobacillus A2*

Reaction system (mg protein)	Total incubation time (min)	Thiosulphate oxidized (µmol)	Oxygen consumed (µmol)	$O_2/Na_2S_2O_3$
Whole cells (0.55 mg dry wt)	4.5	0.58	1.08	1.86
Crude extract (10)*	8	0.46	0.92	2.00
Crude extract (12.5)+	16	0.81	1.76	2.17
'Soluble fraction' (7)+	15	0	0	-
'Membrane fraction' (3)+	15	0	0	-
'Soluble' (5.6) + 'Membrane' (0.6) fraction +	15	0.66	1.31	1.98

Thiosulphate oxidation was measured polarographically as described in the Methods. Residual thiosulphate was determined by the method of Sörbo (1957).

*Prepared using the French pressure cell.

+Prepared using lysozyme treatment for crude extract.

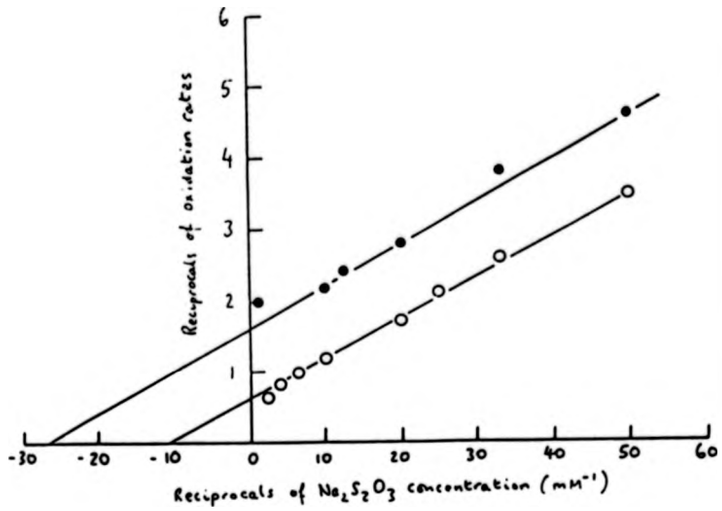

Fig. 2.5 Kinetics of oxidation of thiosulphate by intact organisms (0.9 mg dry wt; ●) and crude extract (9.5 mg protein; ○) of *Thiobacillus* A2 using the oxygen electrode. Lineweaver-Burk plots of reciprocals of thiosulphate concentrations (20 - 400 µM) and oxygen uptake rate (µmol min^{-1} and nmol x 10^{-2} min^{-1} for intact organisms and crude extract respectively) are shown. K_m and V_{max} values were calculated as 37 and 87 µM $Na_2S_2O_3$ and 694 nmol min^{-1} (mg dry wt)$^{-1}$ and 16 nmol min^{-1} (mg protein)$^{-1}$ respectively.

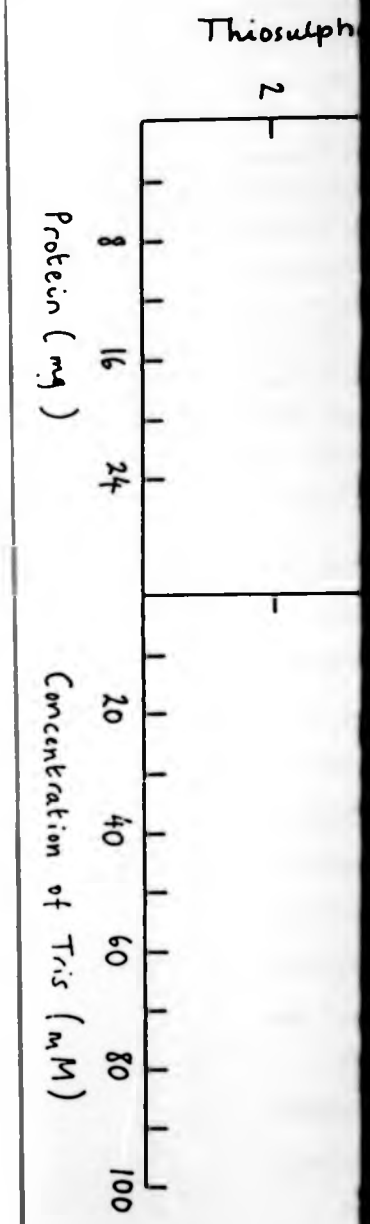

Fig.2.6 Thiosulphate oxidation by crude extracts of *Thiobacillus* A2. (a) Effect of concentration (mg protein ml^{-1}) of crude extract on specific activity of thiosulphate oxidation (nmol O_2 uptake min^{-1} $(mg\ protein)^{-1}$); (b) Effect of concentration (mM) of Tris-HCl buffer, pH 7.4, on specific activity with two amounts of crude extract (5 mg protein ml^{-1}, ●; 10 mg protein ml^{-1}, ○). Oxygen uptake was determined polarographically as described in the Methods.

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in ml^{-1}, ● ; 10 mg protein ml^{-1}, ○).
ically as described in the Methods.

LU and KELLY: Energy metabolism

FIG. 2.6

of thiosulphate oxidation (Fig. 2.6.b).

2.3.4. *Reconstitution and localization of the thiosulphate oxidation system.*

It is illustrated in Table 2.3 that the factors essential for thiosulphate oxidation were totally soluble in the cell and that the membrane fraction was only necessary for the terminal transfer of electrons to oxygen. The membrane fraction could be replaced by mammalian cytochrome c and cytochrome oxidase. The stoicheiometry of thiosulphate oxidation by the reconstituted system also showed an $O_2/S_2O_3^{2-}$ ratio of 2, which suggested that the reaction was complete (equation 1). Moreover, the following experiment revealed that the enzyme system was located in the cytoplasmic rather than periplasmic side, thus the supernatant after spinning down and separation from the spheroplasts produced by lysozyme - EDTA treatment in 0.5M sucrose as described in the Methods (2.2.3) contained no thiosulphate-oxidizing activity, which was shown in the solution after lysis of the sedimented spheroplasts by osmotic shock in 55mM phosphate buffer. The specific activity of the solution was more or less the same as that of crude extract prepared by ultrasonication or French Pressure Cell treatment.

2.3.5. *ATP formation by cell-free preparations during thiosulphate oxidation.*

The oxidation of thiosulphate was coupled to ATP synthesis by the 40,000 xg supernatant fraction from Thiobacillus A2 (Table 2.4). The P/O ratio from four determinations was 1.10 $\pm$ 0.23. This oxidative phosphorylation was not affected by antimycin A or HQNO, in contrast to NADH-dependent oxidative phosphorylation which was severely inhibited (Table 2.4). This confirmed the former finding that thiosulphate entered the electron transport chain at the cytochrome c level and only the terminal site of energy conservation involving the action of cytochrome c:cytochrome oxidase was functional in thiosulphate-grown Thiobacillus A2.

The crude extract (10,000 xg or 20,000 xg supernatant) oxidized thiosulphate less rapidly and exhibited phosphorylation but with a wide variation in P/O ratio (from 0.5 to 1.5).

The 130,000 xg supernatant from French Pressure Cell treated organisms also catalysed phosphorylation coupled to thiosulphate oxidation (Table 2.4).

The same kind of supernatant from lysozyme-treated cells failed to oxidize thiosulphate (Table 2.2). The French Pressure Cell preparation contained cytochrome oxidase activity and was able to oxidize NADH. These results indicate that membrane material was still present in the 130,000 xg

Table 13 *Thiosulphate oxidation by the 'soluble fraction' (7 mg protein) of Thiobacillus A2 supplemented with horse heart cytochrome c (0.17 μmol) and bovine cytochrome oxidase (5 units)*

$Na_2S_2O_3$ added (nmol)	Oxygen consumed (nmol)	$O_2/Na_2S_2O_3$
20	40.3	2.02
50	99.5	1.99
200	385.0	1.93

Experimental conditions were as described in Methods except that oxygen uptake was recorded until thiosulphate oxidation was complete.

Experimental conditions were as described in the Methods using extracts prepared from French pressure cell treatment. Values for ATP formation and oxygen consumption were corrected for values obtained without added substrate.

Some supplementary data of the experiment: (a) $Na_2S_2O_3$ was added last into reaction mixture, which had been incubated for 4 min, to initiate the reaction (i.e. to start the incubation time). (b) This was an average value of four measurements (0.91, 1.3, 1.3 and 0.9 (S.D. $\pm$ 0.19). The average value of endogenous formation of ATP (i.e. in the absence of $Na_2S_2O_3$) for the four measurements was 282 nmol (S.D. $\pm$ 0.19). The value of P/O ratio of 0.9 was obtained from a difference in the values of ATP formation and O_2 uptake between 6 min and 8 min incubation. (c) AMP was used against the ATPase activity (hydrolysis of ATP), which, in a control experiment (the conditions were the same as above except without adding $Na_2S_2O_3$ and adding ATP (0.1 µmol) instead of AMP and ADP), showed an activity of about 9 nmol ATP hydrolysis per min per mg protein of extract during the 8 min incubation. The figure decreased about 10 times in the additional presence of AMP (0.1 µmol). On the other hand, the AMP could enhance the activity of adenylate kinase after ATP was synthesized to certain amounts by the phosphorylation. However, the following control experiments indicated that this role of AMP would not be significant in the experimental condition. Thus, the extract showed an adenylate kinase activity of about 80 nmol ATP formation from ADP (0.5 µmol) per mg protein during a 2 min incubation in the absence of ATP and AMP, whereas only about 25 nmol of ATP disappeared per mg protein during a 2 min incubation with ATP and AMP (0.5 µmol each). (d) The values of P/O ratio assayed by using crude extract, S-130,000 fraction and membrane fraction (without washing) exhibited a great diversity of results, from 0.2 to 1.5 (giving an average value of 0.75 from 12 measurements and S.D. = 0.45). It is not clear why the S-40,000 fraction showed the better result, which might be related with the size of the membrane particles, their orientation (i.e. inside

Table 2.4 O

Preparation tested*	
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(c) AMP was used against the ATPase
n a control experiment (the conditions
adding $Na_2S_2O_3$ and adding ATP (0.1 µmol)
ivity of about 9 nmol ATP hydrolysis
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d that this role of AMP would not be
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iversity of results, from 0.2 to 1.5
12 measurements and S.D. = 0.45). It is
howed the better result, which might be
particles, their orientation (i.e. inside

Table 2.4 *Oxidative phosphorylation by cell-free extracts of Thiobacillus A2*

Preparation tested*	Substrate	Reaction time (min)	Inhibitor (µg(mg protein)$^{-1}$)	ATP formed (nmol)	Oxygen consumed (natom)	$P/_O$
S-40,000[a]	$Na_2S_2O_3$	6	None	258	231	1.12[b]
			Antimycin A			
			HQNO	210	189	1.11
				241	227	1.06
	NADH	0.5	None	167	322	0.52
			Antimycin A	0	12	-
			HQNO	0	10	-
S-130,000	$Na_2S_2O_3$	4	None	80	104	0.76
			2:4-Dinitro-phenol (0.1 mM)	113	0	-

*S-40,000, supernatant fraction from centrifuging at 40,000 xg for 30 min.
S-130,000, supernatant fraction from centrifuging at 130,000 xg for 90 min.

out or right side out). Generally the preparations from EDTA-lysozyme treatment showed poorer results (more diverse and lower P/O ratio) than that from the French cell treatment, which implied that the large membrane fragments (probably right side out) were poor materials.

The assay of ATP synthesis may be done by following the disappearance of free Pi in the presence of a 'glucose-hexokinase trap' system, which rapidly removes ATP formed. In this way the problems of adenylate kinase and ATPase (to catalyse hydrolysis of ATP) could be largely obviated.

supernatant liquid, as was also shown by Loya et al. (1982). Lysozyme treatment presumably produced no membrane material not sedimented at 130,000 xg.

AMP was added to the reaction mixture in order to inhibit the adenylate kinase in the crude extract, which otherwise rapidly catalysed the conversion of about one-third of the added ADP into ATP. ATPase activity was also observed in the crude extract in the absence of fluoride, with an initial hydrolysis rate of approximately 10 nmol ATP min^{-1} (mg protein)$^{-1}$.

2.3.6 Occurrence of rhodanese in extracts

Most rhodanese activity was recovered in the soluble fractions following lysozyme treatment, indicating it not to be membrane-bound. If bovine liver rhodanese (2 units ml^{-1}) was incubated with the Thiobacillus A2 membrane fraction (0.5 mg protein), horse heart cytochrome c (33 nmol) and thiosulphate (1 umol) no oxygen consumption occurred, indicating the soluble fraction to provide components as well as or other than rhodanese that were necessary for thiosulphate oxidation to occur. The addition of reduced lipoate resulted in rapid oxygen uptake even if the absence of rhodanese.

2.3.7 Sulphite oxidation by cell-free preparations

The rate of sulphite oxidation by the crude extract was negligible (1.2 - 1.4 nmol O_2 min^{-1} (mg protein)$^{-1}$) in 0.05M phosphate pH 7.4 or 0.01 M Tris pH 7.4. If horse heart cytochrome c was added at 70 or 140 uM, the rate in 0.01 M Tris was increased to 2.1 and 5.4 respectively. In 0.1 M Tris pH 7.4, sulphite oxidation was increased to 5.2, and further stimulated to 13.3 by 70 uM cytochrome c. The crude extract would oxidize 2 mM thiosulphate in phosphate buffer (9 nmol min^{-1} (mg protein)$^{-1}$) but the prior addition of sulphite (2 mM) to give an initial oxidation rate of 1.4 nmol min^{-1} (mg protein)$^{-1}$ before thiosulphate addition, resulted in a rate of only 1.7 after adding thiosulphate.

Similarly, the oxygen uptake rate by the crude extracts was depressed by 40% when sulphite (2 mM) was added during thiosulphate oxidation. The inhibitory action of sulphite on thiosulphate-dependent oxygen uptake in

phosphate buffer has not yet been explained, but is apparently not due to direct inhibition of cytochrome oxidase.

The soluble fraction (130,000 xg) had a very active sulphite:cytochrome *c* oxidoreductase, exceeding that for thiosulphate by about 40-fold. Sulphite-dependent cytochrome *c* reduction could only be demonstrated in Tris buffer, as phosphate was markedly inhibitory. Thus the rate of reduction of cytochrome *c* (35 μM) by sulphite (1 mM) was 71 nmol min^{-1} $(mg\ soluble\ protein)^{-1}$ in 0.1 M Tris pH 7.5 but only 0.8 in 0.05 M phosphate. Cytochrome *c* reduction by thiosulphate had a K_m of about 200 μM cytochrome *c* and was consequently very slow in this system, but sulphite-dependent reduction was not affected by thiosulphate.

Ferricyanide was also reduced by sulphite using the soluble fraction. Respective rates were 65 and 242 nmol min^{-1} $(mg\ protein)^{-1}$ in phosphate and Tris buffers. Under similar conditions no ferricyanide reduction was effected by thiosulphate.

2.4 DISCUSSION

This is the first time that a cell-free system from *Thiobacillus* A2 has been obtained that is capable of the stoicheiometric oxidation of thiosulphate to sulphate and the coupling of this oxidation to ATP synthesis. Recently Kula *et al.* (1982) reported unstable thiosulphate oxidizing activity in extracts of *Thiobacillus* A2 and a failure to observe ATP synthesis during thiosulphate oxidation. The data obtained are consistent with the following stoicheiometry:

$$S_2O_3^{2-} + 2O_2 + H_2O + 4ADP + 4P_i = 2SO_4^{2-} + 2H^+ + 4ATP \quad \text{.....(2)}$$

The effects of the uncoupling agent 2:4-dinitrophenol and of electron transport inhibitors indicate that thiosulphate oxidation is coupled directly to cytochrome *c* reduction, without involving cytochrome *b* for the oxidation of either the sulphane- or sulphone-sulphur to sulphate, and that ATP synthesis is effected exclusively by electron transport phosphorylation coupled to the

phosphate buffer has not yet been explained, but is apparently not due to direct inhibition of cytochrome oxidase.

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reoxidation of cytochrome *c*. The spectrophotometric observations with NADH, succinate and inhibitors showed that the organism contains a complete electron transport chain allowing electron flow from NADH to oxygen. The thiosulphate oxidizing enzyme system was located in the 'soluble' 130,000 xg fraction of cell-free extracts and the membrane material was required only to provide the cytochrome oxidase system for terminal oxidation processes. This soluble fraction is currently being resolved into component fractions catalysing thiosulphate cleavage and sulphane-sulphur or sulphite oxidation (W.P. Lu, unpublished data). It is noteworthy that the system obtained from *Thiobacillus* A2 is significantly different from that from *T. novellus* (Oh & Suzuki, 1977a,b) which was wholly membrane-associated. Among the enzymes believed by Suzuki (Suzuki, 1965; Suzuki & Silver, 1966; Charles & Suzuki, 1966; Oh & Suzuki, 1977a,b) to be involved in cell-free thiosulphate oxidation were rhodanese, sulphite oxidase and the 'sulphur-oxidizing enzyme'. The former were present in our preparations but our results give positive evidence incompatible with a role for a sulphur oxygenase in *Thiobacillus* A2. The demonstration that electrons from thiosulphate oxidation enter the electron transport chain at cytochrome *c* and the $P/_O$ of 1.0, indicate that each pair of electrons transferred to oxygen supports one phosphorylation. Since four oxygen atoms (equivalent to four electron pairs) are consumed during thiosulphate oxidation and four ATP formed, all oxygen consumption must be used by cytochrome oxidase, and no oxygen could have been used in the direct oxygenation of sulphur.

Our results on thiosulphate-dependent phosphorylation using a 130,000 xg 'soluble' preparation can be compared with those of Cole & Aleem (1970) using a 144,000 xg fraction from *T. novellus*. They believed their system to prove oxidative phosphorylation in a membrane-free soluble system. This is not the case with our data, as our observations indicate that membrane material, albeit very finely degraded, is present even after high-speed centrifuging. Moreover, high-speed supernatant preparations from lysozyme-treated bacteria are inactive as no small membrane fragments are produced by this method in contrast to the French Pressure cell or sonic disintegration methods.

The growth yield data obtained from the chemostat culture can be used for a theoretical comparison with the ATP synthesis observed with the cell-free systems. The 'true growth yield' ($Y^{max}_{S_2O_3}$) of 7.0 and carbon content of 46% of the dry wt indicates the fixation of 0.27 mol CO_2 for each thiosulphate oxidized. Since CO_2-fixation by the Calvin cycle requires 3ATP and 2NADH for each CO_2, a minimum of 0.81 ATP and 0.54 NADH must therefore be produced from the oxidation of one thiosulphate. As 0.54 NAD^+ requires 1.08 reducing equivalents (H) for reduction and thiosulphate oxidation produces 8 H per mol oxidized, energy for the generation of ATP and cytochrome *c* -dependent reduction of NAD^+ comes from the oxidation with oxygen of 6.92 H produced from 0.87 mole thiosulphate. ATP and NADH production from one mole thiosulphate oxidized for energetic purposes via electron transport to oxygen thus becomes 0.93 ATP + 0.62 NADH. Thiosulphate-dependent NAD^+ reduction in thiobacilli requires a minimum of 2ATP per mole NAD^+ reduced (For reviews, see Kelly, 1978, 1982). Consequently the ATP required to effect the CO_2-fixation required for the observed $Y^{max}_{S_2O_3}$ would be 2.17 ATP per mole thiosulphate oxidized for energetic purposes. Since about 30% of the energy available from chemolithotrophic oxidations is probably expended directly in CO_2-fixation and the remaining 20% in biosynthetic and transport processes, the total ATP production is indicated to be of the order of 2.7 ATP per mole thiosulphate. Experimentally the P/O ratio of about one supported by cell-free thiosulphate oxidation indicated that the overall apparent efficiency of growth was lower than assumed in these calculations and could indicate that more than two ATP were consumed per mole of NAD^+ reduced. The maximum experimental ATP production observed of 4.4 per mole thiosulphate oxidized (Table24) could indicate that 4ATP were consumed for reduction of one NAD^+. This problem is discussed in greater detail elsewhere (Kelly, 1982).

Thiobacillus A2 is proving the ideal experimental system for the elucidation of electron transport-dependent phosphorylation during sulphur compound oxidation as it is possible to obtain cell-free systems that are sufficiently stable for biochemical fractionation and are not complicated by the presence of any substrate-level phosphorylation, such as are present in some other thiobacilli (Kelly, 1982).

2.5 Some reconsiderations of the amount of ATP required for reduction of pyridine nucleotides and the energy efficiency of the process

Two energy consumption processes which have not been taken into account in the above calculation (see also Kelly 1982) are the reduction of NADPH required by biosynthesis and thiosulphate transport. Stouthamer (1973) calculated that the formation of amino acids and lipid from glucose and inorganic salts needed 110.3 and 39.2 mol x 10^{-4} NADPH per g cells respectively (i.e. 0.286 and 0.1 mol NADPH per mol of thiobacillus cell carbon if a value of 46% carbon per g dry wt cells is used). The value for biosynthesis of nucleotides is estimated about 5.5 mol x 10^{-4} NADH per g cells from Stouthamer's figures and the knowledge of the biosynthetic pathway. So a total amount of 0.4 mol NADPH is required for 1 mol of cell carbon formation. Stouthamer believed that the production of NADPH during glucose breakdown would be sufficient to meet the demands of biosynthesis and assumed that the formation of NADPH from NADH did not require any ATP. Therefore this value was only considered for the amount of reducing equivalents liberated per mol of thiosulphate oxidized. Thus, formation of 0.27 mol cell carbon from 1 mol thiosulphate needs an extra 0.108 mol NADPH, the total reducing equivalents available from 8e of thiosulphate for energy conservation will be 84%.

Transport of glucose and sucrose by T. versutus was by respiration dependent systems requiring a membrane proton gradient (Wood and Kelly 1982). But we know nothing about thiosulphate transport into this organism. The lysozyme experiment (see 2.3.4.) showed that the thiosulphate-oxidizing system is located inside the cytoplasmic membrane. From the consideration of chemiosmotic theory cations should enter across membranes on bifunctional carriers, or proton symports (Hamilton 1977). This was confirmed by the experimental results so far. Transport of sulphate, phosphate, ammonium and several sugars were shown to be through the action of a proton symport with a 1:1 proton stoichiometry

(Hamilton 1977). A value for the electroneutral symport of $2H^+/SO_4^{2-}$ across the membrane of Paracoccus denitrificans was also reported (Burnell et al. 1975). In Salmonella typhimurium, sulphate and thiosulphate were transported by a single transport system (Dreyfuss 1964). On the basis of these observations, I assume that a similar proton symport is operative for thiosulphate transport in T. versutus with a 2:1 proton/thiosulphate ratio, equivalent to 0.5 ATP per thiosulphate.

Let us refer back to the previous calculation: we have deduced that the observed $Y^{max}_{S_2O_3^{2-}}$ represents fixation of 0.27 mol CO_2 to cell carbon, for which 0.81 ATP and 0.54 NADH are required for CO_2 fixation and 0.243 ATP is required for biosynthesis of cell components using a value of 0.9 mol ATP per mol cell carbon, which was calculated from Stouthamer's value of 347.1×10^{-4} mol ATP per g cells). Assuming that 2ATP are required for reduction of 1 mol NAD^+, we have 2.63 ATP (0.81 + 0.54 x 2 + 0.243 + 0.5 consumed for $S_2O_3^{2-}$ transport) formation from 1 mol of thiosulphate. Because only 84% of the H equivalents are used for energetic purposes, the real value will be 3.13 ATP formed per thiosulphate oxidized. If 3ATP/NAD^+ is assumed, the value becomes 3.8, which is close to the experimental result of a P/o ratio of 1, i.e. 4ATP formation per thiosulphate oxidized. The value will be 4.4 or 50.6 if 4 or 5ATP are needed for reduction of 1 mol NAD^+ respectively.

There may be some errors involved in the above calculations. The Stouthamer value of ATP requirement for the formation of microbial cells from glucose (347×10^{-4} mol ATP/g cells) is a purely theoretical one, from which a Y^{max}_{ATP} of 28.8 is obtained, whereas the experimental value of Y^{max}_{ATP} is only about 11 (E. coli) (Stouthamer 1977). Therefore, the actual value of ATP requirement for biosynthesis is probably much higher than that used here. The ATP requirement for thiosulphate transport is an entirely speculative one, which would be too high if there is a sort of energy-independent efflux pathway exchange between

thiosulphate and sulphate. It is unclear if the same mechanism is operative in the transport of inorganic compounds mainly for energetic purposes and for biosynthetic demands.

We propose that the low redox centres of cytochrome c_{551} (Em7-110 mV) and cytochrome $c_{552.5}$ (Em7 + 25 to -25 mV) might function as electron transfers to reduce NAD^+ in the oxidation of thiosulphate (see Chapters 7 and 9). In this case the energy gap between the two cytochromes and NAD^+/NADH couple will be about 0.22 or 0.32V, from which a free energy difference of 10.12 or 14.7 Kcal is obtained. Therefore, an energy efficiency would be 46% or 67% for 3ATP requirement for NAD^+ reduction, or 35% or 50% for 4ATP/NAD^+ (the G^o of 7.3 Kcal for ATP is used).

It is noted that in their classic paper (Chance and Hollunger 1960) about the energy-dependent reduction of mitochondrial pyridine nucleotide, Chance and Hollunger pointed out that it is not surprising that the energy expenditure in the reduction of NAD^+ by succinate is high (actually about two) relative to the one ATP equivalent obtained in the oxidation of NADH because the reduction reaction must pay the full cost of the reaction.

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CHAPTER 3

PARTIAL PURIFICATION AND RESOLUTION OF A THIOSULPHATE-OXIDIZING SYSTEM FROM THIOBACILLUS A2

3.1 INTRODUCTION

Despite considerable study over many years, a complete understanding of the mechanism of thiosulphate oxidation to sulphate by thiobacilli has not yet been achieved (Kelly 1982). In the previous paper we reported complete thiosulphate oxidation and energy-coupling with high efficiency by crude cell-free preparation from *Thiobacillus* A2 (Chapter 2). The principal unresolved problems of the oxidation pathway are the mechanism of the initial 'activation' reaction of thiosulphate and the nature of the enzymes oxidizing the sulphane-sulphur atom of thiosulphate (and probably also of sulphur and sulphide) to sulphite. A number of hypothetical schemes have appeared (for general review, see Kelly, 1968, 1976, 1982; Suzuki, 1974; Aleem, 1975), but substantive evidence to confirm these hypotheses, including highly purified enzymes and the demonstration of thiosulphate oxidation by reconstituted enzyme systems *in vitro* are still lacking except for the terminal conversion of sulphite to sulphate. Our recent work (Chapter 2) demonstrated that the enzyme system for thiosulphate oxidation was completely soluble (i.e. not sedimented at 130,000 xg) and that membrane material was required only to provide the cytochrome oxidase system for terminal electron transport. The present paper describes the partial purification, resolution and reconstitution of this soluble enzyme system into three major components.

3.2 METHODS

3.2.1 *Organism and chemostat culture.* *Thiobacillus* A2 was grown in continuous culture on a medium as described elsewhere (Chapter 2) except that 200 mM $Na_2S_2O_3$ was used in order to maximize biomass production. A chemostat culture was established in a Gallenkamp glass fermenter vessel with a culture volume of 5.2ℓ, stirred (1000 rpm) with a direct drive shaft stirrer module and aerated (320 ml min^{-1}) with air containing 1% (v/v) CO_2. (Fig.3.1). Temperature was maintained at 30°C with an LH Engineering heater module and pH was maintained at pH 7.7 by automatic titration with 10 M NaOH using an LH Engineering pH controller. Thiosulphate was completely consumed and was the growth-limiting nutrient, giving about 7g dry wt of bacteria per

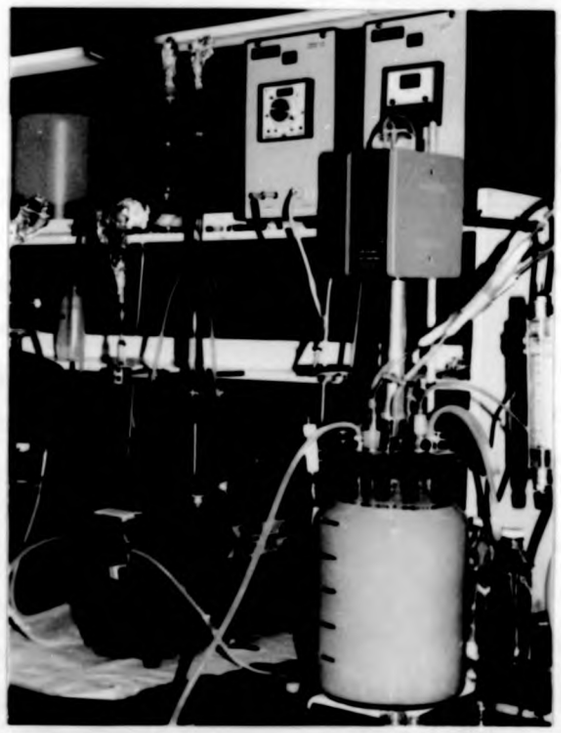


Fig. 3.1 Continuous chemostat cultivation of T. versutus on a 5.2ℓ pot. For details see 3.2.1.

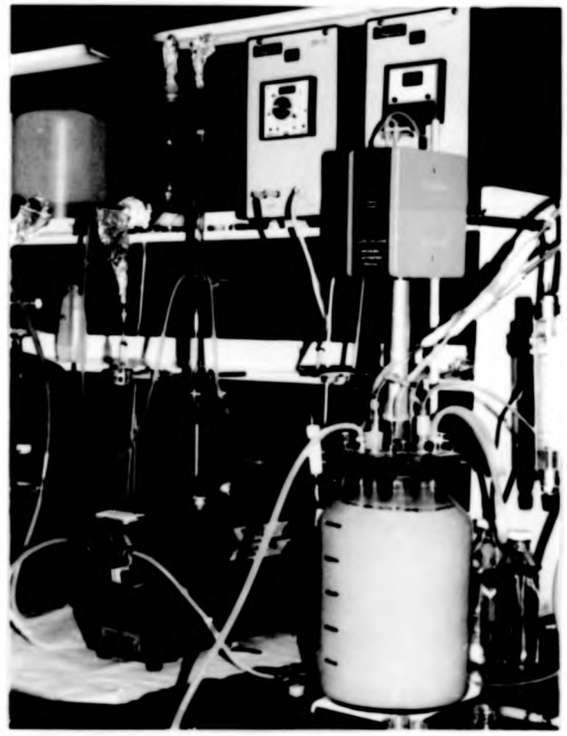


Fig. 3.1 Continuous chemostat cultivation of T. versutus on a 5.2ℓ pot. For details see 3.2.1.

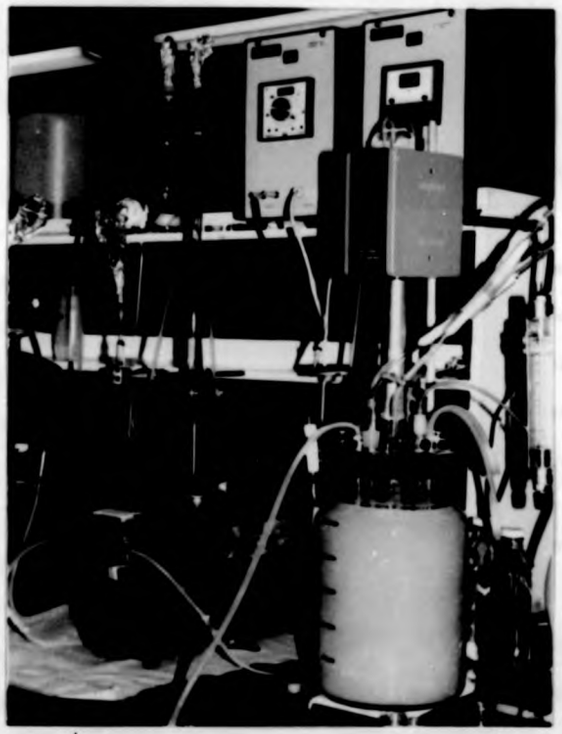


Fig. 3.1 Continuous chemostat cultivation of T. versutus on a 5.2ℓ pot. For details see 3.2.1.

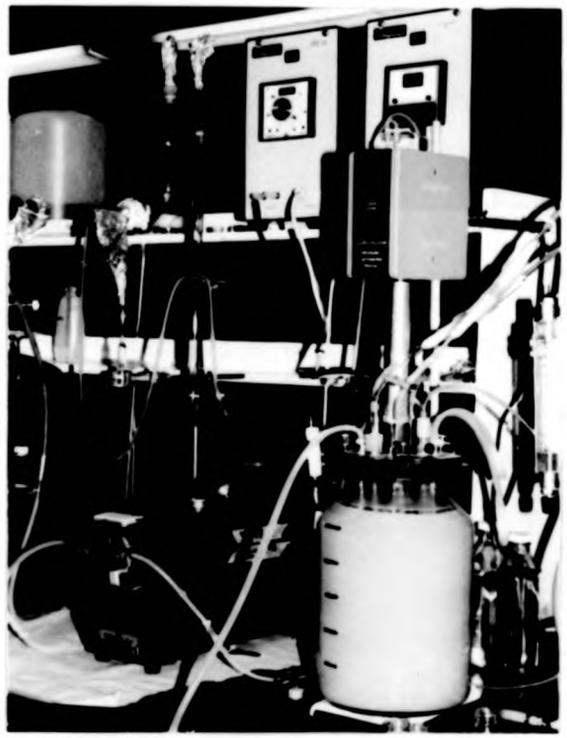


Fig. 3.1 Continuous chemostat cultivation of T. versutus on a 5.2 l pot. For details see 3.2.1.

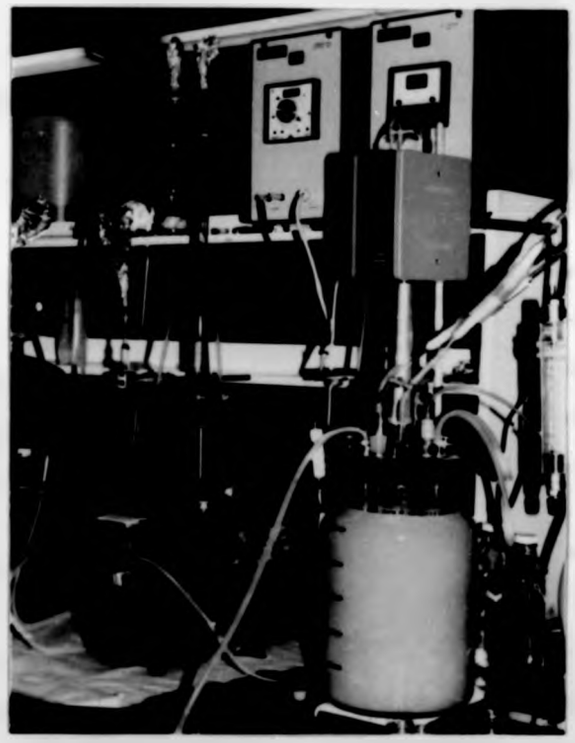


Fig. 3.1 Continuous chemostat cultivation of T. versutus on a 5.2 l pot. For details see 3.2.1.

day at a dilution rate of 0.06 h^{-1}. The effluent from the chemostat was collected in a flask maintained at 2°C and stored at 4°C until centrifuged.

3.2.2

Preparation of cell-free extract. Bacteria were sedimented by centrifuging and the cell pellet suspended to about 80 mg dry wt ml^{-1} in 55 mM phosphate buffer (pH 7.5). Organisms were disrupted by sonication for 5 min at 30 sec intervals in a 100 ml beaker immersed in ice using a 3/4" probe and maximum power output from a M.S.E. ultrasonic disintegrator. The treated cell suspension was centrifuged at 10,000 xg for 15 min and the supernatant centrifuged at 48,000 xg for 30 min to remove any unbroken cells and large membrane fragments. The resultant supernatant was designated the 'crude extract.' 'Membrane fraction' was prepared as described previously (Chapter 2).

3.2.3

Enzyme assays. Thiosulphate and sulphite-oxidizing activities were measured polarographically as described elsewhere (Chapter 2) except that 'membrane fraction' (0.5 mg protein) and various amounts of cytochrome c (horse heart III) were added as indicated. Thiosulphate:cytochrome c oxidoreductase, sulphite: cytochrome c oxidoreductase, rhodanese and succinate-oxidizing activity were assayed as stated elsewhere (Chapter 2). ^{35}S sulphate production from ^{35}S thiosulphate was assayed as described elsewhere (Kelly & Syrett, 1966).

3.2.4

Ammonium sulphate fractionation. Crude extract was fractionated at 4°C by the addition of serially increasing amounts of a saturated (4 M) ammonium sulphate solution. In each step the precipitated protein was centrifuged at 17,000 xg for 10 min, then dissolved in a small volume of phosphate buffer (25 mM, pH 6.5 or 55 mM, pH 7.5) and dialyzed against the same buffer for 5 h. The supernatant at each step was brought to a higher percentage saturation with saturated ammonium sulphate. The fraction precipitating between 44 - 60% of saturation with ammonium sulphate was called fraction A60%. A typical fractionation is shown below: 110g $(NH_4)_2SO_4$ was added into 550 ml crude extract containing 38g protein, which was then stirred for 15 min and centrifuged at 17 kg for 15 min. The pellet was discarded and the supernatant added with 11g $(NH_4)_2SO_4$ and treated as above to obtain second supernatant, to which a final 115g $(NH_4)_2SO_4$ was added and stirred and centrifuged as above. The pellet was collected and dissolved in phospha

3.2.5
DEAE - Sepharose CL-6B chromatography. The A60% fraction (25 ml, 1550 mg protein) was applied to a column (2.6 x 16.5 cm) of DEAE-Sepharose 6B, equilibrated at 2°C with 25 mM phosphate buffer, pH 6.5, containing 2 mM thiosulphate and eluted stepwise with a total of 1600 ml of the same buffer containing NaCl at 0M, 0.1M, 0.2M, 0.3M, 0.35M and 0.4M. Fractions (18 ml) collected at 2°C using a flow rate of 105 ml h^{-1} were monitored for protein, A_{280}, *c*-type cytochrome (by absorbance at 416 nm), and sulphite: cytochrome *c* oxidoreductase. Fractions containing distinct peaks of protein or cytochrome were combined and brought to 63% saturation with ammonium sulphate. The protein precipitated was collected by centrifugation and dissolved in a small volume of 55 mM phosphate buffer, pH 7.4.

Analytical procedures. Thiosulphate was estimated colorimetrically as described by Kelly *et al.* (1969).

Protein was measured according to the procedure of Lowry *et al.* (1951) using crystalline bovine serum albumin as standard.

Materials. Sepharose 4B, Sephacryl 200 superfine and DEAE-Sepharose CL-6B were from Pharmacia Fine Chemicals AB, Sweden. Cytochrome *c* (horse heart III) cytochrome oxidase (bovine heart), rhodanese (liver) and catalase(liver) were from Sigma (London) Ltd.

3.3 RESULTS AND DISCUSSION

3.3.1
Ammonium sulphate fractionation of the crude extract.

The thiosulphate- and sulphite-oxidizing activities were recovered in the fractions above 44% of saturation with ammonium sulphate (Table 11). The specific activity of thiosulphate oxidation was increased about fourfold in the A58% fraction compared with that in the crude extract, and total activity

Table 3.1 *Ammonium sulphate fractionation of the crude cell-free extract prepared by sonication of Thiobacillus A2*

Fraction*	Total protein (mg)	Thiosulphate-oxidizing activity (nmol O_2 (min x mg protein)$^{-1}$) Cytochrome *c* added (μM)				(nmol O_2) (min x total protein)$^{-1}$	Recovery (%)	Sulphite-oxidizing activity (nmol O_2 (min x mg protein)$^{-1}$) Cytochrome *c* added (μM)				Succinate-oxidizing activity (nmol O_2 (min x mg protein)$^{-1}$)
		0	40	70	100			0	70	100	140	
Crude extract	660	12.0	12.0			7920	100	1		2.5		25
A21%	105	0		0		-		0	2.5			51
A39%	192	0		0		-		0	2.6			41
A44%	60	2.5	6.8	9.1		546	7	0	4.2			1
A58%	140	4.4 (13)+	35.0 (34)+	47.0		6580	83	2.2 (4.1)+	12	14	16	0
A64%	10	0	8.5			85	1	-				-

*A21%, etc., refer to protein participated at 21% etc. of saturation with ammonium sulphate. Enzymes were assayed as described in Methods except that 10 mM Tris-HCl, pH 7.4 was used for assay of sulphite or succinate oxidation.

+Activities obtained when A21% or A39% fractions were added (1 mg protein ml^{-1}) instead of the *Thiobacillus* membrane fraction (see Methods)

recovered was about 91%. Fractions A21% and A39% contained cell membrane; because (a) these fractions contained nearly all the succinate-oxidizing activity, which was previously shown to be membrane-bound and (b) could also replace the requirement for the 'membrane fraction' for electron transfer to oxygen (Table 31). The rate of thiosulphate oxidation in fraction A58% was stimulated about ten-fold by the addition of horse-heart cytochrome *c*. This contrasted with the crude extract, to which addition of cytochrome *c* had no effect (Table 31). The stimulation could have been due to the loss of a certain amount of specific *c*-type cytochrome during the fractionation, even though the content of *c*-type cytochrome increased from about 1.4 nmol(mg protein)$^{-1}$ in the crude extract to about 2 nmol(mg protein)$^{-1}$ in fraction A58%. This possibility was supported by the fact that addition of fractions A21% and A39% to the reaction mixture instead of the membrane fraction (prepared as described previously; Chapter 2) showed higher thiosulphate-oxidizing activity in the absence of added horse-heart cytochrome *c*. The thiosulphate:cytochrome *c* oxidoreductase activity of fraction A60% showed a K_m value of about 200 μM for horse heart cytochrome *c*. The fact that a multiplicity of enzymes is involved in this system means, however, that this value might not be highly significant. The A60% fraction oxidized thiosulphate completely to sulphate. This was demonstrated by the consumption of two moles of oxygen for each thiosulphate oxidized and the demonstration, using thiosulphate labelled with ^{35}S in either atom, that both the sulphane- and sulphone-sulphur were oxidized to sulphate.

3.3.2
Gel filtration of the thiosulphate-oxidizing system precipitated by ammonium sulphate between 44 - 60% of saturation (A60%)

Passage of fraction A60% down a column of Sepharose CL-4B or Sephacryl-300 superfine yielded similar elution patterns with one main peak of protein (A_{280}) with which a peak of *c*-type cytochrome coincided. The peak of thiosulphate:cytochrome *c* oxidoreductase activity, however, eluted in fractions slightly behind this main peak. These results indicate that little

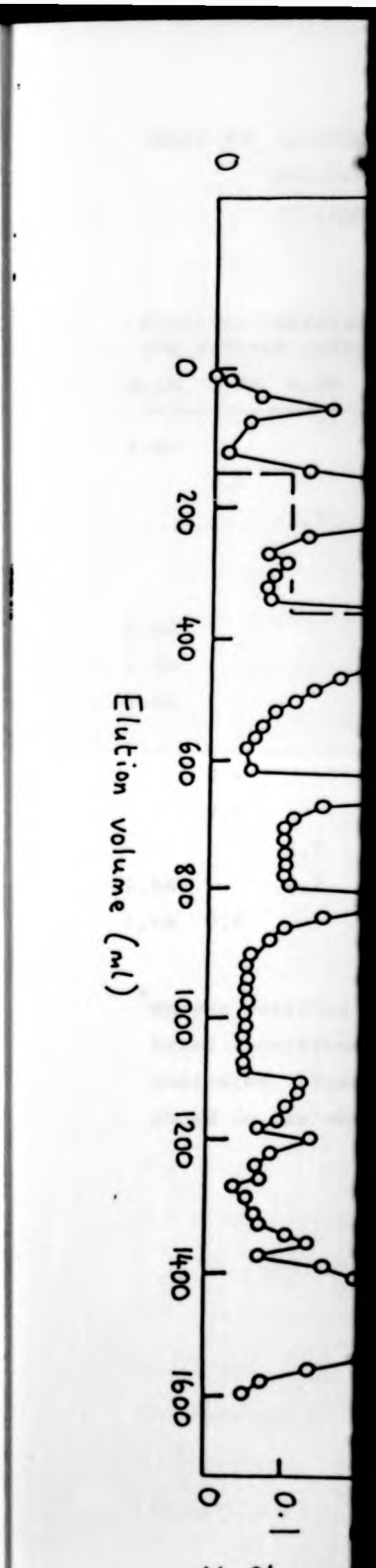
resolution of the complex mixture of enzymes necessary for thiosulphate oxidation by the A60% fraction had been achieved, although the complex was partly resolved from the bulk protein of the preparation.

3.3.3.
Resolution of the thiosulphate-oxidizing system into three main fractions by DEAE-Sepharose CL-6B chromatography.

Chromatography of fraction A60% on a column of DEAE-Sepharose CL-6B yielded seven distinct peaks with absorbance at 280 nm or 460 nm (Fig. 32) The first and last of these (0 M and 0.4 M NaCl) contained little protein when analysed by the Lowry procedure and were not further studied. The 0.1 M NaCl fraction contained no detectable cytochrome, but *c*-type cytochrome (A_{416nm} and characteristic 550 to 552 nm absorbance) was present in the other four peak fractions. The fractions with 0.1 M and 0.2 M NaCl had a greenish colour. The 0.35 M NaCl eluate was resolved into two parts: the first (0.35 M-I) contained all the sulphite:cytochrome *c* oxidoreductase activity and the second (0.35 M-II) mainly consisted of *c*-type cytochrome.

None of the fractions alone showed thiosulphate:cytochrome *c* oxidoreductase activity (Table 32) This activity was, however, recovered when the 0.1 M and 0.35 M-I NaCl fractions were mixed. The 0.2 M NaCl could replace the 0.1 M NaCl fraction, but gave lower activity. Activity of the 0.35 M-I fraction with either the 0.1 M or 0.2 M NaCl fraction was stimulated about five-fold by the addition of the 0.35 M-II NaCl fraction. Boiling the 0.35 M-II fraction destroyed the stimulatory activity. These results demonstrate that a number of components were necessary for the expression of thiosulphate:cytochrome *c* oxidoreductase activity. Thiosulphate-oxidizing activity could also be measured polarographically using reconstituted mixtures of these fractions. Oxygen uptake was observed when a mixture of the 0.1 M, 0.35 M-I and 0.35 M-II fractions were supplied with thiosulphate in the presence of horse heart cytochrome *c* and either bovine cytochrome oxidase or *Thiobacillus* A2 'membrane fraction'. Oxygen was consumed at about 9 nmol min^{-1} (mg protein)$^{-1}$, calculated only for

Fig. 32. Fractionation of the A60% fraction from *Thiobacillus* A2 on DEAE-Sepharose CL-6B. The chromatography was performed as described in the Methods with stepwise elution with increasing concentration of NaCl in Tris-HCl buffer. Sodium chloride concentration is indicated (— — —) for each step. Fractions were assayed for absorbance at 280 nm (○), absorbance at 416 nm (●) and sulphite:cytochrome *c* oxidoreductase (□).



n from *Thiobacillus* A2 on DEAE-
performed as described in the Methods
centration of NaCl in Tris-HCl
indicated (— — —) for each step.
280 nm (○), absorbance at 416 nm
ctase (□).

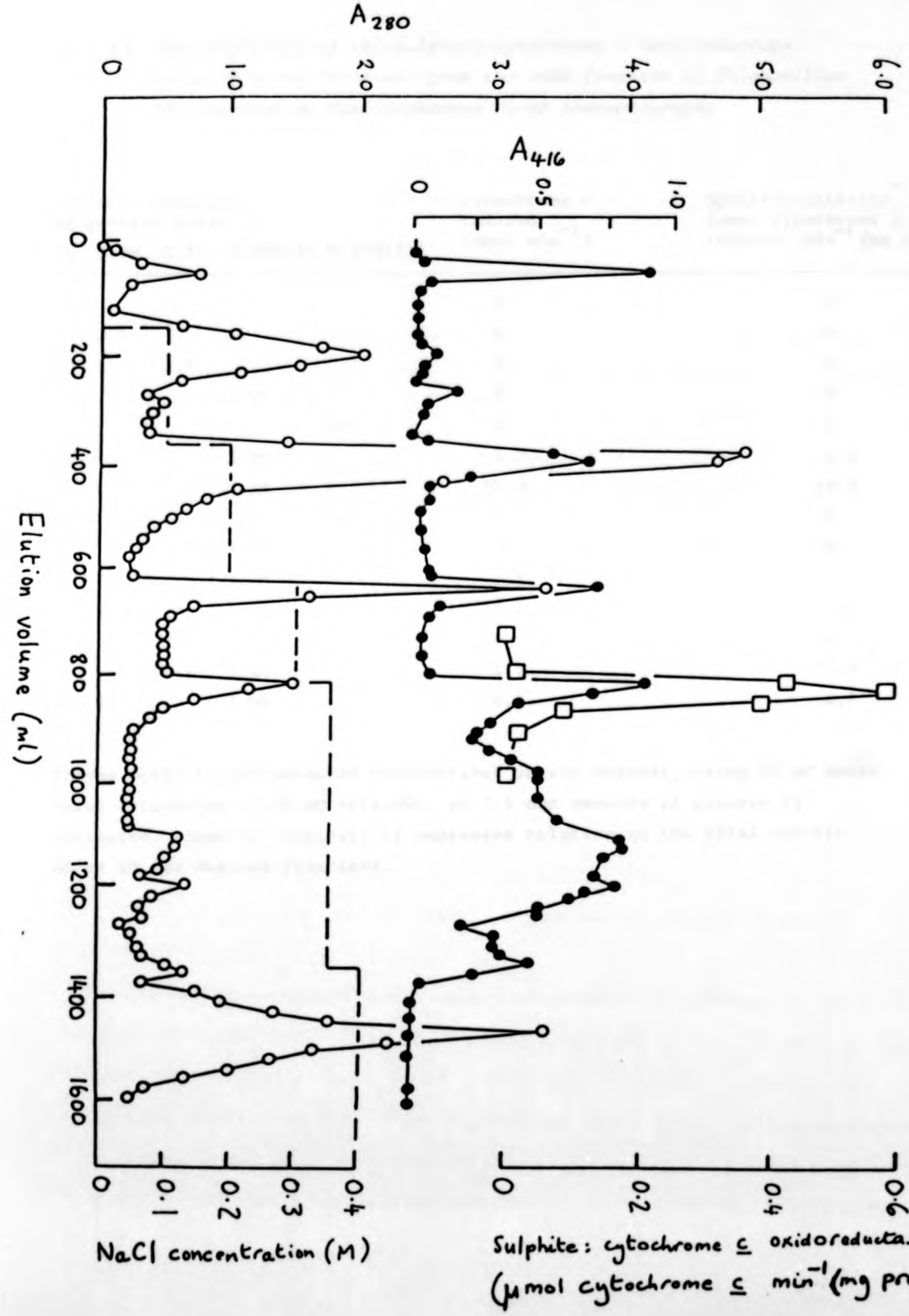


Table 3.2 *Reconstitution of thiosulphate:cytochrome c oxidoreductase activity using fractions from the A60% fraction of Thiobacillus A2 separated by DEAE-Sepharose CL-6B chromatography*

Fractions assayed (mg protein assay^{-1})					Cytochrome *c* reduced (nmol min^{-1})	Specific activity* (nmol cytochrome *c* reduced min^{-1} (mg protein)$^{-1}$
0.1M	0.2M	0.3M	0.35M(I)	0.35M(II)		
1.64					0	0
	2.4				0	0
		2.7			0	0
			1.04		0	0
				0.6	0	0
1.64			1.04		5.3	2.0
1.64			1.04	0.3	25.0	10.0
1.64				0.3	0	0
			1.04	0.3	0	0
	2.4		1.04		1.8	0.5
	2.4		1.04	0.3	13.0	5.8
		2.7	1.04		0	0
1.64		2.7	1.04		4.5	0.8
1.64	2.4		1.04		5.3	0.7

*Enzyme activity was measured as described in the Methods, using 70 μM horse heart cytochrome *c*, 90 mM Tris-HCl, pH 7.4 and amounts of protein as indicated. Specific activity is expressed relative to the total protein added in the various fractions.

the protein added in the three NaCl fractions. This oxidation rate would require cytochrome *c* reduction and reoxidation at a rate of 36 nmol min^{-1} $(mg\ protein)^{-1}$, or rather more than was observed spectrophotometrically. . The reaction mixture contained only 70 μM cytochrome *c* (or only one-third of that for the K_m value), so oxidation could have been stimulated when complete electron transfer to oxygen was possible.

3.3.4
Separation of two cytochrome c components.

Two distinct cytochrome *c* spectra were observed in the different NaCl fractions (Table 3.3). One was that of the predominant cytochrome c_{552}, which was present largely in the oxidized form in fractions 0.35 M-I and 0.35 M-II, and was rapidly reduced by sulphite when the 0.35 M-I fraction was present. The other was cytochrome c_{550} which was mainly present in the 0.2 M NaCl fraction. This was always recovered in the reduced form, presumably because of the presence of thiosulphate as an enzyme protector in the eluate. The existence of cytochrome c_{550} in the crude extract and A 60% fraction was masked by the intense absorbance of cytochrome c_{552}. Two types of cytochrome *c* (550 and 551 nm maxima) were also purified from *T. novellus* (Yamanaka *et al.* 1971, 1981).

3.3.5
Distribution of enzyme activities among the fractions from DEAE-Sepharose CL-6B chromatography.

Thiosulphate:cytochrome *c* oxidoreductase activity in mixtures of the 0.35 M-I, 0.35 M-II and either the 0.1 M or 0.2 M NaCl fractions always showed a lag and acceleration phase of about two minutes if the 0.1 M or 0.2 M NaCl fraction was the component added to initiate the assay. This implied that these two fractions contained an enzyme or enzymes required for the initial attack on thiosulphate, and that this activity was initially rate-limiting. It is likely

Table 3.3 *Separation of two types of cytochrome c during DEAE-Sepharose CL-6B chromatography of the A60% fraction from Thiobacillus A2*

Fraction Assayed	Absorption maxima in fractions (nm)*			Concentration+ (nmol mg protein^{-1})
	α	β	γ	
OM NaCl	550		415(412)	N.D.
0.1M NaCl				
0.2M NaCl	550	522	416	0.4
0.3M NaCl			417(412)	N.D.
0.35M NaCl (I)	552	522	418(410)	3.0
0.35M NaCl (II)	552	522	418(410)	9.0

*Reduced cytochrome absorbance maxima were determined after reduction with dithionite. Oxidized peaks for the γ bands are given in parenthesis.

+Cytochrome *c* concentration in the fractions was calculated as the reduced *vs* oxidized absorbance using a molar extinction coefficient of 28, and expressed relative to the total protein content of the fraction.

(Kelly, 1982) that this initial step is the cleavage of thiosulphate to sulphite and a sulphane-sulphur moiety. Enzymes implicated as possible cleavage enzymes include rhodanese (Silver & Kelly, 1976; Kelly, 1982).

Rhodanese was present at high activity in the 0.1 M NaCl fraction and to a lesser extent in the 0.2 M fraction (Table 3.4). Addition of liver rhodanese instead of the 0.1 M NaCl fraction in the assay mixture with the 0.35 M I and II fractions did not, however, give any thiosulphate:cytochrome c oxidoreductase activity. Pure catalase also exhibited rhodanese activity (Table 3.4), but it could not replace the 0.1 M NaCl fraction either. The cleaving enzyme in this fraction is thus either not rhodanese, or the activity requires enzymes or cofactors other than rhodanese which are provided by the 0.1 M NaCl fraction. The location of the enzyme or enzymes catalysing the oxidation of sulphane-sulphur to sulphite has not yet been established, but the complete absence of absorbance in the 590 nm region in the 0.1 M and 0.2 M NaCl fractions suggests that they did not contain a sirohaem sulphite reductase of the kind found in T. denitrificans, that might in vivo act as a sulphane-oxidase (Schedel & Trüper, 1979; Kelly, 1982).

The specific activity of sulphite:cytochrome c oxidoreductase was increased about tenfold after chromatography and was recovered in the 0.35 M I fraction (Table 3.5). Activity was further enhanced by addition of the 0.35 M II fraction (Table 3.5). The latter stimulation may reflect improved catalysis of the reaction by the additional Thiobacillus cytochrome c.

3.4 CONCLUSIONS

This is the first time that a cell-free thiosulphate-oxidizing system from a thiobacillus has been resolved into distinct fractions and then reconstituted from those fractions. The fractionation so far achieved is summarized in Fig. 3.3. These fractions are, however, still relatively crude and the total number and nature of the enzymes involved is not yet known. The system catalyzing the oxidation of sulphane-sulphur to sulphite has clearly survived the fractionation procedure so far, and it is hoped that further purification of the 0.1 M and 0.2 M NaCl fractions from DEAE-Sepharose

Table 3.4 *Rhodanese activity in the fractions from DEAE-Sepharose CL-6B chromatography of the A60% fraction*

Fraction assayed	Thiocyanate formed (nmol (min x mg protein)$^{-1}$)
A60%	270
0.1M NaCl	420
0.2M NaCl	130
0.3M NaCl	100
0.35M NaCl (I)	0
0.35M NaCl (II)	0
Catalase (bovine liver)	1500
Rhodanese (bovine liver)	1600

Rhodanese was assayed as described in the Methods

Table 15 *Separation of sulphite:cytochrome c oxidoreductase during chromatography of the A60% fraction on DEAE-Sepharose CL-6B*

Fraction assayed	Protein in assay (mg)	Specific activity (nmol cytochrome c_{red} (min x mg protein)$^{-1}$)
A60%	0.30	110
0.2M NaCl	0.24	4
0.3M NaCl	1.65	2
0.35M NaCl (I)	0.10	1200
0.35M NaCl (II)	0.15	7
I + II	0.1 + 0.05	1500

Experimental conditions were as described in the Methods and in Fig. 2, except that 35 µM horse heart cytochrome *c*, 90 mM Tris-HCl buffer, pH 7.4, and the indicated amounts of protein were used in the assay.

Table 35 *Separation of sulphite:cytochrome c oxidoreductase during chromatography of the A60% fraction on DEAE-Sepharose CL-6B*

Fraction assayed	Protein in assay (mg)	Specific activity (nmol cytochrome c reduced (min x mg protein)$^{-1}$)
A60%	0.30	110
0.2M NaCl	0.24	4
0.3M NaCl	1.65	2
0.35M NaCl (I)	0.10	1200
0.35M NaCl (II)	0.15	7
I + II	0.1 + 0.05	1500

Experimental conditions were as described in the Methods and in Fig. 2, except that 35 µM horse heart cytochrome c, 90 mM Tris-HCl buffer, pH 7.4, and the indicated amounts of protein were used in the assay.

Fig. 3.3 Summary of the fractionation procedure employed with the cell-free thiosulphate-oxidizing system from *Thiobacillus* A2

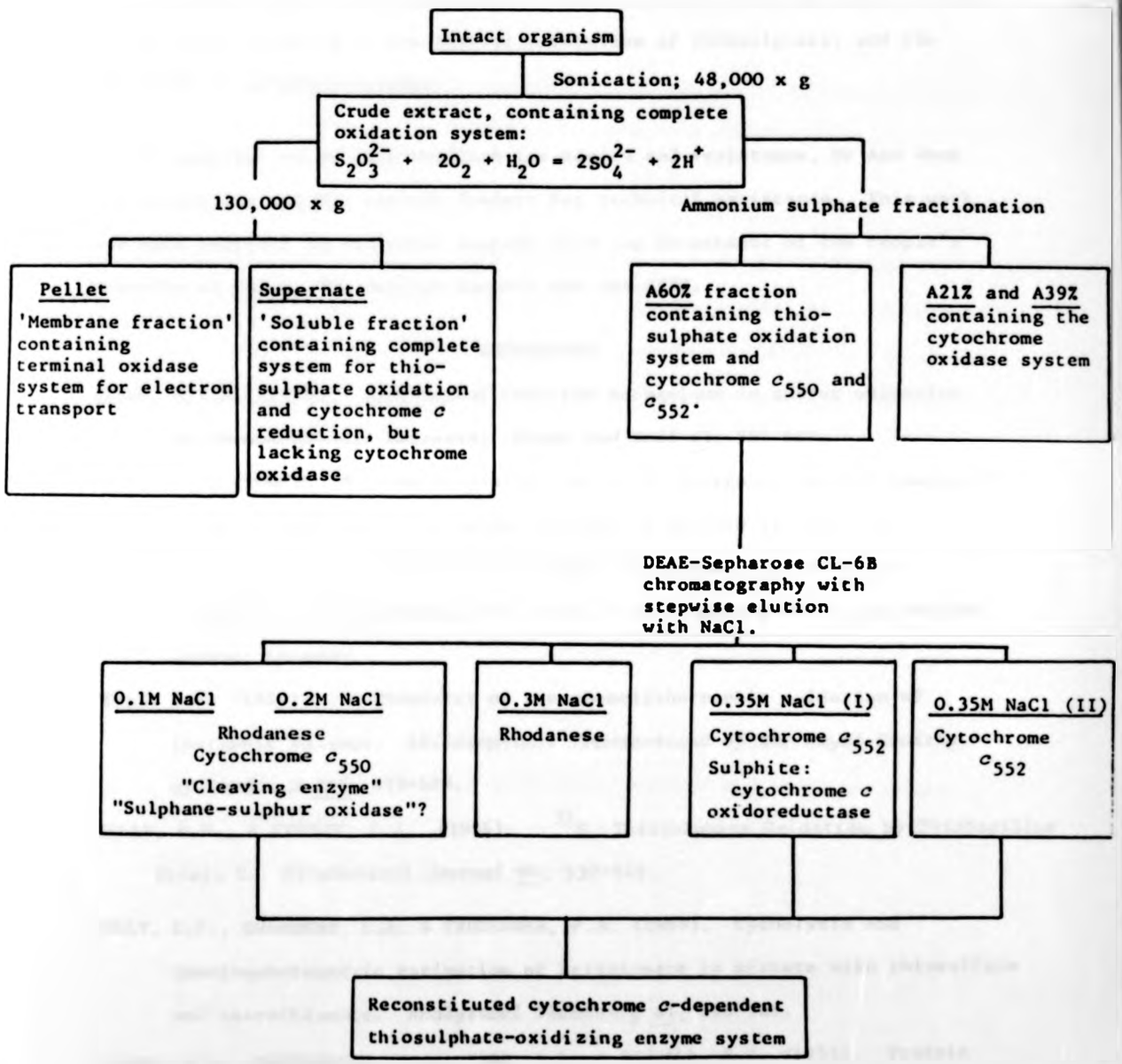
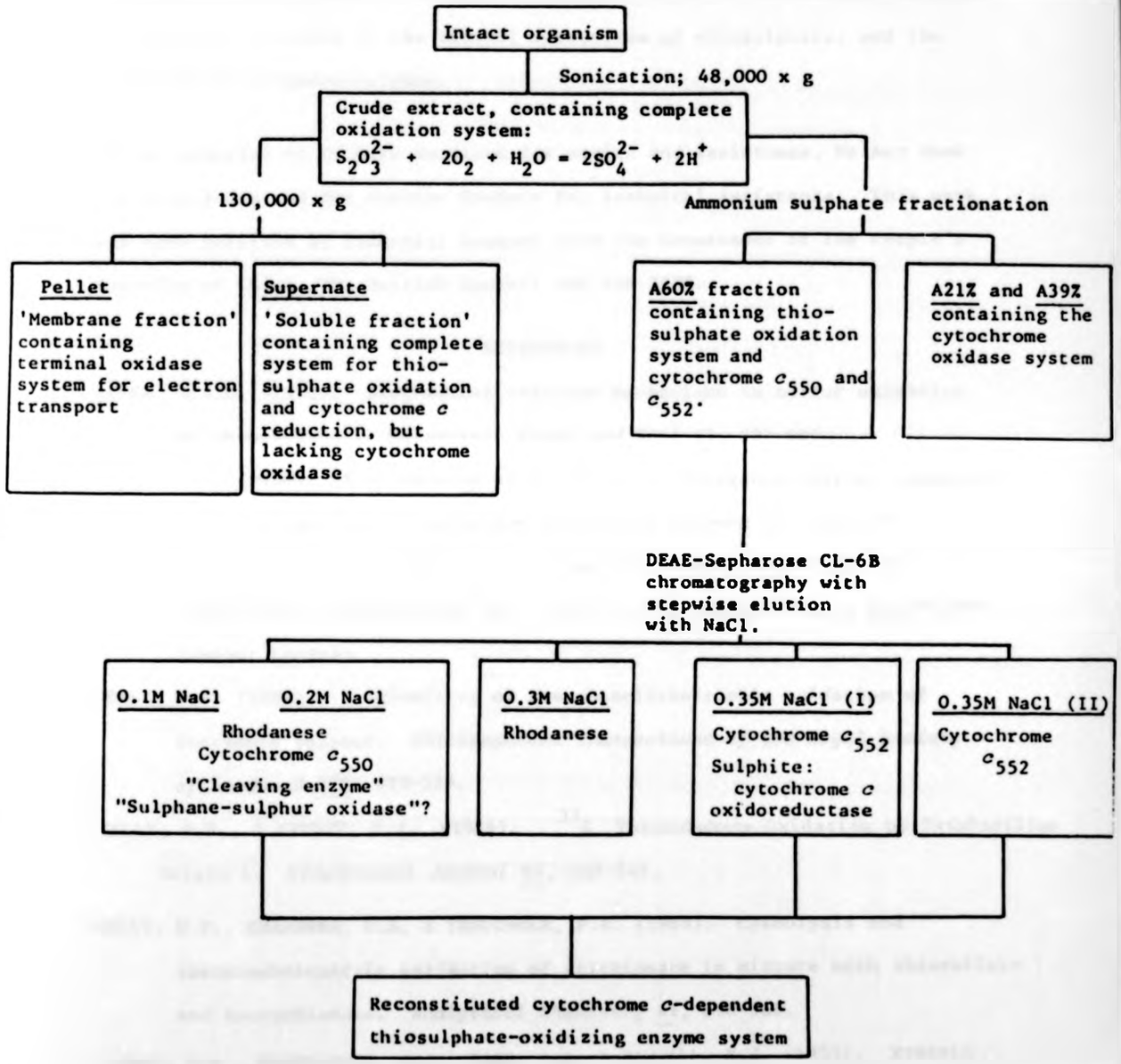


Fig. 3.3 Summary of the fractionation procedure employed with the cell-free thiosulphate-oxidizing system from *Thiobacillus* A2



chromatography may enable unequivocal identification of the enzymes and kind of reactions involved in the initial metabolism of thiosulphate, and the oxidation of sulphane-sulphur.

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3.6 APPENDIX

3.6.1 Choice of experimental conditions for the ion exchange Chromotography of A65% fraction on DEAE-Sepharose CL-6B

The reasons for using DEAE-Sepharose CL-6B as the ion exchanger matrix were simply that most proteins had pI values of less than 7, though we initially knew nothing about the enzymes in this system.

The test-tube method essentially as described by Pharmacia's booklet "Ion exchange chromatography" (1982) was used to select starting pH and buffer. Two ml of the gel and four mg protein of A65% fraction were used for each tube. A range of pH from 5 to 9 with 0.5 pH intervals in 0.1M Tris buffer or 55 mM phosphate buffer were tried. It was found that nearly all of the proteins were bound onto the gel and were difficult to elute with buffer containing NaCl (0.1-0.3M) in the tubes with pH at and over 7. While in the tubes with pH at and below 6.5 some proteins were in the supernatant of the start buffers and the proteins bound on the gels could be eluted out stepwise with the buffers containing 0.1 to 0.5M NaCl which meant that the A65% fraction could be fractioned in these conditions. Since pH less than 6.5 would probably adversely affect the enzyme activity, pH 6.5 was chosen. Obviously phosphate buffer was superior to Tris buffer in this pH range and was used in the chromatography.

Next, a similar test-tube method for selecting starting and eluting ionic strengths of the buffer was done. It was found that proteins and c-type cytochromes (indicative of red colour) absorbed on the gel in phosphate buffer, pH 6.5 were eluted out stepwise with the same buffer containing 0.1, 0.2, 0.3 and 0.35M NaCl and $S_2O_3^{2-}$: cytochrome c oxidoreductase activity appeared.

Finally, a column chromatography was conducted and by trial and error, the final chromatography condition was found as described at 3.2.5.

Regeneration of the ion exchanger was done as introduced by the booklet "Ion exchange chromatography."

3.6.2 A large scale and modified chromatography of A65% fraction on DEAE-Sepharose CL-6B

Fig.34 shows a typical elution pattern of the large scale and modified chromatography of A65% fraction on DEAE-Sepharose CL-6B. The A65% fraction (250 ml, 12g protein) was applied to a column (4.1 cm x 22 cm) of DEAE-Sepharose CL-6B, equilibrated at 4°C with 25 mM phosphate buffer, pH 6.5, containing 2 mM thiosulphate and eluted stepwise with a total of 3.5 ℓ of the same buffer containing NaCl at OM (460 ml); 0.1M (530 ml), 0.12M (270 ml), 0.2M (350 ml), 0.3M (350 ml) and 0.35M (1500 ml) at a flowrate of about 140 ml h^{-1}. Fractions (20 ml) collected at 4°C were monitored for protein, A_{280} c-type chromosomes (A_{416}), enzyme A (see 4.2.2) and sulphite:cytochrome c oxidoreductase. Fractions containing distinct peaks of protein or cytochrome or enzyme activities were combined as shown in the figure and brought to 65% saturation with $(NH_4)_2SO_4$. The protein precipitated was collected by centrifugation and dissolved in a small volume of 50 mM Tris-buffer, pH 7.2.

Fig. 3.5 shows a photograph of the chromatography.

Fig. 3.4 Elution pattern of the A65% fraction on DEAE-Sepharose CL-6B.

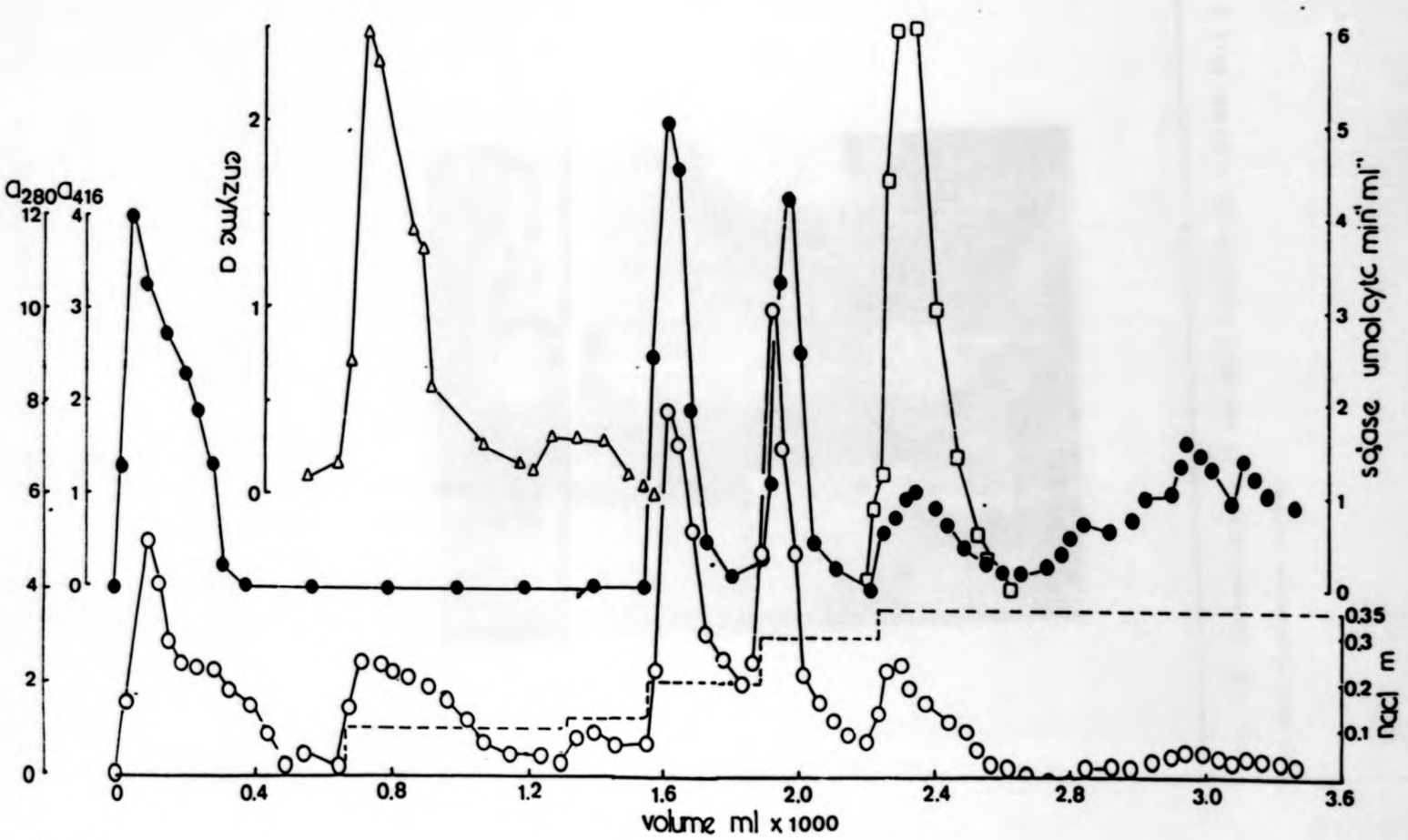

fig 3.4

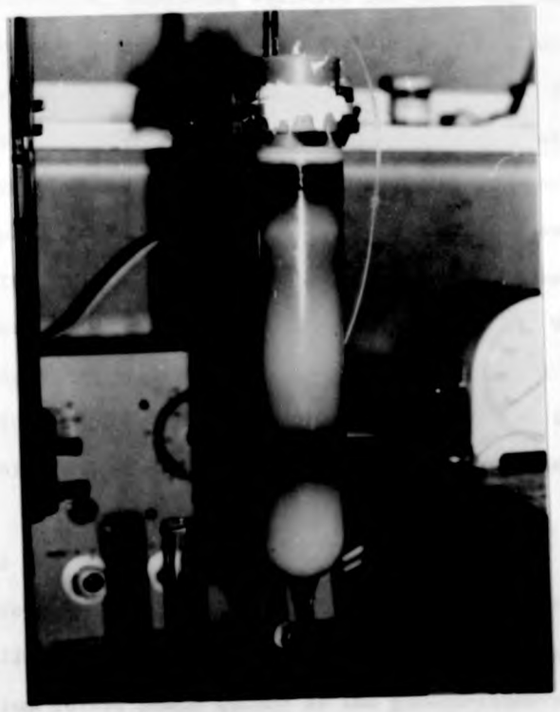

Fig. 3.5 Chromatography of the A65Z fraction on a column (4.1 x 22 cm) of DEAE-Sepharose CL-6B.

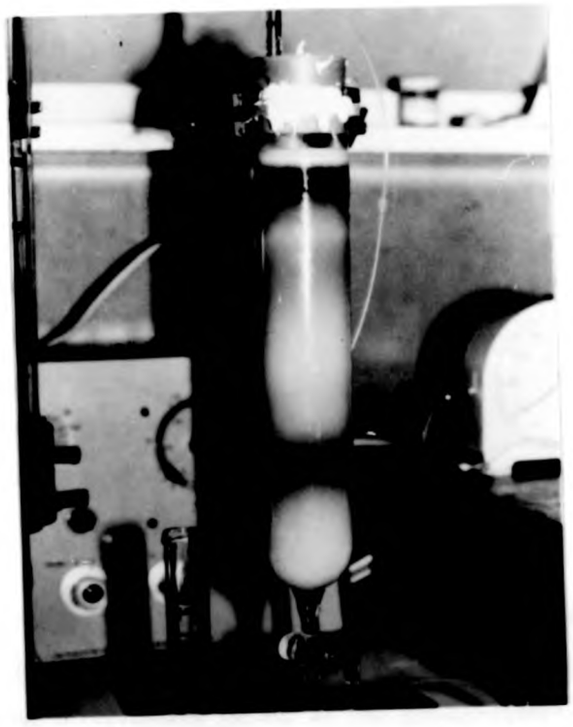

Fig. 3.5 Chromatography of the A65% fraction on a column (4.1 x 22 cm) of DEAE-Sepharose CL-6B.

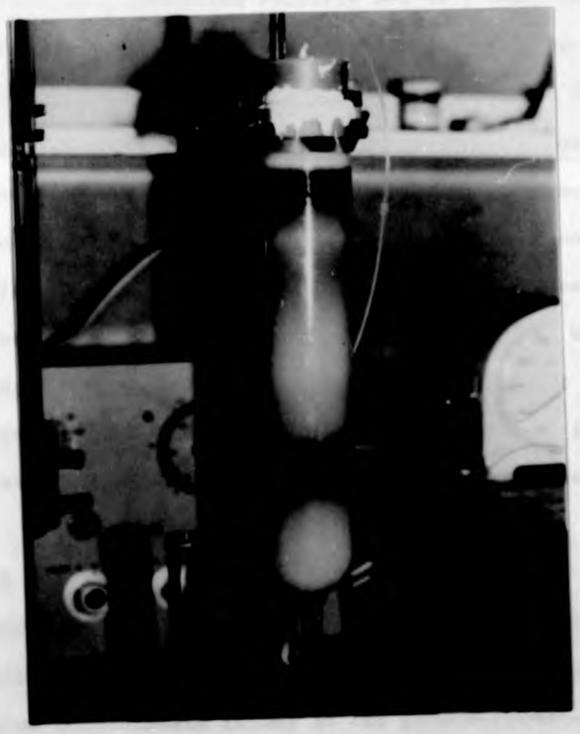

Fig. 3.5 Chromatography of the A65Z fraction on a column (4.1 x 22 cm) of DEAE-Sepharose CL-6B.

CHAPTER 4

RHODANESE: AN ENZYME NOT NECESSARY FOR THIOSULPHATE OXIDATION BY THIOBACILLUS A2

4.1 INTRODUCTION

The oxidation of thiosulphate to sulphate can provide all the energy required for autotrophic growth of the *Thiobacillus* and *Thiomicrospira* species [1]. The first step in thiosulphate oxidation is its enzymatic cleavage to sulphite and a sulphane-sulphur residue [1]. Thiosulphate reductase, producing sulphite and sulphide, has been shown able to fill this role [2]. More generally, rhodanese has come to be regarded as the most likely cleaving enzyme [1,3-7]. Rhodanese cleaves thiosulphate to produce sulphite and thiocyanate or lipoate persulphide with either cyanide or reduced α-lipoic acid as sulphane-acceptor molecules [5,8-11]. Rhodanese appears to be present in all the thiobacilli, often at high specific activity, but its presence is not restricted to organisms grown on thiosulphate [4,12]. We now present evidence showing that in *Thiobacillus* A2, rhodanese is not the cleaving enzyme initiating thiosulphate metabolism and has no role in the complete oxidation of thiosulphate to sulphate by cell-free preparations.

4.2 MATERIALS AND METHODS

4.2.1 *Preparation and fractionation of the thiosulphate-oxidising system.*

Thiobacillus A2 was grown autotrophically in continuous chemostat culture on sodium thiosulphate (0.2M) as the growth-limiting nutrient (Chapter 2). The 48,000 x g crude extract (following sonication) and the protein fraction precipitating between 44-65% saturation with $(NH_4)_2SO_4$

(A65%) were prepared at 4°C as described previously (Chapter 3).

The A65% contained a thiosulphate-oxidizing system effecting the stoichiometric oxidation of thiosulphate to sulphate with the concomitant reduction of cytochrome *c* . This system was fractionated into three major components by chromatography on DEAE-Sepharose-CL6B with elution using a series of concentrations of NaCl (see 3.3). Reconstitution of the system previously required two fractions eluted with 0.35M NaCl (0.35M-I and 0.35M-II) and a component eluted with 0.1M or 0.2M NaCl. The component eluted with 0.1M or 0.2M NaCl will now be referred to as "Enzyme A". In the present study the A65% fraction (12g protein) was fractionated essentially as before using a column 4.1 cm x 22 cm, eluted with about 4ℓ NaCl solutions at 160 ml h^{-1}. Following elution with 0.1M NaCl, elution with 0.12M NaCl was carried out before applying 0.2M NaCl. This resulted in removal of all activity for "Enzyme A" into the 0.1 + 0.12M NaCl fractions (see 3.6.2 for details).

"Enzyme A" was further purified by a second ammonium sulphate precipitation. The combined 0.1 + 0.12M NaCl eluates were brought to 60% of saturation with solid $(NH_4)_2SO_4$, centrifuged at 17,000 x g for 15 min, and the precipitated protein recovered as "second A60% fraction". The supernatant solution was brought to 90% saturation with additional solid $(NH_4)_2SO_4$ and the precipitated protein collected by centrifugation as above and recovered as "second A90% fraction". All procedures were performed at 4°C. Protein precipitates were redissolved in 25mM phosphate, pH 6.5, to a final concentration of about 30 mg ml^{-1} and stored at -20°C.

..2.2 *Assay of "Enzyme A"*

This was based on the assay of thiosulphate: cytochrome *c* oxidoreductase previously described (2.2.6) The reaction mixture (1 ml in a 1 cm light-path cuvette) contained (μmol): $Na_2S_2O_3$, 2; phosphate buffer, pH 7.5, 50; cytochrome *c* (horse heart type III), 0.07;

(A65%) were prepared at 4°C as described previously (Chapter 3).

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0.35M-I fraction (0.9 mg protein); 0.35M-II fraction (0.6 mg protein); and "Enzyme A" solution (0.1 - 0.3 mg protein). Reaction at 30°C was normally initiated by adding the "Enzyme A" solution. Activity was expressed as cytochrome *c* reduction relative to the protein added as "Enzyme A", rather than relating to total protein in the assay.

2.3 *Assay of rhodanese*

Rhodanese was assayed spectrophotometrically essentially as described by Smith and Lascelles [14]. The reaction mixture (1 ml in a 1 cm cuvette) contained (μmol): $Na_2S_2O_3$, 50; Tris-HCl buffer, pH 7.5; 30; NaCN, 50; 2,6-dichlorophenol-indophenol (DCPIP), 0.09; N-methylphenzonium methosulphate (PMS), 0.1 mg; and enzyme solution as indicated, 0.2 - 0.4 mg protein. The reference cuvette contained the complete reaction mixture without thiosulphate and DCPIP. The reaction was initiated with the cyanide and DCPIP reduction recorded at 605 nm using a Pye-Unicam SP1700 spectrophotometer. A millimolar extinction coefficient of 16.4 at 605 nm was used for DCPIP. Rhodanese specific activity was expressed as nmol DCPIP reduced min^{-1} $(mg\ protein)^{-1}$.

2.4 *Chemicals*

DEAE-Sepharose-CL6B was from Pharmacia Fine Chemicals AB, Sweden; cytochrome *c* (horse heart III), catalase (liver) and N-methylphenazonium methosulphate were from Sigma (London) Ltd; DCPIP was purchased from British Drug Houses, Poole, England.

4.3. RESULTS

4.3.1 *Distribution of rhodanese activity from Thiobacillus A2 among the fractions from DEAE-Sepharose-CL6B chromatography and the second ammonium sulphate fractionation*

Most of the rhodanese activity in the A65% fraction was eluted from the column using 0.3 M NaCl (Table 41). The remainder was contained in the OM and 0.1 + 0.12M NaCl eluates (Table 41). The activity

Table 1. Separation of rhodanese during fractionation of cell-free extracts of *Thiobacillus* A2 by DEAE-Sepharose CL6B chromatography and further ammonium sulphate precipitation

Fraction assayed[a]	Total protein (mg)	Rhodanese activity	
		Total recovered (nmol DCPIP reduced min^{-1})	Specific activity (units(mg protein)$^{-1}$)
Crude extract	-	-	34
44-65% $(NH_4)_2SO_4$ (A65% fraction)	12000	660,000	55
DEAE-Sepharose fractions:			
0 M NaCl	780	134,000	172
0.1+0.12M NaCl	2190	52,500	24
0.2M NaCl	1120	3,300	3
0.3M NaCl	2460	524,000	213
0.35M NaCl (I)	1155	0	0
0.35M NaCl (II)	890	0	0
2nd $(NH_4)_2SO_4$ fractionation of 0.1M NaCl components:			
A60% precipitate	-	-	36
A90% precipitate	-	-	0
Catalase (bovine)	-	-	548

[a] see Materials and Methods for details

present in the 0.1 M NaCl fraction was completely precipitated by $(NH_4)_2SO_4$ at 60% of saturation with none being present in the A90% fraction (Table 4.1). For comparison, commercial catalase was also shown to exhibit high rhodanese activity in this assay procedure (Table 4.1) as well as in the standard procedure in which thiocyanate formation is measured (Chapter 3).

Reconstitution of the complete thiosulphate-oxidizing system required components in all three of the 0.1M (or 0.2M), 0.35M-I and 0.35M-II NaCl fractions (3.3.3). Rhodanese activity was absent from the 0.35M-I and 0.35M-II fractions, so if the activity attributed to "Enzyme A" in the 0.1 M fraction were actually due to rhodanese, further purification of rhodanese should increase "Enzyme A" activity in parallel. In fact 94% of the "Enzyme A" activity was recovered in the protein precipitated between 60-90% saturation with $(NH_4)_2SO_4$ of the 0.1 + 0.12M NaCl fractions, whereas all the rhodanese activity was in the 60% saturated $(NH_4)_2SO_4$ precipitate (Table 4.2). Consequently, "Enzyme A" and rhodanese are separate and unrelated enzymes.

4.4 DISCUSSION

This demonstration that the cell-free thiosulphate-oxidizing system from Thiobacillus A2 does not require rhodanese for its activity shows that an as yet unidentified enzyme effects thiosulphate cleavage and that rhodanese plays no part in thiosulphate oxidation. The physiological role of rhodanese is unknown, as is also true for rhodanese in other bacteria. Indeed "rhodanese" appears not to be a single enzyme in Thiobacillus A2 as it occurred in three distinct subcellular fractions (Table 4.1) and is a function also exhibited by unrelated enzymes such as catalase. The non-involvement of rhodanese in thiosulphate oxidations is consistent with its constant specific activity in chemostat cultures exhibiting a threefold range of specific rate of thiosulphate oxidation (2.3.1). The complete oxidation mechanism also involves c-type cytochromes and enzymes for the oxidation of sulphite to sulphate and the sulphane-sulphur residue of

Table 4.2 Distribution of "Enzyme A" after ammonium sulphate fractionation of 0.1 and 0.12M NaCl fractions from DEAE-Sepharose CL-6B chromatography

Fraction assayed [a]	Total protein (mg)	"Enzyme A"[a] Total activity (μmol cytochrome c reduced min^{-1})	"Enzyme A"[a] Specific activity (μmol cytochrome c reduced min^{-1} (mg protein)$^{-1}$)
0.1 + 0.12M NaCl fractions[b]	2190	541	0.247
"Second A60%"	1240	30	0.024
"Second A90%"[b]	940	511	0.540

[a] see Materials and Methods for details of procedures.

[b] Supplementing these assay mixtures with "membrane fraction" prepared as previously described, resulted in complete oxidation of thiosulphate to sulphate, when assayed by the oxygen electrode procedure (see 2.3.4).

thiosulphate to sulphite [1]. Sulphite:cytochrome c oxidoreductase was located in the 0.35M-I fraction (see 3.3.3). "Enzyme A" from the 0.1 + 0.12 NaCl fraction and a second essential enzyme component from the 0.35M-I fraction are currently being purified and characterized. The absence of a requirement for any reduced cofactors for this thiosulphate-oxidizing system also indicates that the enzyme for thiosulphate cleavage is not a reductant-requiring thiosulphate reductase of the kind shown by Peck [2], or if it is, it is fed with electrons recycled internally from subsequent sulphane or sulphite oxidation. This possibility is thermodynamically unlikely. Moreover, a role for reduced lipoate as a sulphane-sulphur acceptor is unlikely both thermodynamically [1] and through the lack of role for rhodanese.

The role of rhodanese in the metabolism- of thiosulphate was also suspected by other observations. The activity of this enzyme in Chromatium was about the same in organisms grown on thiosulphate, succinate or pyruvate and was only about twice that of the Athiorhodaceae, Rhodospirillum rubrum and Rhodopseudomonas spheroides which are unable to utilize thiosulphate [15]. Giani [16] and Bowen et al. [17] showed with Clorobium vinosum and T. denitrificans respectively that the enzyme activity may be found in a variety of fractions and different molecular weights.

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CHAPTER 5

PURIFICATION AND SOME PROPERTIES OF TWO PRINCIPAL ENZYMES OF THE THIOSULPHATE-OXIDIZING MULTIENZYME SYSTEM

5.1 INTRODUCTION

The preparation of a cell-free extract from Thiobacillus A2, capable of the complete oxidation of thiosulphate to sulphate with the consumption of two moles of oxygen for each thiosulphate oxidized, was described previously (Chapter 2). Complete oxidation of thiosulphate required both a membrane system and a soluble fraction. The soluble fraction coupling thiosulphate oxidation to cytochrome c reduction was separated into three major components (Chapter 3). The earlier work indicated the thiosulphate: cytochrome c oxidoreduction process to be effected by a soluble multi-enzyme system. Subsequently, the rhodanese present in the system was shown not to be required for thiosulphate oxidation. Further analysis of this multi-enzyme system was undertaken with an initial objective of establishing the nature of the thiosulphate-cleaving enzyme, believed from earlier work to be a primary step in thiosulphate oxidation, (Suzuki, 1974; Kelly, 1982) and to seek the presence of a 'sulphane-sulphur oxidase' in addition to the sulphite oxidase already demonstrated.

The present one describes the purification and some properties of two principal enzymes from the enzyme system and the reconstitution of the thiosulphate-oxidising activity with the two enzymes, two partially purified c-type cytochromes, mammalian cytochrome c and cytochrome oxidase. The involvement of thiosulphate cleavage and of sulphite oxidase in thiosulphate oxidation is discussed.

5.2 METHODS

5.2.1 Organisms and chemostat culture. Thiobacillus A2, which has recently been designated as a new species, T. versutus (Harrison, 1983), was grown in continuous culture as described previously (Chapters 2, 3). Cell suspensions (80-100 mg dry wt ml^{-1}) collected and concentrated from the culture were stored at -70°C. This storage had no effect on the thiosulphate-oxidising activity of crude extracts prepared from the frozen cells.

Preparation of cell-free extract and A65Z fraction and resolution of the A65Z fraction into three main fractions involved in thiosulphate oxidation.

These procedures were as described previously (Chapter 3) and are summarised in Fig.5.1. The c-type cytochromes in the 0.35M NaCl(I) and (II) fractions were found to have α bands at 551 and 552.5 nm respectively rather than at 552 nm, as was previously thought (Chapter 3). The previous observation was probably due to incomplete separation of the two cytochromes from each other.

Resolution of the A65Z fraction

Enzymes A and B, sulphite:cytochrome c oxidoreductase and cytochrome c_{551} were resolved as summarized in Fig.5.1 .

5.2.2.
Enzyme assays

Thiosulphate:cytochrome c oxidoreductase activity could only be observed using mixtures of the main fractions or of the further purified enzymes (Fig.5.1 ; Chapters 3, 4). The 0.1M NaCl and 0.35 M NaCl (I) fractions contained activities we shall refer to respectively as "Enzyme A" and "Enzyme B". Both were required for thiosulphate-oxidizing activity. The 0.35M NaCl(I) fraction was resolved into two major components, one being enzyme B, the other being cytochrome c_{551}, both of which were required for full activity.

Enzyme A activity was measured as described before (see 4.2.2) except that a smaller amount of enzyme A solution (0.01 - 0.1 mg) was used at the later stages of the purification. Enzyme B was assayed by essentially the same procedure as for enzyme A. The reaction mixture (1 ml in a 1 cm light-path cuvette) contained (μmol): $Na_2S_2O_3$, 2; Tris buffer, pH 7.3, 45; cytochrome c (horse heart type II), 0.07; A65Z fraction (0.3 mg protein, as enzyme A); 0.35M NaCl(II) fraction (0.6 mg protein); G-200-I fraction (0.4 mg protein, containing cytochrome c_{551}; see Results) and enzyme B solution (0.01-0.08 mg protein). Reaction at 30°C was initiated by adding the enzyme B solution.

Activity was expressed as cytochrome c reduction in terms of the protein added as enzyme B. The activities of enzyme A and enzyme B measured and calculated in this way are relative, as the amounts of enzyme B (for assay of enzyme A), enzyme A (for assay of enzyme B), cytochrome c_{551} and cytochrome $c_{552.5}$ used in the assay will affect the activity. In order to obtain a linear relationship between amount of enzyme A assayed and the activity, a ten times or greater excess of enzyme B (in terms of protein) was used. Similarly, enzyme A was used in excess to assay enzyme B. Sulphite: cytochrome c oxidoreductase activity was measured as described before (see 2.2.6) except that less protein (4-50 µg) was used.

5.2.3
Purification of Enzyme A

Protein precipitated from the combined 0.1M and 0.12M NaCl fractions by precipitation between 60-90% saturation with ammonium sulphate was recovered as described previously and referred to as the A90% fraction. This was stored at -20°C. The A90% fraction (420 mg) was thawed and applied to the top of a 2.6 cm x 6.5 cm column of phenyl-Sepharose CL-4B equilibrated with 18 mM phosphate buffer, pH 6.5 containing 2 mM $Na_2S_2O_3$ and $(NH_4)_2SO_4$ at 17% of saturation. After sample addition elution was continued with 1 bed volume of equilibrating buffer, followed by a linear gradient of decreasing ammonium sulphate concentration and increasing ethylene glycol concentration, which was produced by constant-head mixing of 250 ml each of $(NH_4)_2SO_4$ (17% saturation) and 50% (v/v) ethylene glycol, both in 18 mM phosphate buffer, pH 6.5 containing 2 mM $Na_2S_2O_3$ at 4°C and a flow rate of 30 ml h^{-1}. The typical elution pattern is shown in Fig. 2 . The active fractions were combined and then concentrated at 4°C in a 50 ml Amicon ultra-filtration cell over an Amicon PM10 membrane under nitrogen at 0.7 bar (7×10^4 Nm^{-2}). In the presence of ethylene glycol about 30% of the enzyme passed through the membrane in the filtrate. The ultrafiltration had to be repeated three times to obtain 95% of the enzyme.

5.2.4 Gel filtration on Sephadex G-100

The concentrated enzyme A solution (120 mg) was loaded on the bottom of a 2.6cm x 83cm column of Sephadex G-100 equilibrated with 50 mM Tris buffer, pH 7.3, containing 2 mM $Na_2S_2O_3$ and eluted upwards with the same buffer at 4°C and a flow rate of 16 ml h^{-1}. The elution pattern was as seen in Fig. 3. The active fractions were pooled and concentrated by $(NH_4)_2SO_4$ as described before (Lu and Kelly, 1983c).

5.2.5 Preparative isoelectric focusing in Sephadex IEF thin layer

Preparation of plate (gel bed). 45 ml of Sephadex IEF suspension containing 3 ml Pharmalyte™ (pH 2.5-5) prepared according to the instructions (Pharmacia Fine Chemicals) was poured on a 11.5cm x 24cm glass plate and spread by means of a glass rod. The plate was dried in air or by means of a hair dryer with indirect flow until the shiny surface of the gel became opaque like a frosted glass window.

Focusing. The contact between the electrodes and the gel layer was established with electrode strips (11cm x 0.5cm) from LKB soaked in 0.1M NaOH at the cathodic side and 0.1M phosphoric acid at the anodic side. An LKB Multiphor 2117 and constant power supply 2197 were used. The gel was pre-focused for about half an hour, then loaded with sample and focused for four or five hours at constant wattage (10 to 30W) and 1500 volts. Cold water (0°C was circulated through the cooling plate during focusing.

Sample application. Enzyme solution was dialysed against 1% glycine for 2.5h. About 3 ml gel was scraped out from the pre-focused plate with a spatula forming a slot in the position of about 10 cm from the anode and mixed with the dialysed enzyme solution in a small beaker. The mixture was then poured back into the slot. The gel surface was smoothed and allowed to equilibrate for a few minutes before reconnecting the power.

Detection of separated zone. A quick paper print technique as described in the LKB manual was employed. A sheet of Whatman No.1 paper was rolled on the gel layer, allowed to stay in contact with a gel for

0.5 min, then removed from the gel and dried from above with a stream of hot air from a hair dryer. The dried print was placed in staining solution (0.7g page blue in 1 litre destaining solution) for 5 min, then quickly rinsed twice in destaining solution (60 ml of 70% perchloric acid in 940 ml distilled water) and finally placed in preserving solution (mixture of 70 ml acetic acid, 200 ml glycerol, 250 ml methanol and 500 ml distilled water) for 10 min before removing in onto a sheet of aluminium foil to allow it to dry.

Recovery of separated proteins. After location of the proteins using the stained print, the fractions of interest were removed from the gel layer with a spatula and suspended in small amounts of 50 mM Tris buffer, pH 7.3. The proteins were separated from the gel by filtration on No.1 waterman a small column. Enzyme A activity was measured in the solutions. Solution containing enzyme A was desalted by dialysis or gel filtration on Sephadex G-25, followed by dialysis against solid polyethylene glycol to concentrate the sample. The carrier ampholytes could also be removed from the enzyme solution by ammonium sulphate precipitation of the enzyme.

5.2.6a Possible modifications of the purification procedure for enzyme A

The ultrafiltration steps could use membranes with lower molecular weight cut-off levels (e.g. YM5 instead of PM10) in order to avoid passage of the enzyme in the presence of ethylene glycol. Inclusion of thiosulphate in the elution buffer as an enzyme stabilizing agent was necessary as it was subsequently shown that its omission in the elution buffers caused considerable loss of the enzyme activity.

5.2.6
Purification of enzyme B

Resolution of 0.35M NaCl(I) fraction into two major fractions containing enzyme B and cytochrome $\underline{c}_{551}$ respectively.

The 0.35M NaCl(I) fraction (500 mg protein) was applied to the bottom of a column (3.2 x 85.5 cm) of Sephadex G-200 equilibrated with 50 mM Tris buffer, pH 7.3 and eluted upwards with same buffer at 4°C and a flow rate of 35 ml h^{-1}. Active fractions were pooled and then concentrated by ultrafiltration under nitrogen pressure (0.7 bar) through an Amicon PM10 membrane at 4°C.

5.2.7
Hydrophobic interaction chromatography

Concentrated enzyme B solution (60 mg) was brought to about 15% of saturation with $(NH_4)_2SO_4$ loaded on to the top of a 2.6 x 6.5 cm column of Phenyl-Sepharose CL-4B equilibrated with 50 mM Tris buffer, pH 6.5, containing 10% of saturation with $(NH_4)_2SO_4$. After sample application the column was eluted downwards with one bed volume of the same buffer at 4°C and a flow rate of 30 ml h^{-1}, followed by a linear gradient of decreasing ammonium sulphate concentration, which was produced from two 150 ml volumes of 50 mM Tris buffer, pH 7.3, one of which contained $(NH_4)_2SO_4$ (10% of saturation). The active fractions were combined and concentrated by ultrafiltration as stated above.

Preparative isoelectric focusing

The procedure was the same as described for purification of enzyme A (Fig.5.4b).

5.2.8
Assay of stoicheiometry of thiosulphate oxidation by the reconstituted system

This was done in a Clark oxygen electrode cell essentially as described before (see 2.2.6). Reaction mixture (final volume 1 ml) contained: purified enzyme A (0.15 mg), pure enzyme B (0.2 mg), cytochrome $\underline{c}_{551}$ fraction (G-200-I fraction, 0.5 mg), cytochrome $\underline{c}_{552.5}$ (0.35M NaCl(II) fraction, 0.5 mg), horse heart cytochrome $\underline{c}$ (2 mg), bovine heart cytochrome oxidase (5 units), Tris buffer, pH 7.3 (40 µmol) and $Na_2S_2O_3$ (50 or 100 nmol precisely). Oxygen concentration in the experimental conditions was calibrated using the method described by Robinson and Cooper (1970).

5.2.9
Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate was carried out to monitor the protein purification and to determine molecular weight using the method of Laemmli (1970). 12% (w/v) acrylamide in the resolving gel was used. Protein samples were incubated at 60°C for 10 min in Tris buffer, pH 6.5, containing 3% (v/v) 2-mercaptoethanol and 1% (w/v) SDS before loading and electrophoresed at a constant 40 mA for 5 to 6 h at 4°C. The gels were stained overnight in a mixture of 30% (v/v) methanol, 5% (v/v) acetic acid in distilled water containing 0.2% (w/v) Coomassie Brilliant Blue G and destained in the same solution without the dye. Standard marker proteins (M_r in parentheses) used for calibration were bovine albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100) and α-Lactabumin (14,200). Fig. 5.10 shows such a determination for enzyme A and enzyme B.

Purity of the samples was also examined by discontinuous polyacrylamide gel electrophoresis under nondenaturing conditions essentially as described by Davis (1964). 8% (w/v) acrylamide in the resolving gel was used. The electrophoresis was performed at a constant 30 mA for 4 h at 4°C. The staining procedure was the same as for the SDS-gel.

5.2.10
Determination of the molecular weight of enzyme B by gel filtration. A method based on Andrews (1965) was used with bovine serum albumin (mol wt 67,000), egg albumin (mol wt 43,000), bovine pancreas chymotrypsinogen A (mol wt 25,000) and bovine ribonuclease A (mol wt 13,700) as marker proteins. Enzyme B (4 mg protein) and marker proteins (5 mg protein each) were run separately on a 2.6 cm x 84 cm column of Sephadex G-100 with 50 mM Tris buffer, pH 7.3 at 4°C. The positions of the marker proteins and enzyme B were determined by measuring absorbance at 280 nm and enzyme activity (Fig. 10b)

5.2.11 Determination of isoelectric point. The isoelectric points of the purified proteins were measured by flat bed electro-focusing in polyacrylamide gels using Pharmalyte, pH 2.5-5 and LKB Multiphor 2117 and constant power supply 2197. Preparation of gel (100 x 50 x 1mm), application of samples, running conditions and staining of gel were based on the instruction for PI calibration kits from Pharmacia Fine Chemicals. The pH gradient profile across the IEF gel was calibrated by using a low pI calibration kit.

5.2.12 Reaction of purified enzymes with $^{35}S_2O_3^{2-}$. Reaction mixtures (0.5 ml) in Tris HCl, pH 7.3, contained either $Na_2{}^{35}S\text{-}SO_3$ or $Na_2S\text{-}{}^{35}SO_3$ (2 µmol at 2-10 x 10^6 dpm μmol^{-1}) and other components as indicated in the Results. After incubation at 30°C, samples were analysed by paper chromatography using a butanol-pyridine-acetic acid-water (20:30:6:24) solvent with and without treatment with iodine to convert residual thiosulphate to tetrathionate (Kelly and Syrett, 1966). Whatman No.1 sheet was used. The R_f values for SO_4^{2-}, $S_2O_3^{2-}$ and $S_4O_6^{2-}$ are 0.12, 0.12 and 0.46 respectively. Chromatograms were assayed for ^{35}S by cutting strips into segments and counting in scintillation vials filled with 0.5% (w/v) butyl PBD in toluene. Tris buffer was replaced by phosphate buffer 25 mM, pH 7.2 later, as it was found that Tris buffer caused false formation of small amounts of sulphate (i.e. certain amount of $^{35}SSO_3$ in the iodine-added solution appeared at R_f 0.12 on the chromatographed paper sheet). The thin layer chromatographic method (Kelly 1970) (using solvents systems S_8 or S_{15}) failed to do the job due to the fact that a substantial amount of thiosulphate non-specifically associated with proteins in the experimental conditions, which confused with the calculation of sulphate formation and certain amounts of $S_2O_3^{2-}$ moved to the spot of $S_4O_6^{2-}$.

Chemicals. See Chapter 6.

RESULTS

5.3.1 Purification of enzyme A

Enzyme A was purified some 100-fold by the procedures described (Fig. 5.1; Table 1). The progress of the separation, monitored by SDS polyacrylamide gel electrophoresis is shown in Fig. 5.5.

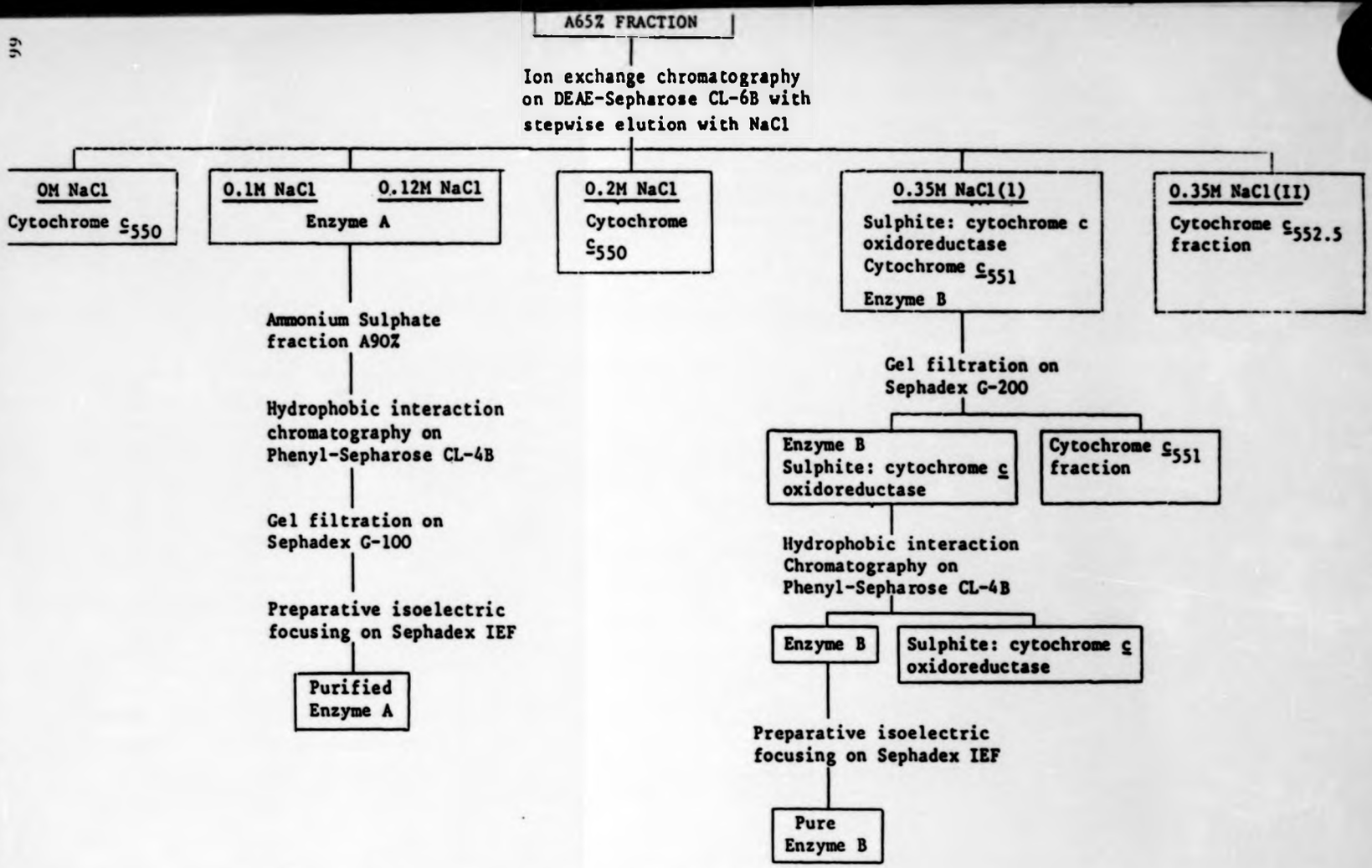


Fig.51 Fractionation of the A65% ammonium sulphate fraction into constituent enzymes and cytochromes.

TABLE 51. Summary of the purification of enzyme A

Procedure	Volume (ml)	Protein (mg)	Specific Activity (μmol cytochrome c reduced min^{-1} mg $protein^{-1}$)	Total Activity (μmol cytochrome c reduced min^{-1})	Purification (fold)	Yield (%)
Crude extract	545	49,100	–	–	1*	100*
First ammonium sulphate fractionation (A65%)	251	12,000	–	–	4*	91*
DEAE-Sepharose-CL6B	74	2,192	0.25	543	18	74
Second ammonium sulphate fractionation (A90%)	34	946	0.54	511	39	70
Phenyl-Sepharose CL-4B	16	409	1.16	474	80	65
Sephadex G-100	14	302	1.5	453	110	62
Isoelectric focusing in Sephadex IEF	–	140	1.4	196	102	27

* These figures were derived from results measured by the oxygen electrode method (Lu & Kelly, 1983b)

Enzyme A was assayed as described in the Methods

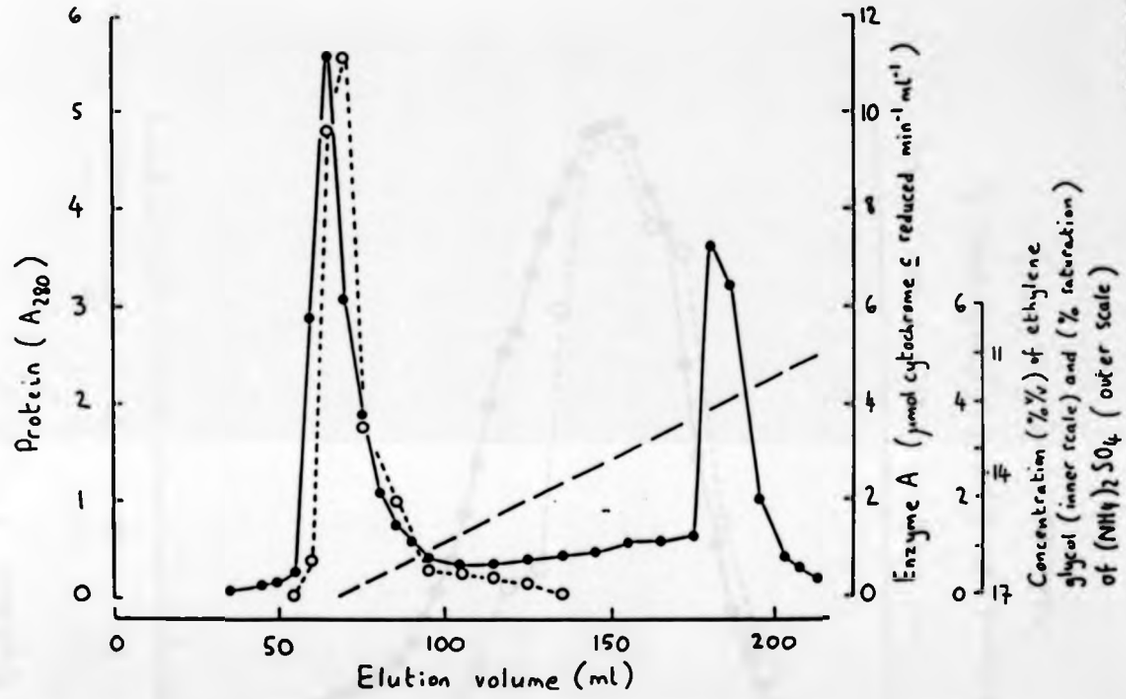


Fig.52 Elution pattern of the A90% fraction on phenyl-sepharose CL-4B. For details see Methods. ● , Protein (A_{280}); ○, enzyme A activity; – – – ammonium sulphate and ethylene glycol gradient.

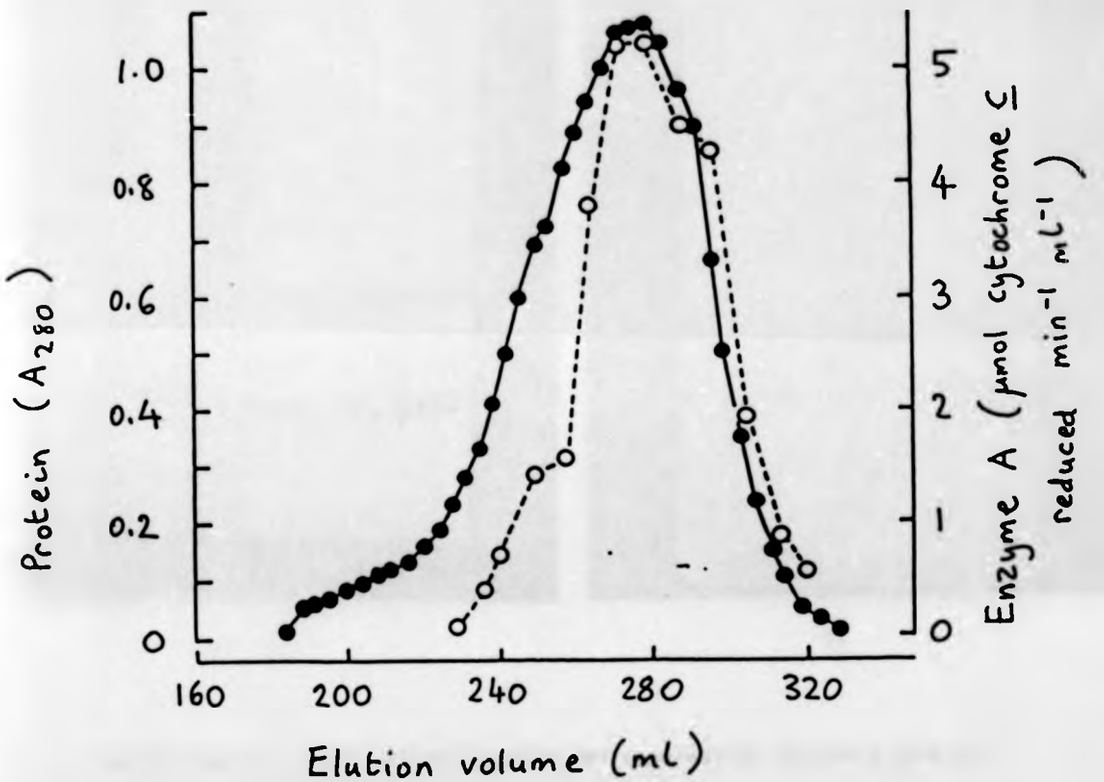
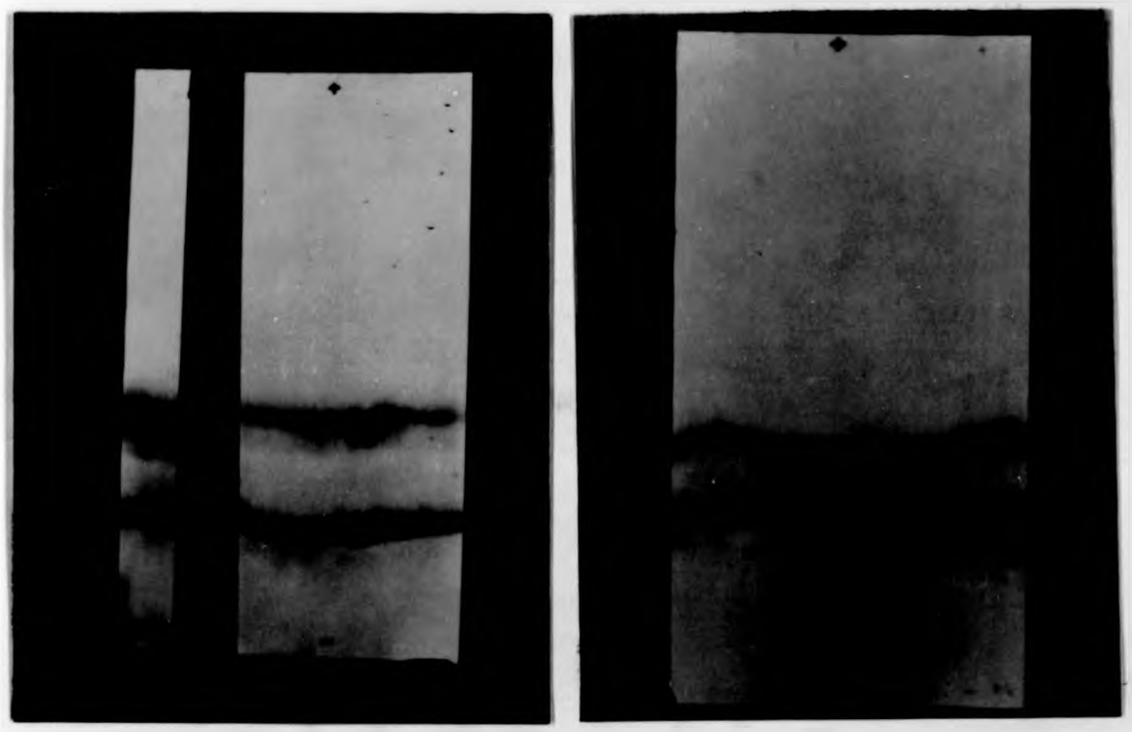


Fig.53 Elution pattern on Sephadex G-100 of the active fraction from Phenyl-sepharose CL-4B chromatography. For details see Methods. ●, Protein (A_{280}); ○ enzyme A activity. Void volume was 160 ml.



a b

Fig.34a and b. Paper prints of flat bed isoelectric focusing gels of purified enzymes A and B.

(a) IEF of active material (enzyme A) from Sephadex G-100 chromatography (see Methods). Sample (12 mg protein) was loaded and focused for 6 h at 12W, constant power, 1,500V maximum. The gel was pre-run for 1 h.

(b) IEF of the enzyme B fraction (HIC-II) from phenyl-sepharose CL-4B chromatography (see Methods and Results). Sample (15 mg protein) was loaded and focused from 6 h at 30W, constant power, 2000V maximum. The gel was pre-run for 1 h.

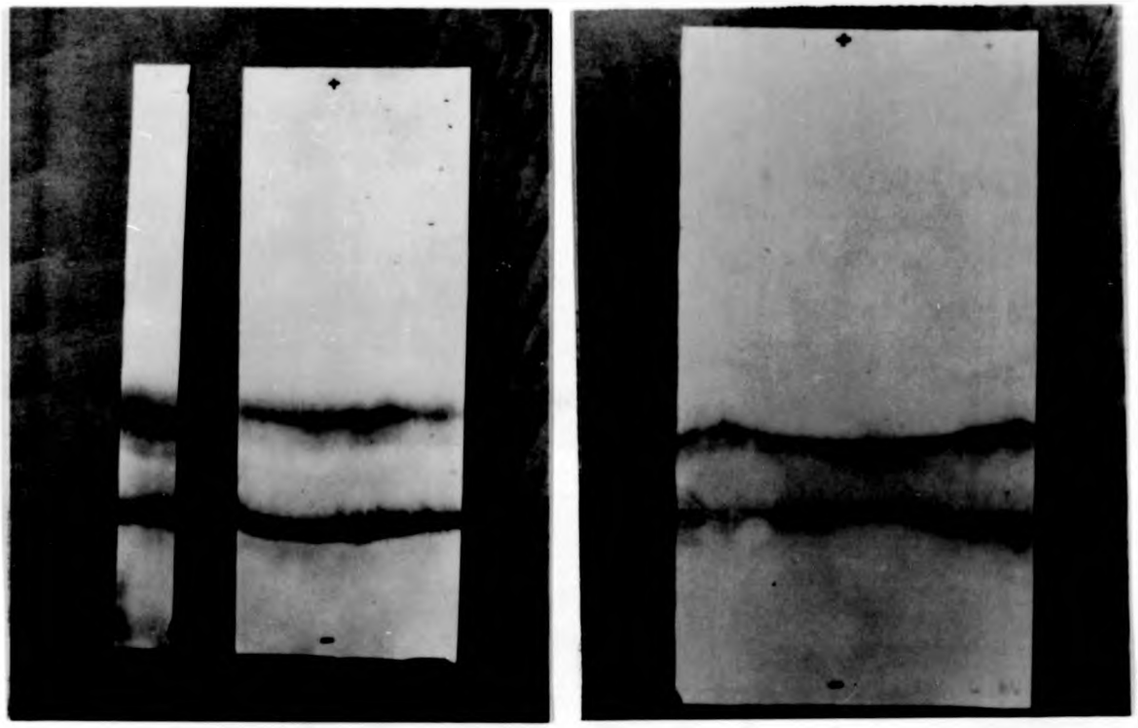


Fig.34a and b. Paper prints of flat bed isoelectric focusing gels of purified enzymes A and B.

(a) IEF of active material (enzyme A) from Sephadex G-100 chromatography (see Methods). Sample (12 mg protein) was loaded and focused for 6 h at 12W, constant power, 1,500V maximum. The gel was pre-run for 1 h.

(b) IEF of the enzyme B fraction (HIC-II) from phenyl-sepharose CL-4B chromatography (see Methods and Results). Sample (15 mg protein) was loaded and focused from 6 h at 30W, constant power, 2000V maximum. The gel was pre-run for 1 h.

Fig.34a and b. Paper prints of flat bed isoelectric focusing gels of purified enzymes A and B.

(a) IEF of active material (enzyme A) from Sephadex G-100 chromatography (see Methods). Sample (12 mg protein) was loaded and focused for 6 h at 12W, constant power, 1,500V maximum. The gel was pre-run for 1 h.

(b) IEF of the enzyme B fraction (HIC-II) from phenyl-sepharose CL-4B chromatography (see Methods and Results). Sample (15 mg protein) was loaded and focused from 6 h at 30W, constant power, 2000V maximum. The gel was pre-run for 1 h.

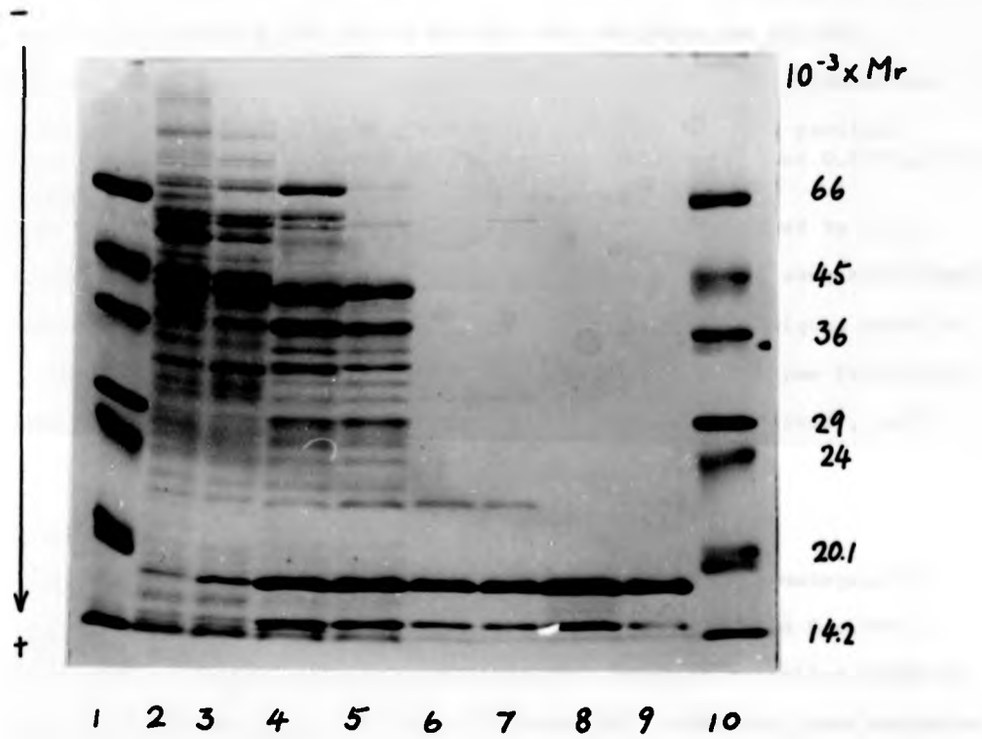


Fig.55 SDS polyacrylamide slab gel electrophoresis of the fractions in the purification of enzyme A. (1) Marker proteins (55 µg); (2) Crude extract (60 µg); (3) A65% fraction (60 µg); (4) 0.1M and 0.12M fraction (60 µg); (5) A90% fraction (60 µg); (6) Pooled active fractions (30 µg) from Phenyl-sepharose CL-4B column; (7) Pooled active fractions (30 µg) from Sephadex G-100; (8,9) Purified enzyme A after preparative isoelectric focusing (30 and 20 µg resp.); (10) Marker proteins (45 µg). For details see Methods and Results. Molecular weights of markers and direction of migration are indicated.

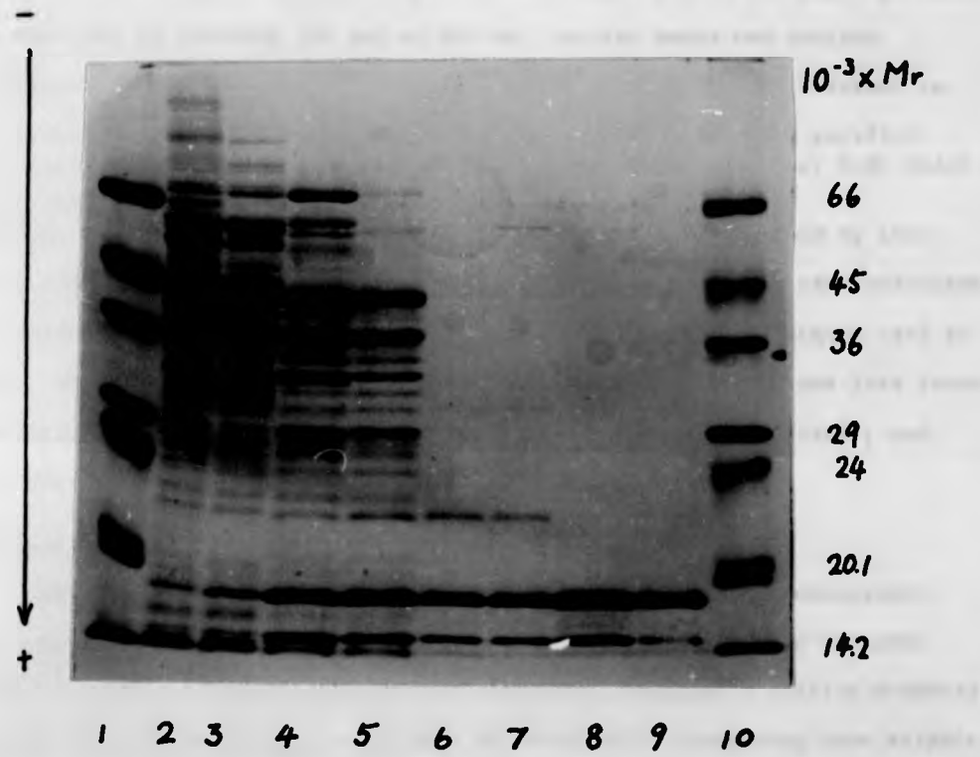


Fig.55 SDS polyacrylamide slab gel electrophoresis of the fractions in the purification of enzyme A. (1) Marker proteins (55 µg); (2) Crude extract (60 µg); (3) A65% fraction (60 µg); (4) 0.1M and 0.12M fraction (60 µg); (5) A90% fraction (60 µg); (6) Pooled active fractions (30 µg) from Phenyl-sepharose CL-4B column; (7) Pooled active fractions (30 µg) from Sephadex G-100; (8,9) Purified enzyme A after preparative isoelectric focusing (30 and 20 µg resp.); (10) Marker proteins (45 µg). For details see Methods and Results. Molecular weights of markers and direction of migration are indicated.

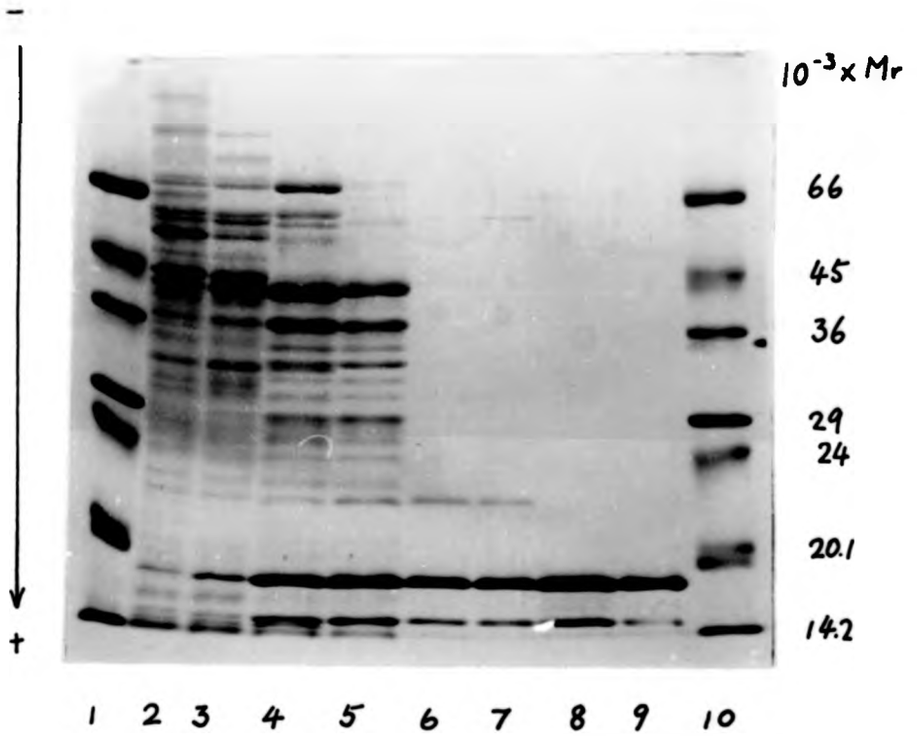


Fig.55 SDS polyacrylamide slab gel electrophoresis of the fractions in the purification of enzyme A. (1) Marker proteins (55 µg); (2) Crude extract (60 µg); (3) A65% fraction (60 µg); (4) 0.1M and 0.12M fraction (60 µg); (5) A90% fraction (60 µg); (6) Pooled active fractions (30 µg) from Phenyl-sepharose CL-4B column; (7) Pooled active fractions (30 µg) from Sephadex G-100; (8,9) Purified enzyme A after preparative isoelectric focusing (30 and 20 µg resp.); (10) Marker proteins (45 µg). For details see Methods and Results. Molecular weights of markers and direction of migration are indicated.

5.3.2
Purity and some properties of enzyme A

As shown in Fig. 5.5 enzyme A, after the final purification, still appeared as one major band with a molecular weight of 16,200 and another minor band of 14,600. The minor protein was about 15% of the major protein as measured by scanning the gel at 600 nm. The two bands had similar densities in the crude extract. On the basis of the 100-fold increase in specific activity and its concentration, the major band in the purified preparation is presumed to enzyme A. The enzyme comprised about 0.8% (w/w) of the crude extract. Because both proteins had very similar molecular weights, nearly the same PI values, determined by isoelectric focusing in polyacrylamide gel, and more or less the same hydrophobic properties, it is very difficult to separate them by the techniques used so far. The isoelectric point of enzyme A is about 4.2. The enzyme lost about 50% and 80% of its activity at 20°C after 6 h and 20 h respectively, and 30% and 50% at 4°C after one day and two days respectively.

5.3.3
Purification of enzyme B

The 0.35M NaCl(I) fraction from DEAE-Sepharose-CL-6B chromatography was separated into two major protein peaks by gel filtration on Sephadex G-200 (Fig 5.6). Three fractions were collected, fraction I (called G-200-I) containing cytochrome c_{551}; fraction II (G-200-II), containing some sulphite: cytochrome c oxidoreductase; and fraction III (G-200-III) containing enzyme B and most of the sulphite:cytochrome c:oxidoreductase activity (Table 5.2).

Enzyme B and sulphite:cytochrome c oxidoreductase in the G-200-III fraction were further separated into two fractions by Phenyl-Sepharose-CL-4B chromatography (Fig. 5.7 and Table 5.3). Most sulphite:cytochrome c oxidoreductase activity was recovered in the first fraction (called HIC-I). Most of the enzyme B activity was in the second fraction (called HIC-II) but the specific activity was not increased and the total activity recovered (yield) was low. The specific activity of enzyme B was, however, increased about 40% by including a small amount of the HIC-I fraction, to give a ratio in the reaction mixture of HIC-I:HIC-II protein equivalent to that in the separated fractions. Yield was also restored to about 95% of that originally present.

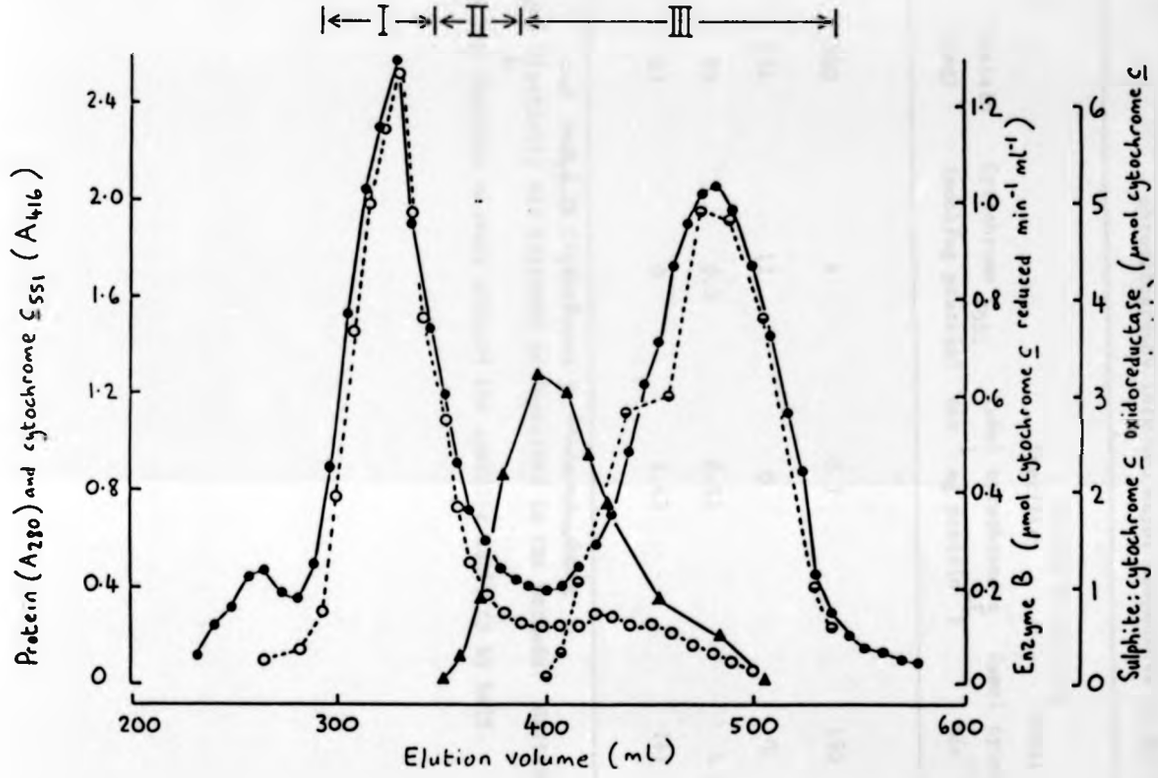


Fig.56 Elution profile of the 0.35 M NaCl(I) fraction on Sephadex G-200 (see text for detail). ● , protein (A_{280}); ○ , cytochrome c_{551} (A_{416}); ◒ enzyme B activity; ▲ , sulphite:cytochrome c oxidoreductase. Void volume was 265 ml.

TABLE 52. Resolution of the 0.35 M NaCl(I) fraction from DEAE-Sepharose CL-6B chromatography into two major components by gel filtration on Sephadex G-200 (See Fig. 56)

Fractions	Protein (mg)	Cytochrome $\underline{c}_{551}$ (nmol/mg protein)	Enzyme B activity Specific (μmol cytochrome $\underline{c}$ min^{-1} mg $protein^{-1}$)	Enzyme B activity Total (μmol cytochrome $\underline{c}$ min^{-1})	Sulphite:cytochrome $\underline{c}$ oxidoreductase activity Specific (μmol cytochrome $\underline{c}$ min^{-1} mg $protein^{-1}$)	Sulphite:cytochrome $\underline{c}$ oxidoreductase activity Total (μmol cytochrome $\underline{c}$ min^{-1})
Sample (0.35M I)	500	4	0.3	150	0.86	428
Fraction I	134	12	0	0	0.02	3
Fraction II	50	0.5	0.03	1.6	0.56	28
Fraction III	103	0	1.43	147	2.97	308

* Enzyme B and sulphite:cytochrome $\underline{c}$ oxidoreductase activity was measured as described in the Methods. Omitting Fraction I (i.e. cytochrome $\underline{c}_{551}$) from the reaction mixture reduced the specific activity by half.

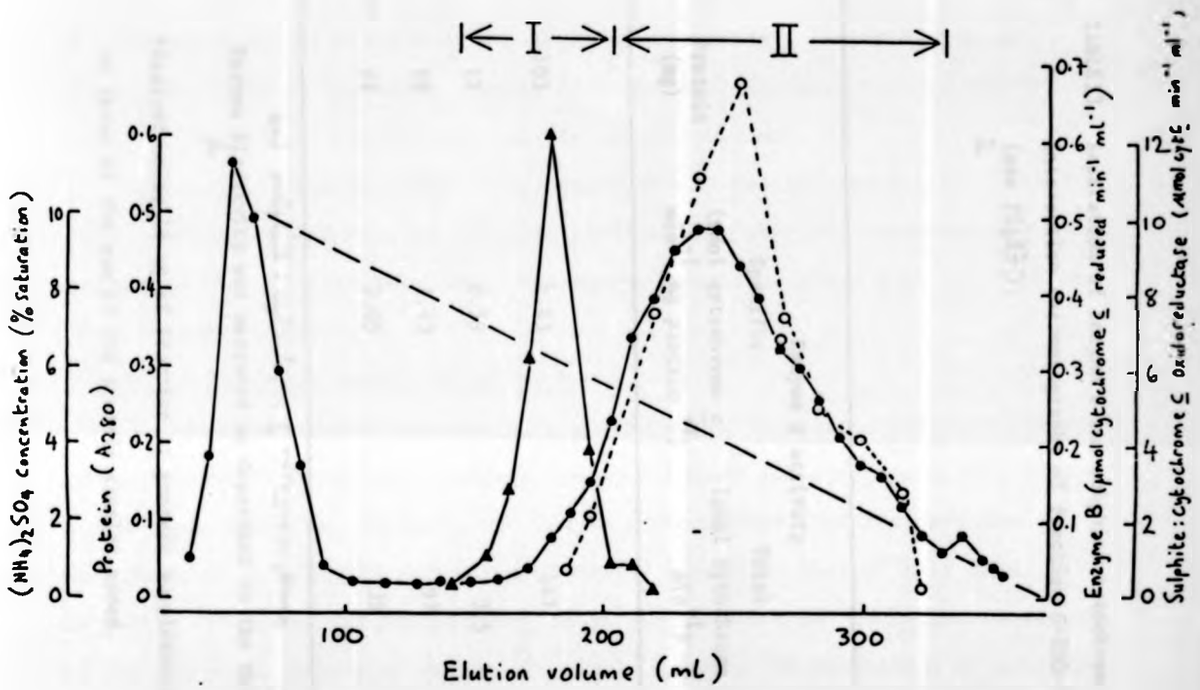


Fig.57 Elution profile of the further resolution of the G-200 (III) fraction on phenyl-sepharose CL-4B (see Fig. 6 and text for detail). ● , protein (A_{280}); ○ , enzyme B; ▲ , sulphite: cytochrome c oxidoreductase; — — , ammonium sulphate concentration gradient.

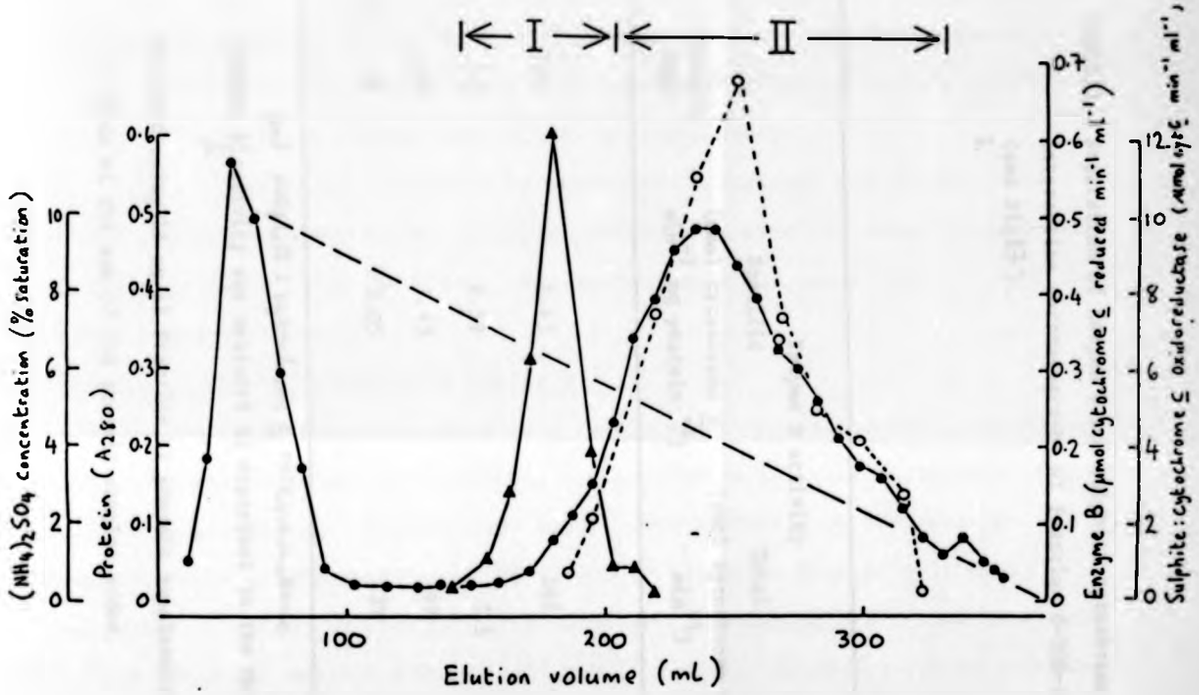


Fig.57 Elution profile of the further resolution of the G-200 (III) fraction on phenyl-sepharose CL-4B (see Fig. 6 and text for detail). ● , protein (A_{280}); ○ , enzyme B; ▲ , sulphite: cytochrome c oxidoreductase; — — , ammonium sulphate concentration gradient.

TABLE 53. Separation of Enzyme B and sulphite:cytochrome c oxidoreductase by hydrophobic interaction chromatography of fraction G-200-III (see Fig. 6) on Phenyl-Sepharose-4B (see Fig. 7).

Fractions assayed	Protein (mg)	Enzyme B activity		Sulphite:cytochrome c oxidoreductase activity	
		Specific (μmol cytochrome c min^{-1} mg protein $^{-1}$)	Total (μmol cytochrome c min^{-1})	Specific (μmol cytochrome c min^{-1} mg protein $^{-1}$)	Total (μmol cytochrome c min^{-1})
Sample (G-200-III)	103	1.43	147	2.97	308
Fraction I	12	0.59	3.5	21.1	247
Fraction II	68	1.43	97	0.97	30
Fraction I + II*	68	2.05	139	-	-

and sulphite : cytochrome c oxidoreductase

Enzyme B activity was measured as described in the Methods.

*Assayed using 16 μg of Fraction II protein supplemented with 3 μg Fraction I protein in terms of the sum of the protein samples added.

Enzyme B in the HIC-II fraction was finally purified by preparative isoelectric focusing in Sephadex IEF. Two major protein bands were obtained on a paper print of the gel (Fig.54b). Both of them contained enzyme B activity (Table54). The SDS-polyacrylamide gel electrophoresis disclosed that they were the same protein although the protein from the bottom band had less enzyme activity and one or two minor contaminants. Enzyme B accounted for at least 90% of the total protein in the HIC-II fraction and the double-banding observed was probably due merely to overloading.

The purification of enzyme B is summarized in Table54 and Fig.51. The final product represents some 280-fold purification over the crude extract with an overall recovery of about 50%. The enzyme comprised about 0.6% (w/w) of the crude extract protein.

5.3.4
Purity and molecular weight of enzyme B

The purified enzyme appeared as a single sharp band after SDS-polyacrylamide gel electrophoresis and discontinuous polyacrylamide gel electrophoresis (Fig.58a and58b respectively). However, the enzyme band shifted from the position equivalent to a molecular weight of 63,000 ($\pm$ 2,000) to that of half this molecular weight on the SDS polyacrylamide gel, indicating the enzyme to consist of two subunits, molecular weight, 32,000 ($\pm$ 2,000). Determination of molecular weight on Sephadex G-100 also confirmed that the native enzyme possessed a molecular weight of about 64,000. Gel electrophoresis of the crude extract and A65% fraction showed a band at the position expected for enzyme B (M_r about 63,000; Fig.58a), but most of enzyme B in the 0.35M NaCl(I) fraction moved down to the subunit position. Since all of the samples were prepared in the same way before being loaded on the gel, it is unclear why the enzyme behaved so differently. Treatment with SDS at 100°C did not cleave the 63,000 M_r enzyme into smaller units. Boiling the purified enzyme from IEF (Fig.58a, lane 6) resulted in most of the protein running as M_r 63,000, although the milder treatment gave the smaller unit seen in Fig.58a. This behaviour is anomalous and cannot be further explained at present.

5.3.5
Some other properties of enzyme B

The enzyme had a PI value about 4.25 and had an absorption spectrum only in the UV region, with a sharp maximum at 280 nm and a broad absorbance below 250 nm.

TABLE 4. Summary of the purification of Enzyme B

Procedure	Total protein (mg)	Specific activity (μmol cytochrome c reduced min^{-1} mg $protein^{-1}$)	Total activity (μmol cytochrome c reduced min^{-1})	Purification (fold)	Yield (%)
Crude extract	49,100	—	—	1*	100*
1st ammonium sulphate fraction (A65%)	12,000	—	—	4*	91*
DEAE-Sepharose CL-6B (0.35M NaCl(I) fraction)	1,155	0.30	346	34	75
Sephadex G-200 (G-200-III) (see Fig. 6)	240	1.43	343	162	73
Phenyl-Sepharose CL-4B (HIC-II) (see Fig. 7)	158	2.05†	323	231	69
Preparative iso-electric focusing	(I)** 67	2.5†	167)	280	53
	(II)** 81	0.9†	) 247 73)	101	

* Figures derived from results obtained using the oxygen electrode procedure (Lu & Kelly, 1983b). Enzyme B was assayed as described in the Methods.

† For these assays some of the HIC-I fraction (3 μg protein) was added to the reaction mixture. For details see Table 3 and the text.

** I and II are the upper and lower bands respectively of Fig. 6.

Fig. 8 (a) SDS-polyacrylamide slab gel electrophoresis of the fractions in the purification of enzyme B. (1) Crude extract (60 µg); (2) A65% fraction (60 µg); (3) 0.35 M NaCl(I) fraction (60 µg); (4) G-200 (III) fraction (see Fig. 6) (30 µg); (5) HIC-II fraction (see Fig. 8) (30 µg); (6) Pure enzyme B from preparative isoelectric focusing (20 µg); (7) Marker proteins (45 µg).

(b) Discontinuous polyacrylamide slab gel electrophoresis of the fractions in the purification of enzyme B. (1) 0.35 M NaCl(I) fraction (60 µg); (2) G-200 (III) fraction (see Fig. 6) (25 µg); (3) HIC-II fraction (see Fig. 7) (25 µg); (4) Pure enzyme B from preparative isoelectric focusing (20 µg). For details see Methods and Results.

trophoresis of the fractions in
1) Crude extract (60 µg); (2) A65%
Cl(I) fraction (60 µg); (4) G-200
µg); (5) HIC-II fraction
enzyme B from preparative isoelectric
oteins (45 µg).

ıb gel electrophoresis of the
enzyme B.
(1) 0.35 M NaCl(I) fraction
ion (see Fig. 6) (25 µg); (3) HIC-II
(4) Pure enzyme B from preparative
For details see Methods and Results.

The enzyme was more stable than enzyme A. It lost about 30% and 50% activity at 20°C after one and two days respectively, and 4% and 20% at 4°C after one and two days respectively. Storage at -20°C for at least three months, with freezing and thawing had no significant effect on the enzyme activity.

5.3.6
Sulphite-cytochrome c oxidoreductase and the stimulation of enzyme B activity by the HIC-I fraction

The specific activity of sulphite:cytochrome c oxidoreductase was increased over 600 times during purification even though the recovery was rather low (Table55). However, the final product (HIC-I fraction) gave more than 6 protein bands on SDS polyacrylamide gel (Fig.59), in which the top major band was most probably enzyme B and the rest had more or less the same density.

The HIC-I fraction stimulated enzyme B specific activity in the HIC-II fraction (Table53) and pure enzyme B (Table54) by 20-40%. Fractions from previous steps were also stimulated. The stimulation could not simply be accounted for by the presence of the small amount of enzyme B in the HIC-I fraction.

5.3.7
Reconstitution of thiosulphate-oxidizing activity with the purified components

Thiosulphate was completely oxidized to sulphate by the reconstituted system with a consumption of 1.95 ± 0.05 moles of oxygen for each thiosulphate added (four determinations). The reaction was negligible in the absence of any one of enzyme A, enzyme B, or the cytochrome c_{551} and cytochrome $c_{552.5}$ fractions.

5.3.8
Attempt to demonstrate thiosulphate cleavage by the purified enzyme(s)

Incubation of partially purified enzyme A (0.35 mg) or enzyme B (1.5 mg) or mixtures of enzyme A, enzyme B and the cytochrome c_{551} and cytochrome $c_{552.5}$ fractions with thiosulphate labelled in the inner or outer sulphur atom with ^{35}S, in the absence of electron acceptors for as long as 45 min at 30°C demonstrated no significant formation of sulphite or sulphate. In other words the S-S bond of thiosulphate was not split by any one of the enzymes or the enzyme system

TABLE 55. Purification of sulphite:cytochrome c oxidoreductase

Procedure	Total Protein (mg)	Specific activity (μmol cytochrome c reduced min^{-1} mg $protein^{-1}$)	Total activity (μmol cytochrome c reduced min^{-1})	Purification (fold)	Yield (%)
Crude extract	-	0.04	-	1*	-
1st ammonium sulphate fraction (A65%)	12,000	0.22	2,640	5.6*	100
DEAE-Sepharose CL-6B	1,155	1.10	1,270	28	48
Sephadex G-200 (G-200-III) (see Fig 56)	240	2.97	713	93	27
Phenyl-Sepharose CL-4B (fraction I, see Fig 57)	28	21.10	591	660	22

*The figure was calculated from that measured by the oxygen electrode procedure (Lu & Kelly, 1983b). The enzyme was assayed as described in the Methods.

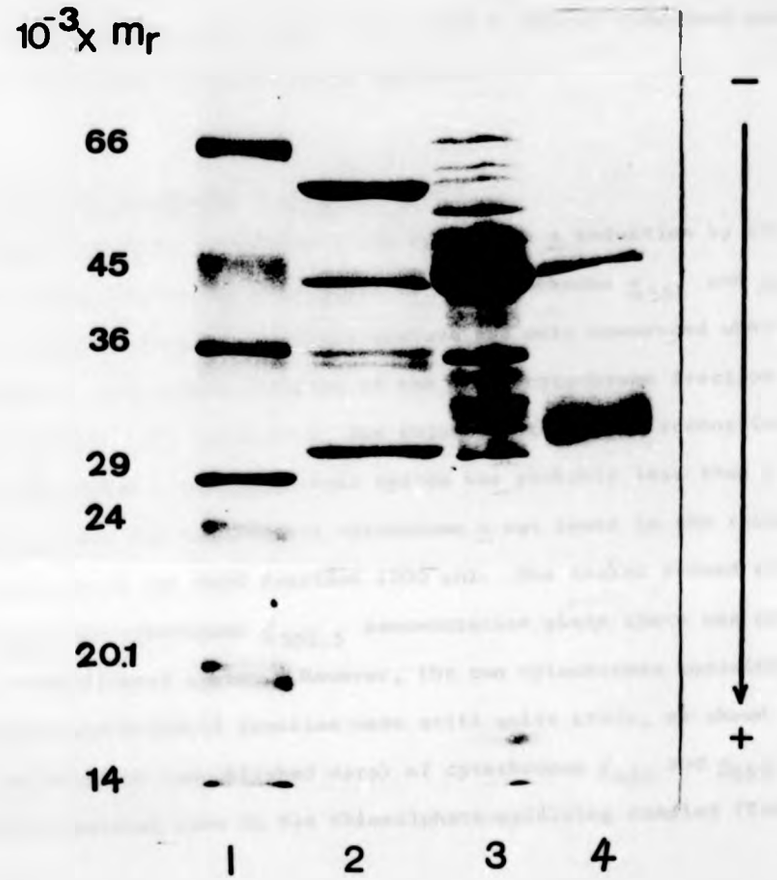


Fig.59 SDS-polyacrylamide gel electrophoresis of fractions in the separation of sulphite:cytochrome c oxidoreductase and cytochrome c_{551}. (1) Marker proteins (45 µg); (2) Fraction HIC-I (see Fig.57) (30 µg); (3) Fraction G-200-I (see Fig.56) (25 µg); (4) 0.35 M NaCl(II) fraction (40 µg).

under these experimental conditions. However, the experiment did show that thiosulphate appeared to be associated with enzyme B (HIC-II fraction) with a molar ratio of about two thiosulphate:one protein.

5.3.9

Cytochrome $\underline{c}_{551}$ and cytochrome $\underline{c}_{552.5}$

Thiosulphate oxidation and horse-heart cytochrome c reduction by the reconstituted enzyme system was negligible if the cytochrome $\underline{c}_{551}$ and $\underline{c}_{552.5}$ fractions were omitted from the reaction mixture and only commenced when one of them was added. Subsequent addition of the other cytochrome fraction increased the activity further. The apparent K_m for thiosulphate in the reconstituted thiosulphate:cytochrome c oxidoreductase system was probably less than 2 μM. The apparent K_m value for horse heart cytochrome c was lower in the reconstituted system (40 μM) than in the A65% fraction (200 μM). The causes seemed related to cytochrome $\underline{c}_{551}$ and cytochrome $\underline{c}_{552.5}$ concentration since there was more of them in the reconstituted system. However, the two cytochromes contained in the G-200-I fraction and 0.35M-II fraction were still quite crude, as shown in Fig. 9. Subsequent purification (unpublished data) of cytochromes $\underline{c}_{551}$ and $\underline{c}_{552.5}$ enabled proof of their essential role in the thiosulphate-oxidizing complex (Table 56).

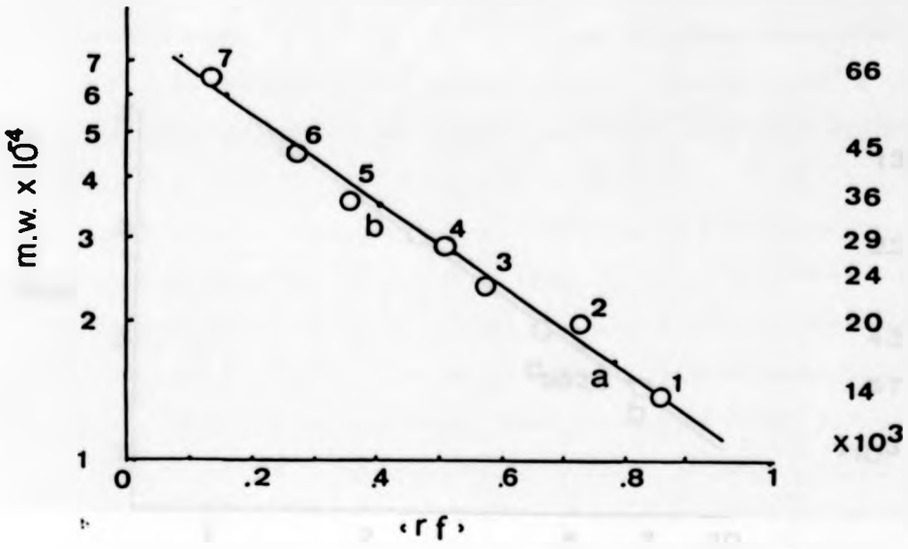
5.4 DISCUSSION

The conclusions to be drawn from our results to date indicate that enzymes thought to have some role in thiosulphate oxidation by thiobacilli (Oh & Suzuki, 1977; Kelly, 1982) do not seem to have central functions in Thiobacillus A2. These include rhodanese, which although very active in Thiobacillus A2 (Silver & Kelly, 1976; Wood & Kelly, 1981; Chapter 2) is not required for complete oxidation of thiosulphate by extracts (Chapter 3), while the "thiosulphate-oxidizing enzyme" (Trudinger, 1961), adenylyl sulphate (APS) reductase and the sulphur-oxidizing oxygenase enzyme (Suzuki, 1965; Charles & Suzuki, 1966a; Suzuki & Silver, 1966) are either absent from Thiobacillus A2 or present only at low levels (Kelly & Tuovinen, 1975; Silver & Kelly, 1976).

TABLE 56. A typical assay of thiosulphate: cytochrome c oxidoreduction activity with the four highly purified components.

Additions (mg protein)				Cytochrome c reduction
Enzyme A	Enzyme B	Cytochrome $c_{552.5}$	Cytochrome c_{551}	(nmol reduced min^{-1} $mg\ protein^{-1}$
0.1	0.08			3
0.1	0.08	0.04		35
0.1	0.08	0.04	0.085	85
0.1	0.08		0.085	22

Enzyme activity was measured spectrophotometrically as described in the Methods except that the components were added into the reaction mixture in the order and the amount as listed above, following the addition of horse-heart cytochrome c and thiosulphate. Activity was calculated in terms of the total protein of the components added. The detailed purification and characterization of cytochrome $c_{552.5}$ and "cytochrome c_{551}" are to be published elsewhere (Lu & Kelly, in preparation).



(Fig. 5.10a)

Fig. 5.10a Molecular weight estimation of enzyme A and enzyme B after SDS gel-electrophoresis.

Marker proteins were:

1.	α-Lactabumin	Mr	14,200
2.	trypsin inhibitor		20,100
3.	trypsinogen		24,000
4.	carbonic anhydrase		29,000
5.	glycerol dehyde-3-phosphate dehydrogenase		36,000
6.	egg albumin		45,000
7.	bovine albumin		66,000

Enzyme A - Molecular weight = 16,000

Enzyme B - subunit molecular weight = 33,000

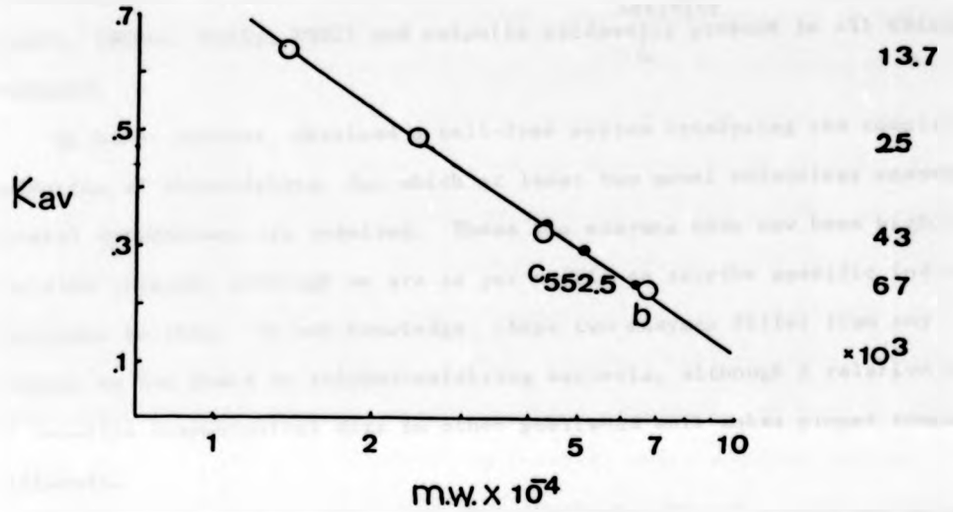


Fig. 5.10b Molecular weight estimation of enzyme B and cytochrome $\underline{c}_{552.5}$ by gel filtration on Sephadex G-100.

Marker proteins were:

	Mr
bovine serum albumin	66,000
egg albumin	43,000
bovine pancrease chymotrypsinogen A	25,000
bovine ribonuclease A	13,700

The molecular weight of enzyme B = 64,000

The molecular weight of cytochrome $\underline{c}_{552.5}$ = 56,000

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Ve = elution volume

Vt = bed volume

Vo = void volume

So far we have also failed to demonstrate a thiosulphate-cleaving enzyme (Peck, 1960; Kelly, 1982) or a major role for free sulphite as a substrate for sulphite oxidase in the system. It is generally believed that free sulphite is the penultimate intermediate in sulphate formation (Charles & Suzuki, 1966b; Kelly, 1982) and sulphite oxidase activity is present in all thiobacilli examined.

We have, however, obtained a cell-free system catalysing the complete oxidation of thiosulphate, for which at least two novel colourless enzymes and several cytochromes are required. These two enzymes have now been highly purified (Fig.5.2) although we are as yet unable to ascribe specific individual functions to them. To our knowledge, these two enzymes differ from any enzymes so far found in sulphur-oxidizing bacteria, although a relative lack of detailed enzymological data in other published work makes proper comparison difficult.

The present work confirms the view (Chapter 3) that the thiosulphate: cytochrome c oxidoreduction system in Thiobacillus A2 is a soluble multienzyme complex. The complete oxidation of thiosulphate by the reconstituted purified components proves that no small cofactor molecules are needed. The relatively easy separation of the components suggests their association in vivo to be weak. Many multienzyme systems show much stronger association, although some (e.g. tryptophan synthetase) are easily separated into subunits, with a considerable decrease in overall activity (Miles, 1979). Dissociation of the complex could help explain why thiosulphate-oxidizing activity in the crude extract from Thiobacillus A2 was about one hundredfold less than in intact cells (Kula et al, 1982; Chapter 2). Thus, rapid and stoicheiometric oxidation of thiosulphate in vivo depends on the integrity of the well-organized multienzyme complex and its association with the membrane system. Any disturbance of the structure, such as caused by disruption of the cell could thus dramatically affect oxidative ability. In fact, one advantage of a multienzyme complex is that it provides a very short transit time for passage of intermediates from

one enzyme to another. This could be particularly important if unstable compounds such as sulphite and other reduced sulphur species are produced as intermediates in thiosulphate oxidation. Such intermediates might always be enzyme-bound in the multienzyme system. For thiosulphate to be oxidized to sulphate, three main processes should occur. These are cleaving of the S-S bond, the oxidation of the sulphane-sulphur group to sulphate and that of the sulphone-group to sulphate. Cleavage has come to be regarded as the primary step (Kelly, 1982), followed by oxidation of the sulphur (or sulphide) and sulphite formed thereby. Our reconstituted system seems to contain two colourless enzyme proteins and two essential c-type cytochromes (of which "cytochrome c_{551}" may in fact be a third enzyme of thiosulphate oxidation), which together can effect all three essential processes. We have so far failed to show a thiosulphate-cleaving function using purified enzymes or the enzyme system in the absence of the electron transport system. This might mean that cleavage only proceeds at the rate of overall oxidation, so that in the absence of electron transport, thiosulphate binding to an enzyme can occur, but cleavage either does not occur or is so slight as not to be detectable. This could be because the products of cleavage are not released in the free state, but have to be transferred to acceptor-enzyme components of the multienzyme system and further cleavage ceases as oxidation of the transferred groups cannot occur. Alternatively, oxidation of sulphane-sulphur to sulphite might occur on the thiosulphate-binding enzyme with cleavage occurring only when an enzyme-bound intermediate analogous to dithionate ($^{-}O_3S-SO_3^{-}$) has been formed. At present it is not possible to decide on the exact nature of the partial reactions of sulphate formation or even the exact timing of the sulphur-sulphur bond cleavage. Certainly, however, thiosulphate cleavage is not a simple primary reaction of the rhodanese type.

In current work we are attempting to evaluate the midpoint potentials of the various c-type cytochromes, which should enable an assessment of the sequence of their involvement as electron transport carriers.

Fig. 92 gives a schematic representation of the probable interrelations of the multienzyme complex and the membrane system in effecting thiosulphate oxidation.

The sulphite:cytochrome c oxidoreductase in Thiobacillus A2 compares with that in T. novellus (Yamanaka et al., 1981), in that cytochrome c_{551} appears to be the electron acceptor for sulphite oxidation and separation of the cytochrome from the enzyme considerably reduced its activity (unpublished observations). Nevertheless, the significance of the sulphite-cytochrome c oxidoreductase in Thiobacillus A2 is still obscure. One phenomenon of potential significance to understanding the system was the observed stimulation of enzyme B activity by the fraction containing the sulphite-cytochrome c oxidoreductase activity. A more critical analysis of the HIC-II fraction is essential before further conclusions can be drawn.

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CHAPTER 6

PURIFICATION AND CHARACTERIZATION OF TWO ESSENTIAL CYTOCHROMES OF THE THIOSULPHATE-OXIDIZING MULTIENZYME SYSTEM

6.1 Introduction

c-type cytochromes are generally regarded as essential components in the chemolithotrophic oxidation of thiosulphate from the facts that they are the primary acceptors for electrons from the substrate oxidation in nearly all of the thiobacilli except probably T. denitrificans [1], and the contents of c-type cytochromes are much higher in autotrophically grown than heterotrophically grown Thiobacillus A2 and T. novellus [2,3]. This type of study was mainly carried out with either crude extract or membrane systems. Although numerous workers have reported the identification and purification of the c-type cytochromes from thiobacilli over more than 20 years [1,4] the investigations were limited to the small, basic c-type cytochromes resembling the mammalian cytochromes and provided little information related directly to the role of c-type cytochromes in thiosulphate oxidation.

Recently we reported [5,6] that the thiosulphate-oxidizing system of Thiobacillus A2 was a multi-enzyme complex composed of up to five soluble components, namely enzyme A, enzyme B, cytochrome $c_{552.5}$, cytochrome c_{551} and sulphite:cytochrome c oxidoreductase, apart from membrane particles which functioned as electron chain components and could be replaced by mammalian cytochrome c and cytochrome oxidase. Enzyme A and enzyme B were highly purified and characterized [6] and an investigation on sulphite: cytochrome c oxidoreductase is to be published elsewhere. The present Chapter describes the purification and some properties of cytochrome $c_{552.5}$ and cytochrome c_{551} as well as two small cytochrome c_{550}. The role of cytochrome $c_{552.5}$ and cytochrome c_{551}, functioning as special redox carriers or effectors, in the thiosulphate-oxidizing system was also investigated and discussed.

6.2 MATERIALS AND METHODS

6.2.1

Growth of the organism and preparation of cell free extract. These were described previously [5-7].

Purification of enzyme A and enzyme B. These were as stated before [6] (Chapter 5)

6.2.2

Enzyme assay. Thiosulphate:cytochrome c-oxidoreductase activity was measured by following the reduction of cytochrome c (horse heart III) essentially as described previously [5-7] (Chapters 3 and 4)

The reaction mixture contained, unless otherwise stated, $Na_2S_2O_3$, 2 μmol; horse heart cytochrome c, 1 mg; enzyme A, 0.05 to 0.1 mg; enzyme B, 0.03 to 0.06 mg; cytochrome $c_{552.5}$, 0.02 to 0.04 mg; cytochrome c_{551}, 0.04 to 0.08 mg; Tris-HCl buffer, pH 7.3, 35 to 45 μmol to give a final volume of 1 ml.

Activities of cytochrome c_{551} and cytochrome $c_{552.5}$ in the thiosulphate: cytochrome c oxidoreducing system were assayed as above except that cytochrome $c_{552.5}$ was omitted for assay of cytochrome c_{551} and vice versa. It was virtually impossible to evaluate the activities of these two cytochromes quantitatively in the course of the purifications mainly due to the facts that (a) the two cytochromes were not completely separated from each other in the 0.35M NaCl (I) and 0.35M NaCl (II) fractions and very small amounts (0.002 mg or less) of cytochrome $c_{552.5}$ (or cytochrome c_{551}) in the reaction mixture for assay of cytochrome c_{551} (or for assay of cytochrome $c_{552.5}$) greatly enhanced the activity and (b) both of them were probably activators or electron carriers to assist the whole enzyme system rather than functioning as enzymes (see Results for further description).

Thiosulphate-oxidizing activity was measured polarographically with a Clark oxygen electrode as described previously [3,6]. The reaction mixture was essentially the same as the spectrophotometric method above except that higher amounts of the two enzymes and the two cytochromes were used as indicated in the individual experiment and bovine heart cytochrome oxidase (5 units) was added.

6.2.3

Terminology. Thiosulphate-oxidizing multi-enzyme system (or complex) means the mixture containing enzyme A, enzyme B, cytochrome $c_{552.5}$, cytochrome c_{551} and sulphite:cytochrome c oxidoreductase or means the mixture containing enzyme A, enzyme B and both or one of the two cytochromes, which are able to start the reaction either spectrophotometrically in the presence of thiosulphate and horse heart cytochrome c (also called thiosulphate:cytochrome c oxidoreductase system) or polarographically in the additional presence of cytochrome oxidase. Sulphite:cytochrome c oxidoreductase was not normally included in the reaction mixture due to the fact that this enzyme was not essential in the assay of thiosulphate oxidation activity, but which did not mean the enzyme was less important than others of the multi-enzyme system *in vivo* (Lu and Kelly, in preparation).

6.2.4

Preliminary purification of c-type cytochromes. As reported before, the crude extract of *Thiobacillus* A2 was fractionated by ammonium sulphate to obtain A65% fraction [5] which was then resolved into five major fractions by chromatography on DEAE-Sepharose CL-6B [5-7] where 0.35M NaCl (I), 0.35M NaCl (II), 0M NaCl and 0.2M NaCl fractions contained cytochrome c_{551}, cytochrome $c_{552.5}$, cytochrome c_{550} (basic) and cytochrome c_{550} (acidic) respectively. From this stage these c-type cytochromes were further purified and detailed in the present paper.

6.2.5

Purification of cytochrome c_{551}

Gel filtration on Sephadex G-100. The 0.35M NaCl (I) fraction (550mg) from the DEAE-Sepharose CL-6B chromatography was loaded on to the bottom of a column (3.2 x 89.5 cm) of Sephadex G-100 equilibrated with 50 mM Tris buffer, pH 7.3, and eluted upwards with the same buffer at 4°C and a flow rate of 30 ml h^{-1}. Cytochrome c_{551}, coming out at the void volume (Fig6.1a) was combined and concentrated by salt out with ammonium sulphate. The concentrated material was called the G-100 preparation. The reasons for using Sephadex G-100 for this step instead

Fig 6.1. Purification of cytochrome $\underline{c}_{551}$ (see Methods for previous procedures and detail). ●, protein (A_{280}); O cytochrome $\underline{c}_{551}$ (A_{416}).

(a) Elution profile of 0.35M NaCl (I) fraction on Sephadex G-100. Void volume was 280 ml. Sulphite:cytochrome c oxidoreductase and enzyme B activities, eluted at the fractions from 310 ml to 390 ml and from 390 ml to 470 ml respectively, are not shown. Fractions containing cytochrome $\underline{c}_{551}$ from 275 ml to 305 ml were pooled and named G-100 fraction.

(b) Elution pattern of the G-100 fraction on Sephadex G-200. Void volume was 165 ml.

(c) Elution profile of the G-100 fraction on Phenyl-Sepharose CL-4B. Cytochrome $\underline{c}_{551}$ existed in all the protein peaks except the first one which contained an unidentified c- type cytochrome.

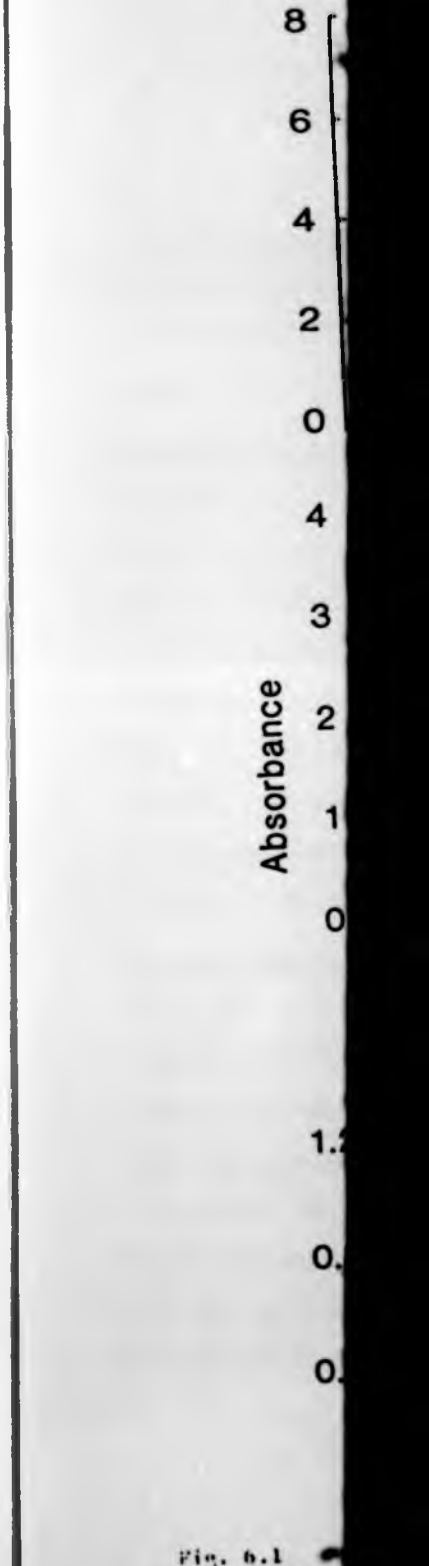

Fig. 6.1

Methods for previous procedures

cytochrome c_{551} (A_{416}).

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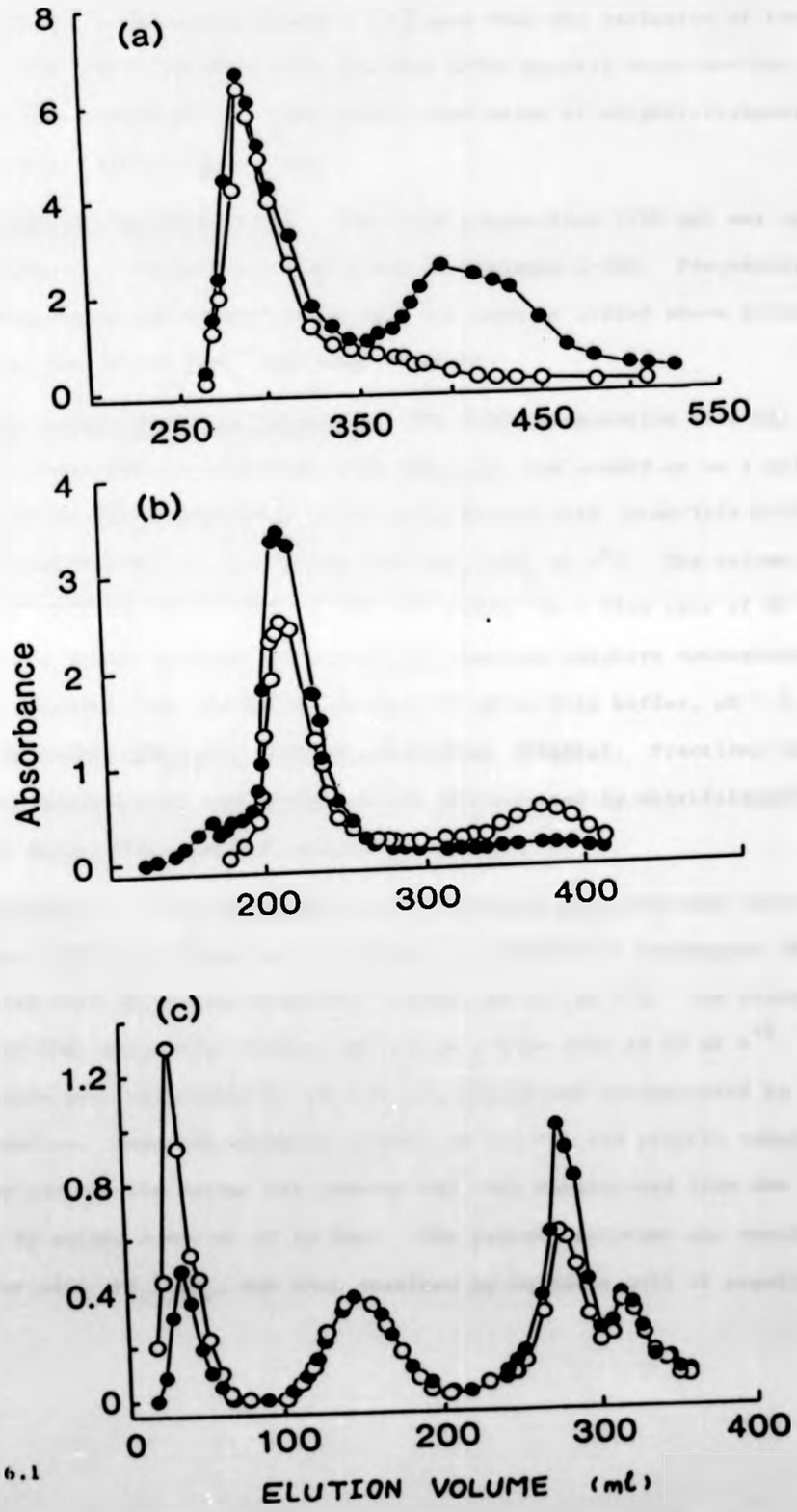


Fig. 6.1

of the Sephadex G-200 used previously [6] were that the exclusion of the cytochrome (Mr about 300,000) from Sephadex G-100 greatly decreased the tailing of the cytochrome and gave better separation of sulphite:cytochrome c oxidoreductase from the cytochrome.

Gel filtration on Sephadex G-200. The G-100 preparation (150 mg) was applied to the bottom of a column (2.6 x 88.5 cm) of Sephadex G-200. Procedures of the chromatography and concentration were the same as stated above except that a flow rate of 18 ml h^{-1} was used (Fig1b).

Hydrophobic interaction chromatography. The G-100 preparation (112 mg) was brought to about 15% of saturation with $(NH_4)_2SO_4$ and loaded on to a column (2.6 x 6 cm) of Phenyl-Sepharose CL-4B equilibrated with 50 mM Tris buffer, pH 7.3, containing 10% of saturation with $(NH_4)_2SO_4$ at 4°C. The column was then eluted with one bed volume of the same buffer at a flow rate of 30 ml h^{-1}, followed by a linear gradient of decreasing ammonium sulphate concentration, which was produced from two 150 ml volumes of 50 mM Tris buffer, pH 7.3, one of which contained $(NH_4)_2SO_4$ (10% of saturation) (Fig1c). Fractions belonging to the same protein peak were combined and concentrated by ultrifiltration through an Amicon PM10 membrane under N_2 pressure.

Chromatofocusing. 25 mg of concentrated cytochrome c_{551} from gel filtration on Sephadex G-200 was loaded on to a column of Polybuffer exchangers PBE 94 equilibrated with 25 mM piperazine-HCl buffer, pH 5.4, at 4°C. The column was eluted with 10% Polybuffer 74-HCl, pH 3.5, at a flow rate of 24 ml h^{-1}. One major protein peak was eluted at pH 3.9-3.7, pooled and concentrated by ultrafiltration. When the effluent reached pH 3.5 the red protein remaining on the top part of the column was removed and then dissociated from the exchanger by adding a few ml of 1M NaCl. The protein solution was concentrated by salt out with $(NH_4)_2SO_4$, and then desalted by Sephadex G-25 if required.

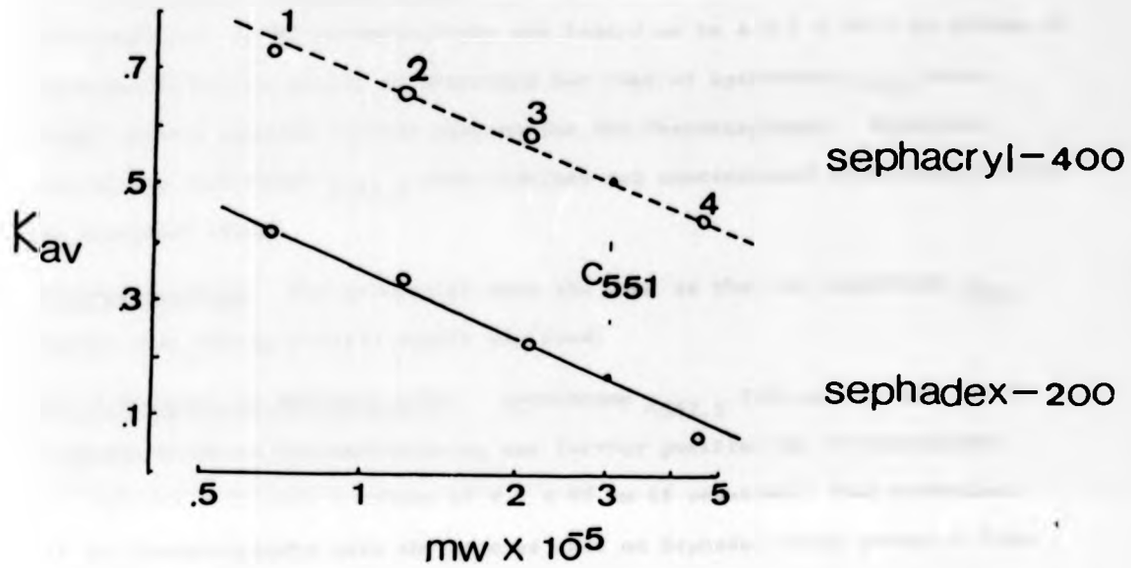


Fig. 6.1d Molecular weight estimation of cytochrome c_{551} by gel filtration on Sephadex G-200 or on Sephacryl S-400.

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Ve = elution volume

Vt = bed volume

Vo = void volume

Molecular weight markers were:

1.	bovine serum albumin	Mr	67,000
2.	lactate dehydrogenase		120,000
3.	catalase		210,000
4.	ferritin		440,000

The molecular weight of cytochrome c_{551} = 300,000

(for the true molecular weight of cytochrome c_{551}, see the text).

6.2.6

Purification of cytochrome $\underline{c}_{552.5}$

Gel filtration on Sephadex G-100. 420 mg of 0.35M NaCl (II) fraction from DEAE-Sepharose CL-6B chromatography was loaded on to a 3.2 x 88.5 cm column of Sephadex G-100 and eluted as described for that of cytochrome $\underline{c}_{551}$ above. Fig62 shows a typical elution pattern for the chromatography. Fractions containing cytochrome $\underline{c}_{552.5}$ were combined and concentrated by ultrafiltration as described above.

Chromatofocusing. The procedures were the same as that of cytochrome $\underline{c}_{551}$ except that 120 mg protein sample was used.

Gel filtration on Sephadex G-75. Cytochrome $\underline{c}_{552.5}$ from gel filtration on Sephadex G-100 or chromatofocusing was further purified by chromatography on Sephadex G-75 with a column of 2.2 x 87 cm if required. The conditions of the chromatography were the same as that on Sephadex G-100 except a flow rate of 18 ml h^{-1} was used.

6.2.7

Purification of cytochrome $\underline{c}_{550}$ (basic). During the chromatography of the A65% fraction on DEAE-Sepharose CL-6B the fractions of the second half of the OM NaCl elution, containing cytochrome $\underline{c}_{550}$, were combined and concentrated by salt out with $(NH_4)_2SO_4$. The concentrated fraction was applied on to a column (2.2 x 87 cm) of Sephadex G-75 and eluted as described above (Fig63a). Fractions containing cytochrome $\underline{c}_{550}$ (with a ratio of A416 to A280 greater than 3.0) were pooled and concentrated by salt out with $(NH_4)_2SO_4$.

6.2.8

Purification of cytochrome $\underline{c}_{550}$ (acidic). Cytochrome $\underline{c}_{550}$ in the 0.2M NaCl fraction from chromatography on DEAE-Sepharose CL-6B was further purified by gel filtration on Sephadex G-100 (Fig.63b) and then on Sephadex G-75 (Fig63c) with the same procedures as stated before. After each gel filtration, fractions containing cytochrome $\underline{c}_{550}$ (with a ratio of A416 to A280 greater than 2 or 2.5) were pooled and concentrated by ultrifiltration as described before.

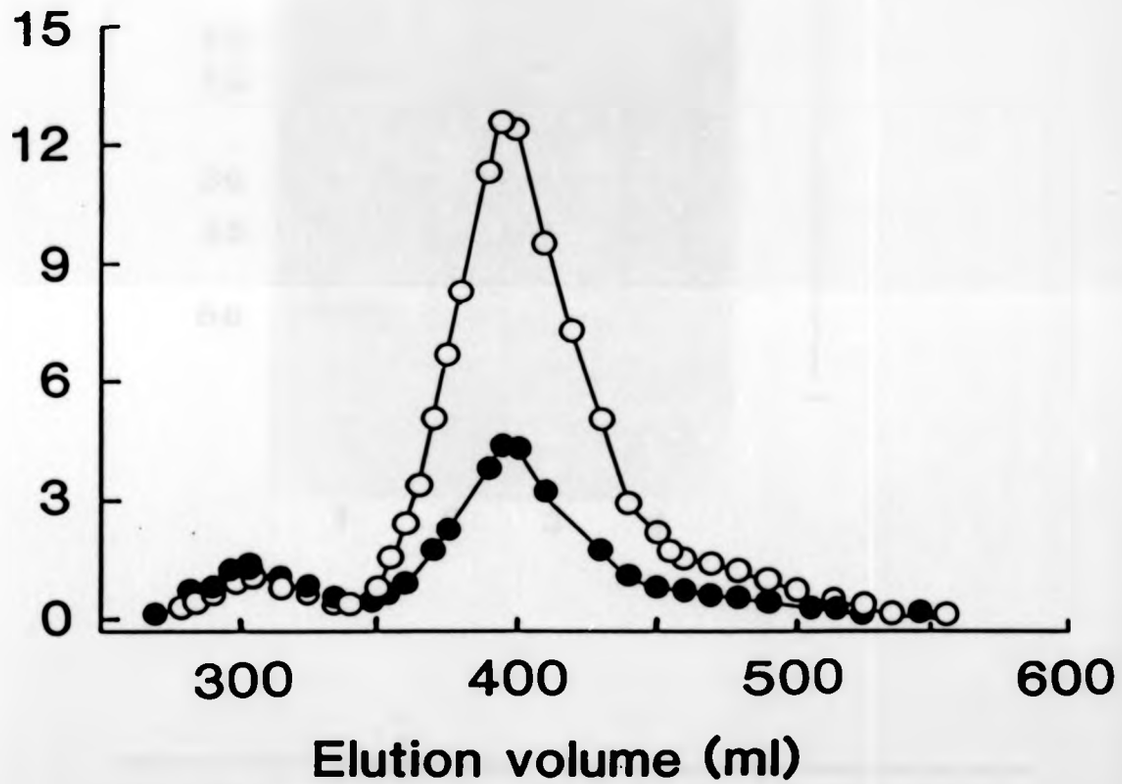


Fig62. Elution profile of cytochrome $c_{552.5}$ (0.35M NaCl II fraction) on Sephadex G-100 (see Methods for details). ● protein, (A_{280}); ○ cytochrome $c_{552.5}$ (A_{416}).

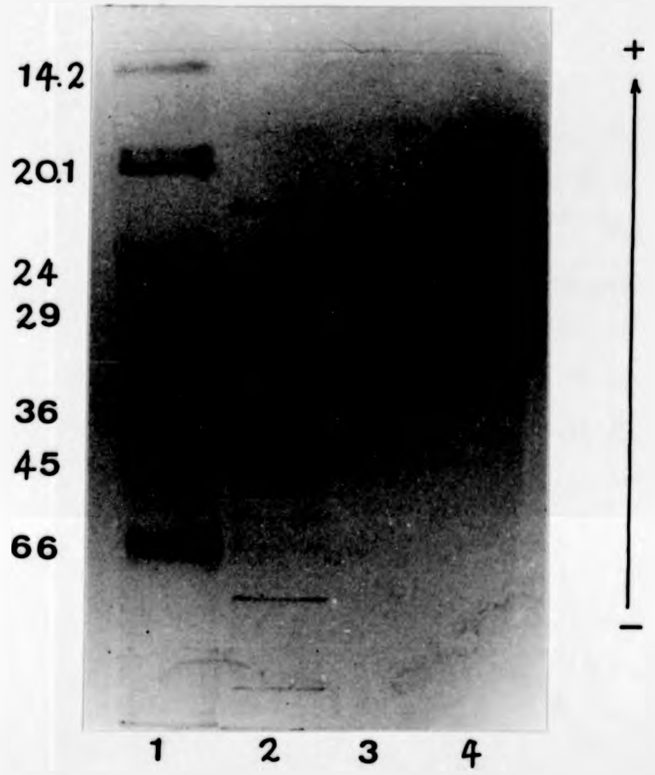


Fig. 6.2b SDS-gel electrophoresis of the fractions in the purification of cytochrome $c_{552.5}$. (1) Protein markers MW (2) 0.35M (II) fraction (3) Sephadex G-100 fraction (4) preparation from chromatofocusing.

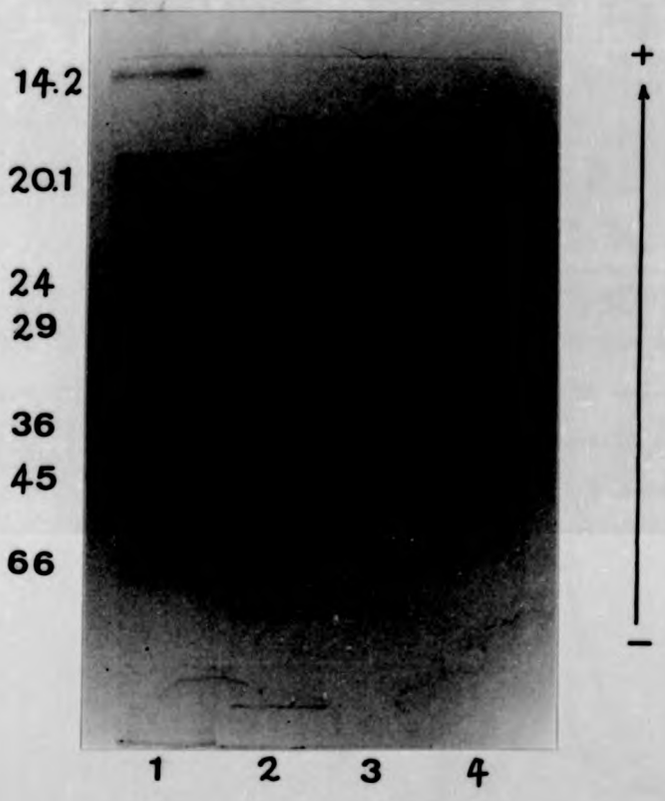


Fig. 6.2b SDS-gel electrophoresis of the fractions in the purification of cytochrome $c_{552.5}$. (1) Protein markers MW (2) 0.35M (II) fraction (3) Sephadex G-100 fraction (4) preparation from chromatofocusing.

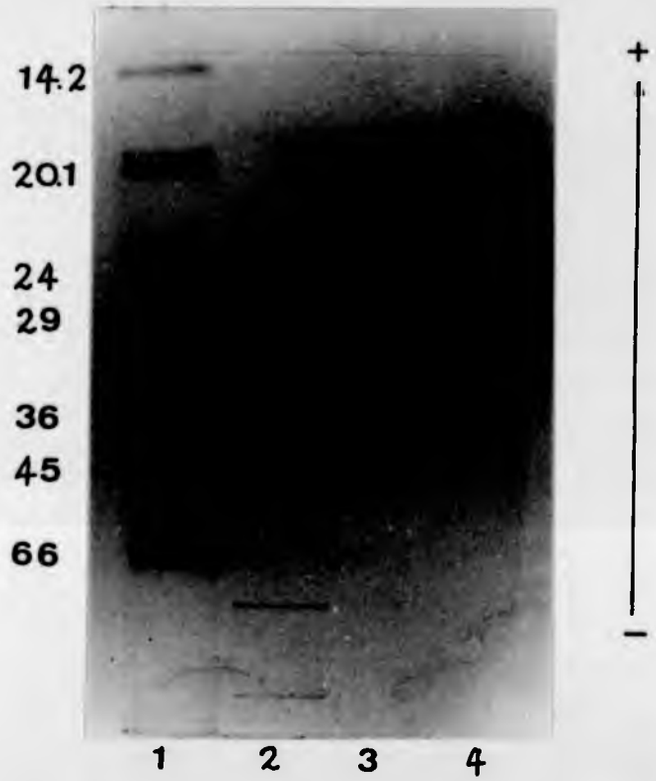


Fig. 6.2b SDS-gel electrophoresis of the fractions in the purification of cytochrome $c_{552.5}$. (1) Protein markers MW (2) 0.35M (II) fraction (3) Sephadex G-100 fraction (4) preparation from chromatofocusing.

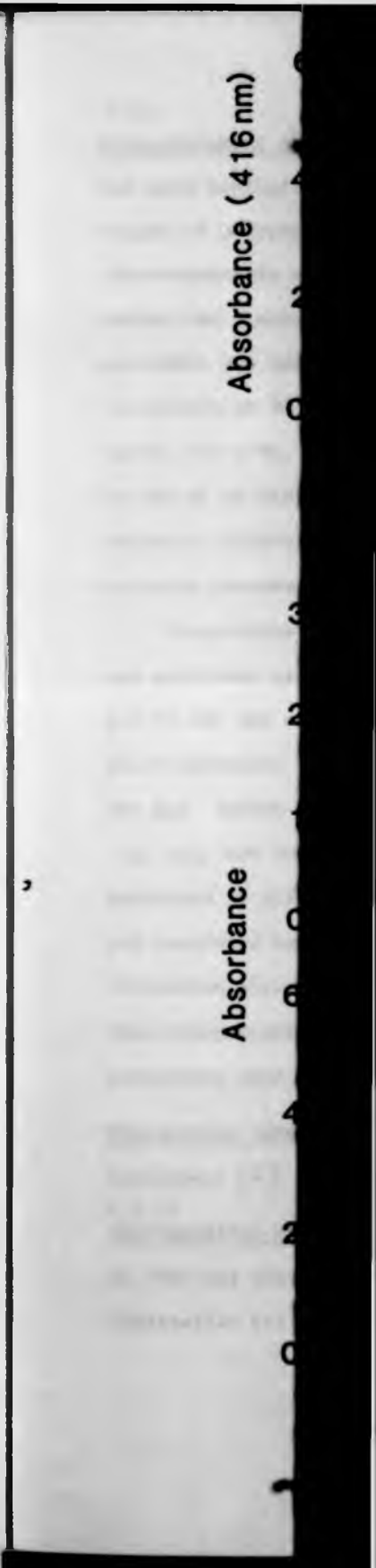
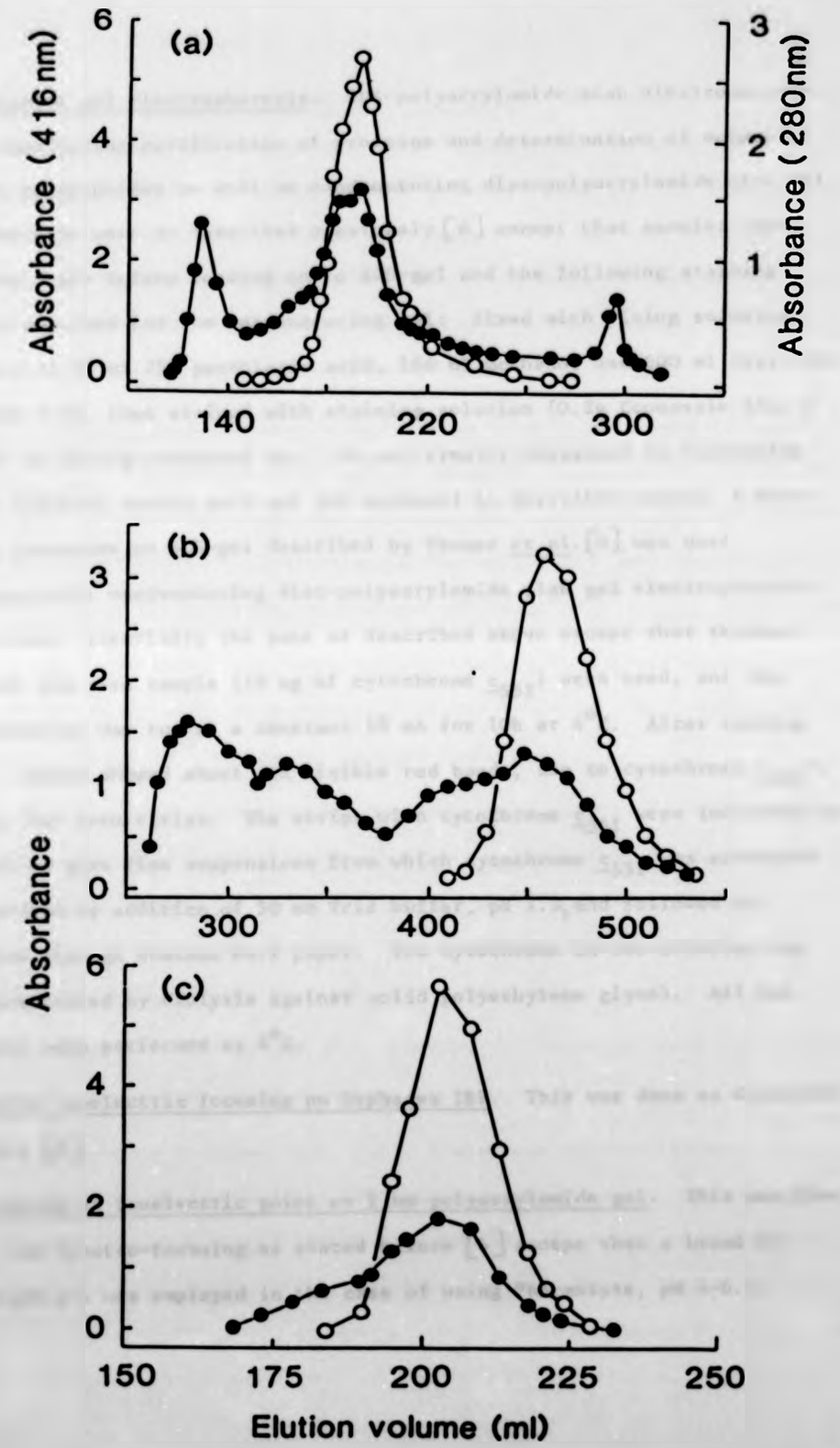


Fig 63 (a) Elution pattern of cytochrome $\underline{c}_{550}$ (basic) (OM NaCl II fraction) on Sephadex G-75. ● protein (A_{280}) ○ cytochrome $\underline{c}_{550}$ (A_{416}) (b) and (c) Purification of cytochrome $\underline{c}_{550}$ (acidic) (b) Gel filtration of 0.2M NaCl fraction on Sephadex G-100 followed by (c) Gel filtration on Sephadex G-75. ● protein (A_{280}); ○ cytochrome $\underline{c}_{550}$ (A_{416}) (see Methods for details).



c_{550} (basic) (OM NaCl II fraction)

(A_{280}) ○ cytochrome c_{550} (A_{416})

tochrome c_{550}(acidic) (b) Gel

on on Sephadex G-100 followed by

G-75. ● protein (A_{280}); ○

thods for details).

6.2.9

Polyacrylamide gel electrophoresis. SDS-polyacrylamide slab electrophoresis for both monitoring purification of proteins and determination of molecular weight of polypeptides as well as nondenaturing disc-polyacrylamide slab gel electrophoresis were as described previously [6] except that samples were boiled for 5 min before loading on to SDS-gel and the following staining procedure was used for the nondenaturing gel: fixed with fixing solution (a mixture of 28 ml 75% perchloric acid, 160 ml methanol and 600 ml distilled water) for 0.5h, then stained with staining solution (0.2g Coomassie Blue G in 400 ml of fixing solution) for 1.5h and finally destained in destaining solution (5%(v/v) acetic acid and 10% methanol in distilled water). A haem-staining procedure on SDS-gel described by Thomas et al.[8] was used.

Preparative nondenaturing disc-polyacrylamide slab gel electrophoresis was performed essentially the same as described above except that thicker gel (3 mm) and more sample (18 mg of cytochrome c_{551}) were used, and the electrophoresis was run at a constant 18 mA for 10h at 4°C. After running the gel (which showed about six visible red bands, due to cytochrome c_{551}), it was cut into strips. The strips with cytochrome c_{551} were individually macerated to give fine suspensions from which cytochrome c_{551} was extracted and separated by addition of 50 mM Tris buffer, pH 7.3, and followed by filtration through Whatman No.1 paper. The cytochrome in the solution was then concentrated by dialysis against solid polyethylene glycol. All the procedures were performed at 4°C.

Preparative isoelectric focusing on Sephadex IEF. This was done as described previously [6]

6.2.10

Determination of isoelectric point on 1 mm polyacrylamide gel. This was done by flat bed electro-focusing as stated before [6] except that a broad PI calibration kit was employed in the case of using Pharmalyte, pH 4-6.5.

6.2.11

Determination of molecular weight by gel filtration. Molecular weight of cytochrome $c_{552.5}$ was determined on Sephadex G-100 using the same procedures as detailed before [6]. Molecular weight of cytochrome c_{551} was measured on Sephadex G-200 and Sephacryl S-400 with essentially the same procedures as that on Sephadex G-100 except that the following molecular weight markers were used: ferritin (Mr 440,000), catalase (Mr 210,000), lactate dehydrogenase (Mr 120,000) and bovine serum albumin (Mr 67,000), (Fig. 6.1d)

6.2.12

Spectrophotometry. Room temperature absorption spectra were done as stated previously [3]. Pyridine haemochromogen spectra were obtained in a mixture containing 10 to 30 ml of cytochrome and equal parts of pyridine and 0.2N KOH to give a final volume of 1 ml after reduction with a few grains of dithionite [9]. A Pye-Unicam SP1700 spectrophotometer was used.

6.2.13

Haem content. The number of c-type haem groups per cytochrome molecule was determined by the pyridine haemochromogen method based on a millimolar extinction coefficient of 29.1 cm^{-1} at 550 nm (reduced band) [10].

6.2.14

Determination of iron. Iron content was measured by atomic absorption spectroscopy using a Rank Hilger instrument (Atomspek H1550). Samples (0.25 mg protein) were prepared by digesting at 100°C (15 min) in 0.5 ml H_2SO_4 + HNO_3 (1 + 1.8 v/v each) and diluted to 2.5 ml for assay. Blanks without protein and with bovine serum albumin (which contains no iron) were used.

Proteins estimation. Protein was determined by the standard Lowry Folin method, using bovine serum albumin as a standard.

Reagents. Cytochrome c (horse heart III), cytochrome oxidase (bovine heart), all of the protein markers used for determination of molecular weight by gel filtration and molecular weight marker kits (MW-SDS-70L), were obtained from Sigma. Sephadex G-75, G-100, G-200, Sephacryl S-400, Phenyl-Sepharose CL-4B, Sephadex IEF, Pharmalyte (pH 2.5-5 and pH 4-6.5). Low and broad PI calibration kits, Polybuffer exchanger PBE94 and Polybuffer 74-HCl were purchased from Pharmacia.

6.3 RESULTS

6.3.1 Purification of cytochrome c_{551}.

Cytochrome c_{551} was purified by the procedures described in the Methods, and the results are summarized in Table 6.1. The reasons why the procedures of HIC and chromatofocusing were not used in the large-scale purification and therefore not included in the summary are presented later. The final purification showed about 85% purity of the cytochrome judged by SDS-gel. Based on this, and assuming a yield of 50%, cytochrome c_{551} represented approximately 1% of the total protein in the crude extract. The purified cytochrome c_{551} was completely free of enzyme B and sulphite:cytochrome c oxidoreductase activities.

6.3.2 Aggregation of cytochrome c_{551}.

One of the peculiar characteristics of cytochrome c_{551} was its polymerization as found during the purification. The cytochrome solution (G-100 fraction) was separated on hydrophobic interaction chromatography (HIC) into four major protein peaks: three of which mainly consisted of the cytochrome with some minor contaminators as shown on SDS-gel (Fig.6.5). Both spectral properties and enzyme assay also confirmed that they were cytochrome c_{551}. The recovery of cytochrome c_{551} after HIC was very low, only about 20% of the sample loaded. Hence, the procedure was not used in the large-scale purification of cytochrome c_{551}.

Whereas cytochrome c_{551} purified after gel filtration and HIC gave only one major band on SDS-gel (Fig.6.4a), the same samples showed multiple bands (about six) on nondenaturing disc-gel (Fig.6.6b) which implied that they might be a sort of isoenzyme . To solve the problem, a large amount of purified cytochrome c_{551} was run by preparative electrophoresis on nondenaturing disc-polyacrylamide gel and then removed from the gel as stated in the Methods. The cytochromes from the six red bands were electrophoresed again on a nondenaturing disc-gel. Each sample continued to show four to six bands with more or less the same pattern on the gel (Fig.6.6b). However, they only gave one major band on SDS-gel (Fig.6.4a). The results strongly suggested that isoenzymes were not involved, otherwise only one original band would appear on the nondenaturing gel. Every

Fig64(a)SDS-gel electrophoresis of the fractions in the purification of cytochrome c_{551} and the fractions from the preparative nondenaturing gel electrophoresis. Marker proteins (1), molecular weights were indicated. Cytochrome c_{551} fractions pooled from Sephadex G-100 (2), from Sephadex G-200 (3), from Sephacryl S-400 (4), from HIC II fraction (5), from HIC III fraction (6) and from HIC IV fraction (7). Lanes (8) to (13) were fractions from the top to the bottom band after preparative nondenaturing gel electrophoresis. Samples contained 30 µg to 80 µg protein.

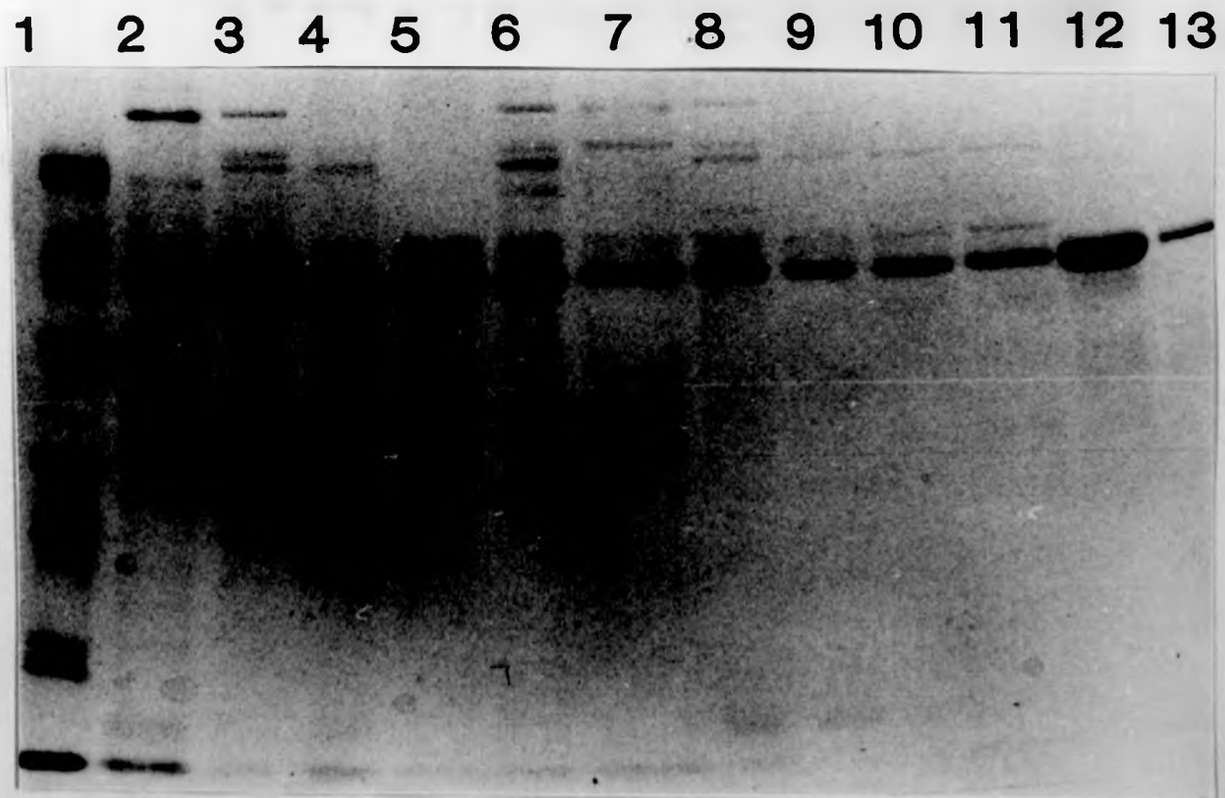


Fig64(a)SDS-gel electrophoresis of the fractions in the purification of cytochrome c_{551} and the fractions from the preparative nondenaturing gel electrophoresis. Marker proteins (1), molecular weights were indicated. Cytochrome c_{551} fractions pooled from Sephadex G-100 (2), from Sephadex G-200 (3), from Sephacryl S-400 (4), from HIC II fraction (5), from HIC III fraction (6) and from HIC IV fraction (7). Lanes (8) to (13) were fractions from the top to the bottom band after preparative nondenaturing gel electrophoresis. Samples contained 30 µg to 80 µg protein.

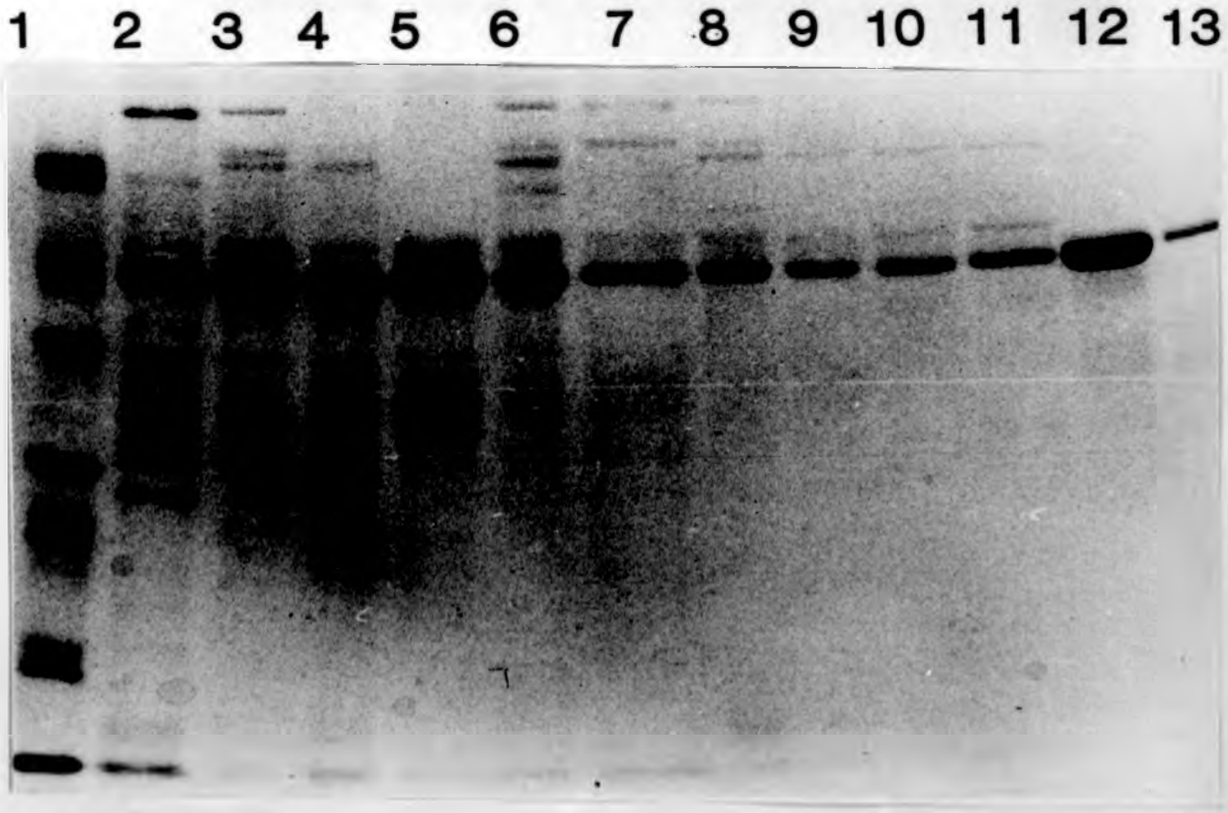
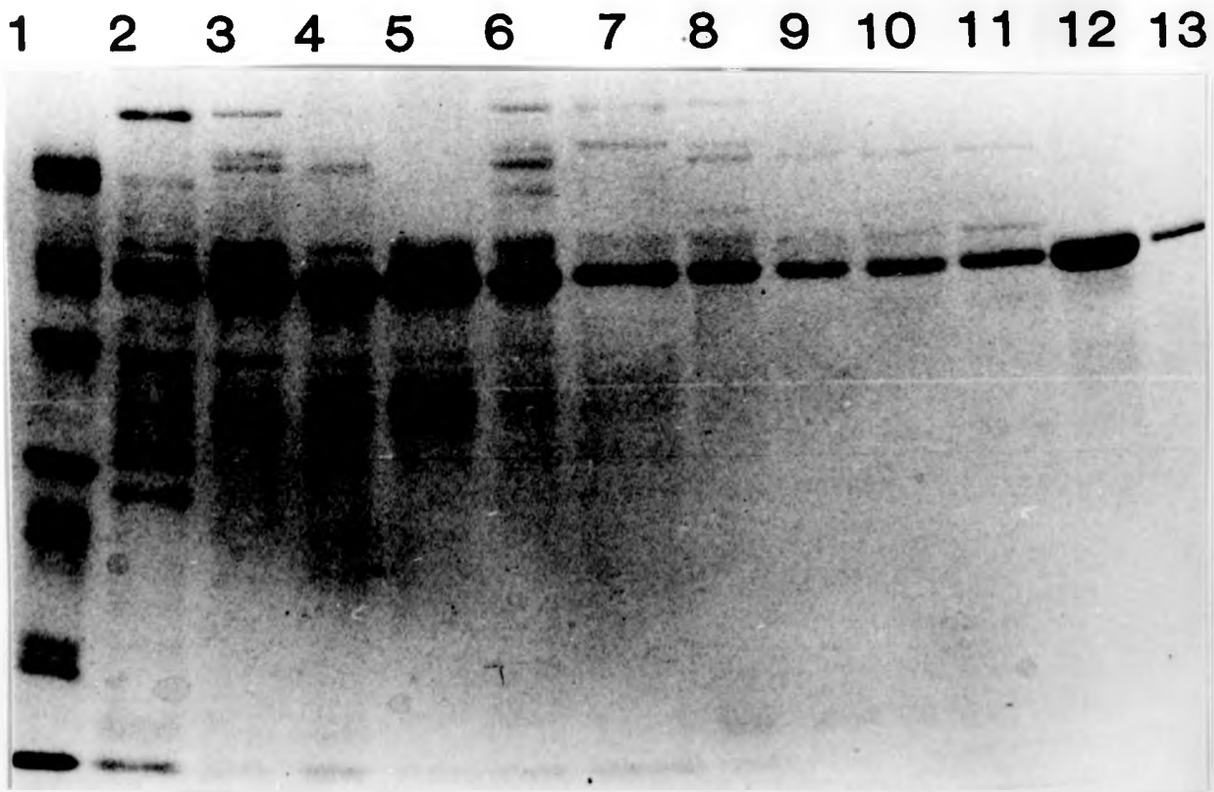


Fig 64 (a) SDS-gel electrophoresis of the fractions in the purification of cytochrome c_{551} and the fractions from the preparative nondenaturing gel electrophoresis. Marker proteins (1), molecular weights were indicated. Cytochrome c_{551} fractions pooled from Sephadex G-100 (2), from Sephadex G-200 (3), from Sephacryl S-400 (4), from HIC II fraction (5), from HIC III fraction (6) and from HIC IV fraction (7). Lanes (8) to (13) were fractions from the top to the bottom band after preparative nondenaturing gel electrophoresis. Samples contained 30 µg to 80 µg protein.



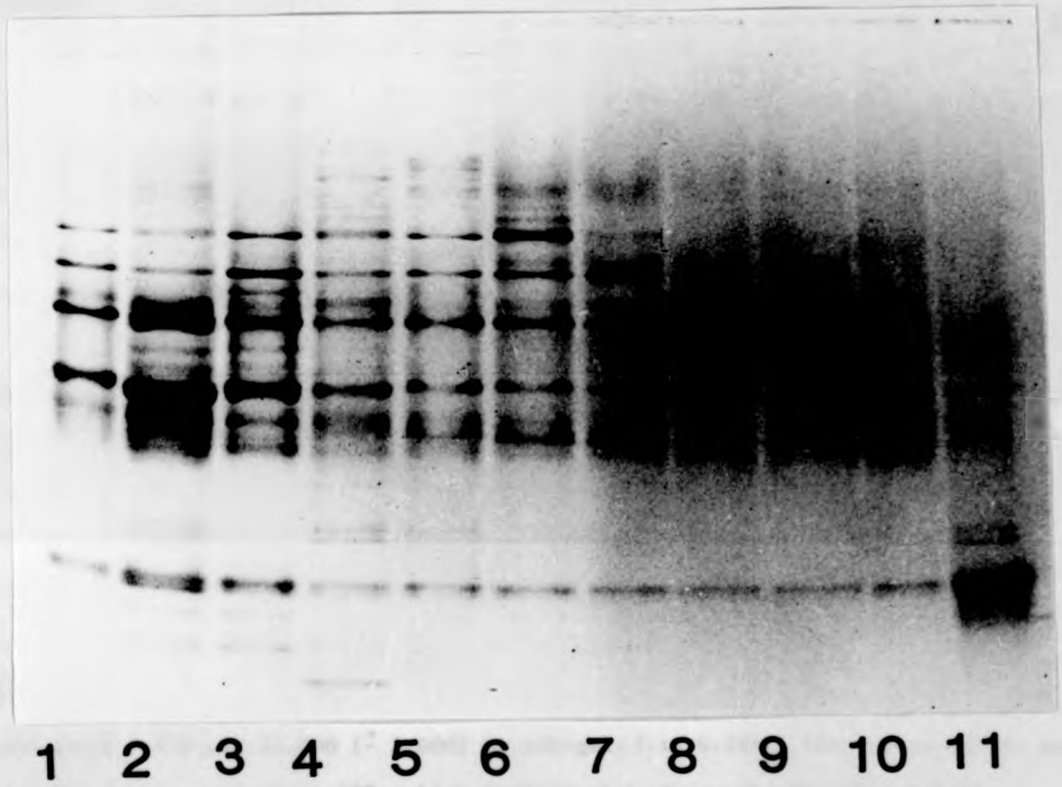


Fig. 6.4 (b) Nondenaturing gel electrophoresis of the fractions in the purification of cytochrome $\underline{c}_{551}$ and the fractions from the preparative nondenaturing gel electrophoresis. Cytochrome $\underline{c}_{551}$ fraction pooled from Sephadex G-100 (1), from Sephadex G-200 (2), from HIC II fraction (3), from HIC III fraction (4), and from HIC IV fraction (5). Lanes (6) to (11) were fractions from the top band to the bottom band after the preparative nondenaturing gel electrophoresis. Samples contained 30 µg to 80 µg protein. See Methods and Results for details.

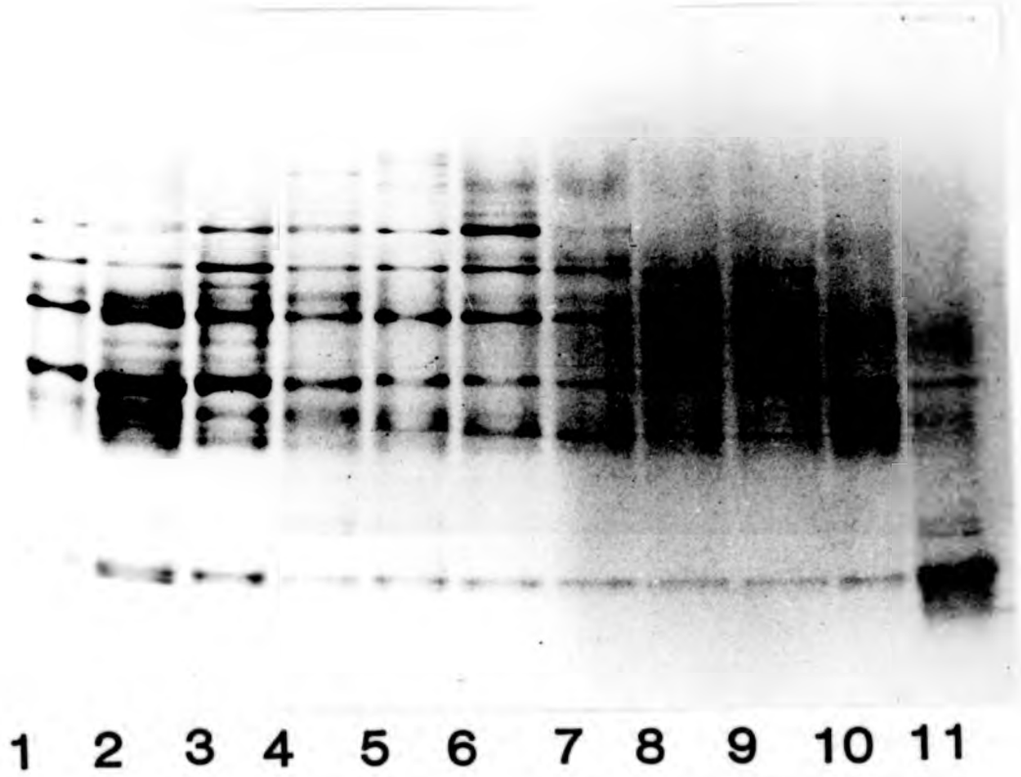


Fig. 6.4 (b) Nondenaturing gel electrophoresis of the fractions in the purification of cytochrome $\underline{c}_{551}$ and the fractions from the preparative nondenaturing gel electrophoresis. Cytochrome $\underline{c}_{551}$ fraction pooled from Sephadex G-100 (1), from Sephadex G-200 (2), from HIC II fraction (3), from HIC III fraction (4), and from HIC IV fraction (5). Lanes (6) to (11) were fractions from the top band to the bottom band after the preparative nondenaturing gel electrophoresis. Samples contained 30 µg to 80 µg protein. See Methods and Results for details.

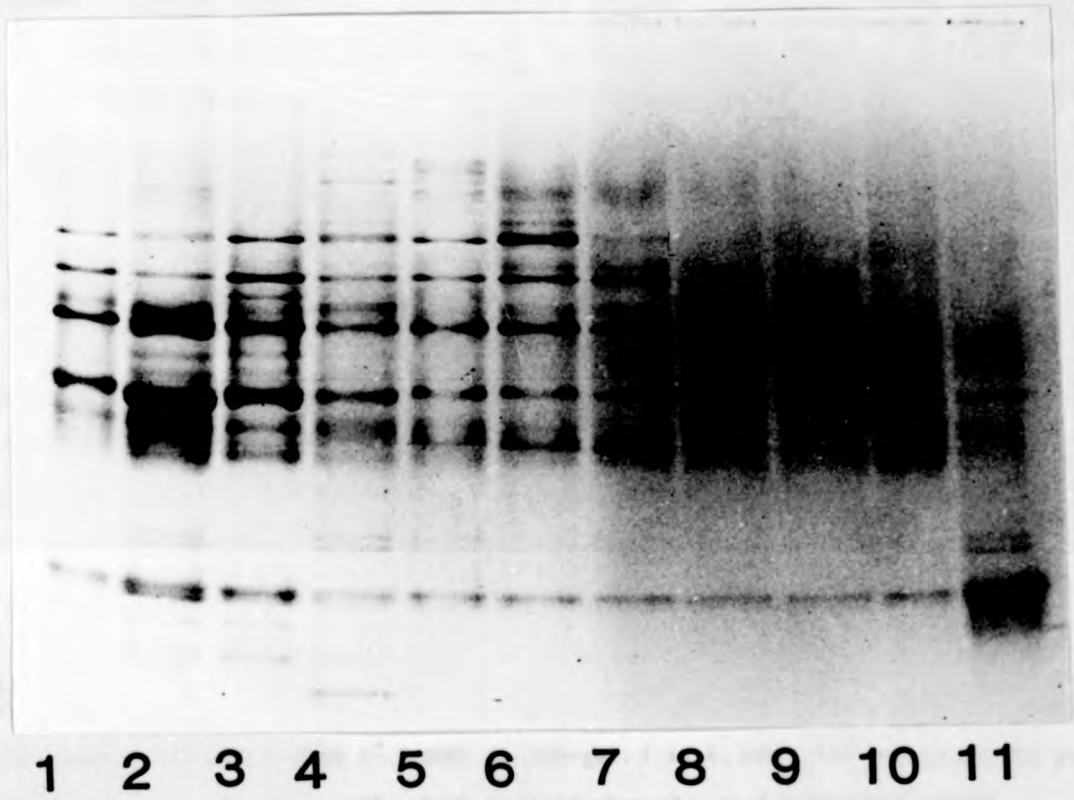


Fig. 6.4 (b) Nondenaturing gel electrophoresis of the fractions in the purification of cytochrome $\underline{c}_{551}$ and the fractions from the preparative nondenaturing gel electrophoresis. Cytochrome $\underline{c}_{551}$ fraction pooled from Sephadex G-100 (1), from Sephadex G-200 (2), from HIC II fraction (3), from HIC III fraction (4), and from HIC IV fraction (5). Lanes (6) to (11) were fractions from the top band to the bottom band after the preparative nondenaturing gel electrophoresis. Samples contained 30 µg to 80 µg protein. See Methods and Results for details.

fraction also showed some stimulatory activity to the thiosulphate:cytochrome c oxidoreductase system.

It appeared, therefore, that cytochrome c_{551} formed a series of aggregates from monomer to hexamer. A plot of the Rf values of the six bands on the nondenaturing disc-gel against the molecular weights of the monomer to the hexamer on semi-logarithmic paper exhibited a straight line. This finding also meant that the subunits were non-covalently bound to each other and their association and dissociation were in a constant equilibrium.

The formation of the aggregates and their equilibrium also help to explain the diverse elution pattern of cytochrome c_{551} on HIC; thus different sizes of aggregates probably had different hydrophobic interaction strengths with the column and were eluted at different times, and the further purification of the cytochrome was difficult simply because of the trapping of contaminating proteins during the polymerization.

6.3.3

Molecular weight of cytochrome c_{551}. The molecular weight of cytochrome c_{551} was about 300,000 estimated by gel filtration on Sephadex G-200 or on Sephacryl S-400 and 43,000 ($\pm$ 2,000) by SDS-gel. (Fig.6.1d). The purity of the purified cytochrome c_{551} was about 85%, which implied that the real molecular weight of the aggregated cytochrome c_{551} was about 260,000. The figure was in good agreement with the molecular weight of the aggregate composed of six polypeptides each of the same size, 43,000.

6.3.4

Some observations on the haem prosthetic groups of cytochrome c_{551}. Acid acetone (0.012 N HCl in acetone) did not extract the haem groups from cytochrome c_{551}, judged by the observation that the red colour stayed with the precipitated proteins, which indicated that the haem groups were covalently attached to the apoprotein. However, as shown on Fig 1b, a certain amount of haem (about 10 - 20%), recognised by absorbance at 416 nm, was eluted at the end of the chromatography on Sephadex G-200 with very little protein (A_{280}) in these fractions. The observations accounted for the very low increase of purity index ($A_{551\ red}/A_{280\ ox}$; Table 1) and might have indicated release of haem

TABLE 6.1. PURIFICATION OF CYTOCHROME c_{551}

Stage	Protein (mg)	Purity Index* ($A_{551\ red}/A_{280\ ox}$)
Crude extract	39,500	-
Ammonium Sulphate fraction (A65%)	11,400	-
DEAE-Sepharose CL-6B (0.35M NaCl (1))	1,150	0.13
Sephadex G-100	355	0.24
Sephadex G-200	185	0.26

*Owing to the presence of nucleic acids and other c-type cytochromes, assay of the purity index was impossible before the ion exchange chromatography stage.

from the cytochrome. The possibility that this haem came from other sources could not be completely ruled out.

The loss of haem from cytochrome c_{551} was also exhibited in SDS-gel electrophoresis, where the green haem band stained as described in the Methods appeared at the front line. However, if the sample was treated without boiling, a certain amount of haem was seen at the band position characteristic of the apoprotein.

Cytochrome c_{551} (G-100 fraction) was separated into two major fractions after chromatofocusing, one fraction eluted at pH 4.5 to 4.2 containing colourless proteins, and another eluted after addition of 1M NaCl containing haem group as indicated by the red colour. Neither the single fractions nor a mixture of the two retained ability to stimulate the activity of the thiosulphate:cytochrome c oxidoreductase system. On SDS-gel the colourless fraction showed a band the same as that of native cytochrome c_{551}, and the haem-containing fraction gave several bands, one of them with the same position as that of native cytochrome c_{551}.

6.3.5

Spectral properties and haem content of cytochrome c_{551}. Fig65a shows the absorption spectrum of the purified cytochrome c_{551}. Ascorbate reduced cytochrome c_{551} to about 70% of the fully reduced state whereas dithionite completely reduced the cytochrome. The absorption maxima were 551, 552 and 418 nm in the reduced form with both reductants and 410 nm in the oxidized form. On the basis of a molecular weight of 260,000 and a purity of 85% the millimolar extinction coefficients were calculated to be 40 cm^{-1} from $A_{551-540}$ and 64 cm^{-1} from absolute absorbance at A_{551} (Fig65a).

The pyridine ferrohaemochromogen of cytochrome c_{551} exhibited an absorption spectrum typical of c-type cytochrome with maxima at 550, 521 and 414 nm (Fig65b). By the same method of calculation, the millimolar extinction coefficient was found to be 96 cm^{-1} at 550 nm, from which the number of haem groups per cytochrome c_{551} molecule was estimated to be 3.5. The iron content per molecule of cytochrome c_{551} was determined to be 5.1 $\pm$ 0.8 (4) atoms/mole. From these

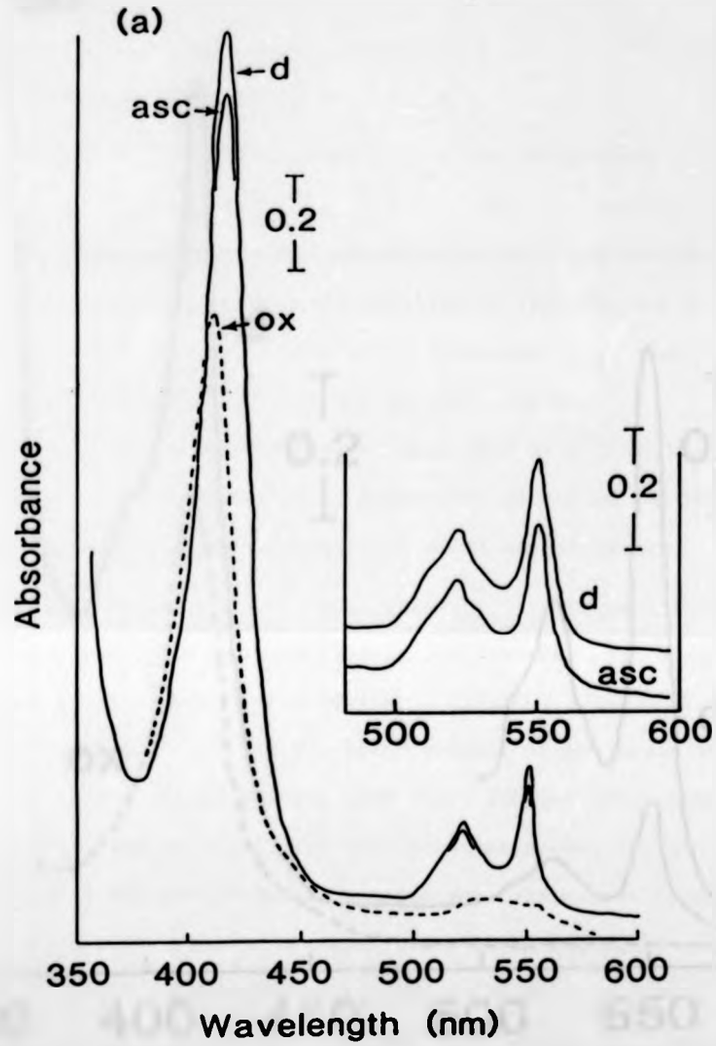


Fig65(a)Absorption spectrum of cytochrome c_{551} (---ox) oxidized; (——asc) ascorbate-reduced;(——— d) dithionite-reduced. 1 cm cuvette contained 1.36 mg of purified cytochrome c_{551} in 50 mM Tris-HCl buffer, pH 7.3 in a final volume of 1 ml. The ascorbate-reduction and dithionite-reduction were allowed to proceed for 25 min and 5 min respectively before recording the spectra.

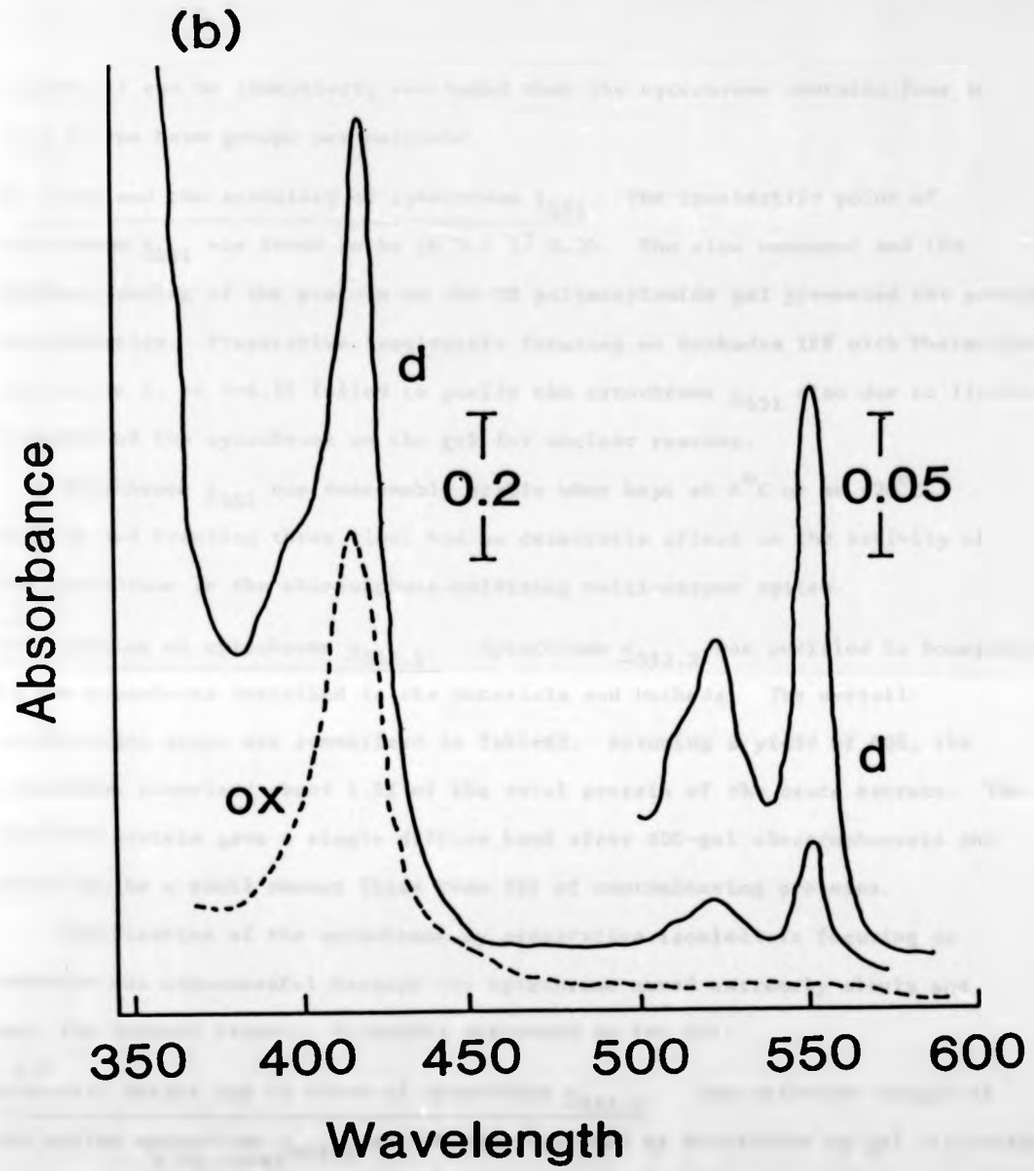


Fig. 6.5 (b) Absorption spectrum of pyridine haemochromogen of cytochrome c_{551} (– – – ox) oxidized, (——— d) dithionite-reduced. 1 cm cuvette contained 0.6 mg of purified cytochrome c_{551} in 1 ml of alkaline-pyridine solution.

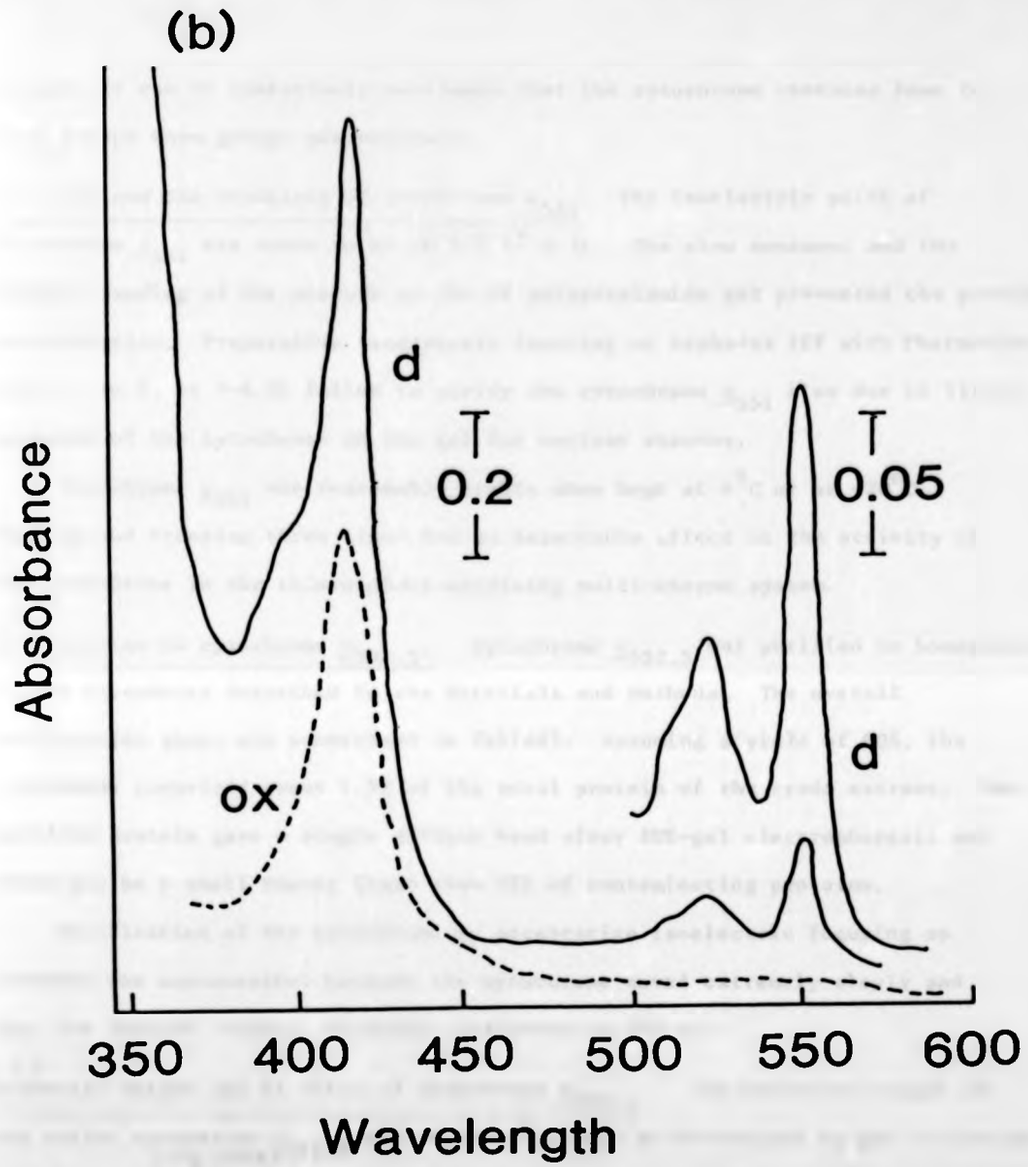


Fig. 6.5 (b) Absorption spectrum of pyridine haemochromogen of cytochrome c_{551} (– – – – ox) oxidized, (——— d) dithionite-reduced. 1 cm cuvette contained 0.6 mg of purified cytochrome c_{551} in 1 ml of alkaline-pyridine solution.

results it can be tentatively concluded that the cytochrome contains four to five c-type haem groups per molecule.

6.3.6
PI value and the stability of cytochrome c_{551}. The isoelectric point of cytochrome c_{551} was found to be pH 5.2 (± 0.3). The slow movement and the diffuse banding of the protein on the 5% polyacrylamide gel prevented the precise determination. Preparative isoelectric focusing on Sephadex IEF with Pharmalyte (pH 2.5 to 5, or 4-6.5) failed to purify the cytochrome c_{551} also due to little movement of the cytochrome on the gel for unclear reasons.

Cytochrome c_{551} was reasonably stable when kept at 4°C or at -20°C. Thawing and freezing three times had no detectable affect on the activity of the cytochrome in the thiosulphate-oxidizing multi-enzyme system.

6.3.7
Purification of cytochrome $c_{552.5}$. Cytochrome $c_{552.5}$ was purified to homogeneity by the procedures described in the Materials and Methods. The overall purification steps are summarized in Table 62. Assuming a yield of 60%, the cytochrome comprised about 1.5% of the total protein of the crude extract. The purified protein gave a single diffuse band after SDS-gel electrophoresis and there may be a small amount (less than 2%) of contaminating proteins.

Purification of the cytochrome by preparative isoelectric focusing on Sephadex was unsuccessful because the cytochrome moved extremely slowly and was, for unknown reasons, virtually unfocused on the gel.

6.3.8
Molecular weight and PI value of cytochrome $c_{552.5}$. The molecular weight of the native cytochrome $c_{552.5}$ was 56,000 (± 2,000) as determined by gel filtration on Sephadex G-100 (Fig. 5.10b). After SDS-gel electrophoresis a single polypeptide species was found, corresponding to a subunit molecular weight of about 29,000 (± 2,000). Thus the cytochrome appeared to be a dimer of subunits of equal molecular size.

The isoelectric point of cytochrome $c_{552.5}$ was measured by isoelectric focusing on polyacrylamide gel and a value of 4.8 (± 0.2) was obtained.

TABLE 62. PURIFICATION OF CYTOCHROME $c_{552.5}$

Purification step	Protein (mg)	Purity index* ($A_{552.5\ red}/A_{280\ ox}$)
Crude extract	39,500	-
Ammonium sulphate fraction (A65%)	11,400	-
DEAE-Sepharose CL-6B (0.35M NaCl (II))	962	0.45
Sephadex G-100	568	0.73
Chromatofocusing	378	0.77

*Due to the presence of nucleic acids and other c-type cytochromes determination of the purity index was impossible before the ion exchange chromatography.

6.3.9

Spectral properties and haem content of cytochrome $\underline{c}_{552.5}$. Absorption maxima of ascorbate- or dithionite-reduced cytochrome $\underline{c}_{552.5}$ were at 552.5, 523 and 418 nm and that of the oxidized form at 415 nm as shown in Fig 6.6. But ascorbate only partially reduced the cytochrome (about 65% of totally reduced state) whereas dithionite completely reduced the cytochrome. On the basis of a molecular weight of 56,000 the millimolar extinction coefficient of 28 at $A_{552.5-540}$ and of 44 at $A_{552.5}$ were obtained for fully reduced cytochrome $\underline{c}_{552.5}$.

Pyridine haemochromogen of dithionite-reduced cytochrome $\underline{c}_{552.5}$ revealed a typical c-type absorption spectrum with maxima at 550, 520 and 415 nm. The millimolar extinction coefficient of the band was determined to be 67 cm^{-1}, from which a value of 2.3 haem groups per molecule of cytochrome $\underline{c}_{552.5}$ was estimated. The iron content was found to be 2.2 Fe per cytochrome $\underline{c}_{552.5}$. Therefore it appears that the cytochrome contains two c-type haem group per molecule.

Acid acetone (0.012N HCl in acetone) did not extract the haem groups from cytochrome $\underline{c}_{552.5}$, which indicated that they were covalently attached to the apoprotein.

Cytochrome $\underline{c}_{552.5}$ was reasonably stable when kept at 4°C or -20°C. Thawing and freezing three times had no detectable affect on the activity of the cytochrome in the thiosulphate-oxidizing multi-enzyme system.

6.3.10

Studies on the involvement of cytochrome $\underline{c}_{552.5}$ and cytochrome $\underline{c}_{551}$ in thiosulphate oxidation. Both cytochrome $\underline{c}_{552.5}$ or cytochrome $\underline{c}_{551}$ could effect the reduction of mammalian cytochrome c by thiosulphate in the presence of enzyme A and enzyme B as shown previously [6]. The rate of the reduction with cytochrome $\underline{c}_{552.5}$ as catalyst showed a progressive increase while cytochrome $\underline{c}_{551}$ gave a progressively decreasing rate, i.e. the rate reached the highest level immediately after the start of the reaction, then slowly declined. A possibly related phenomenon was shown on Table 4 where adding cytochrome $\underline{c}_{552.5}$ into the reaction mixture as the last component gave lower activity than did adding cytochrome $\underline{c}_{551}$ last. It seems that both cytochromes interacted with enzyme A and enzyme B in some way and the interaction with cytochrome $\underline{c}_{551}$ gave lower activity, which also

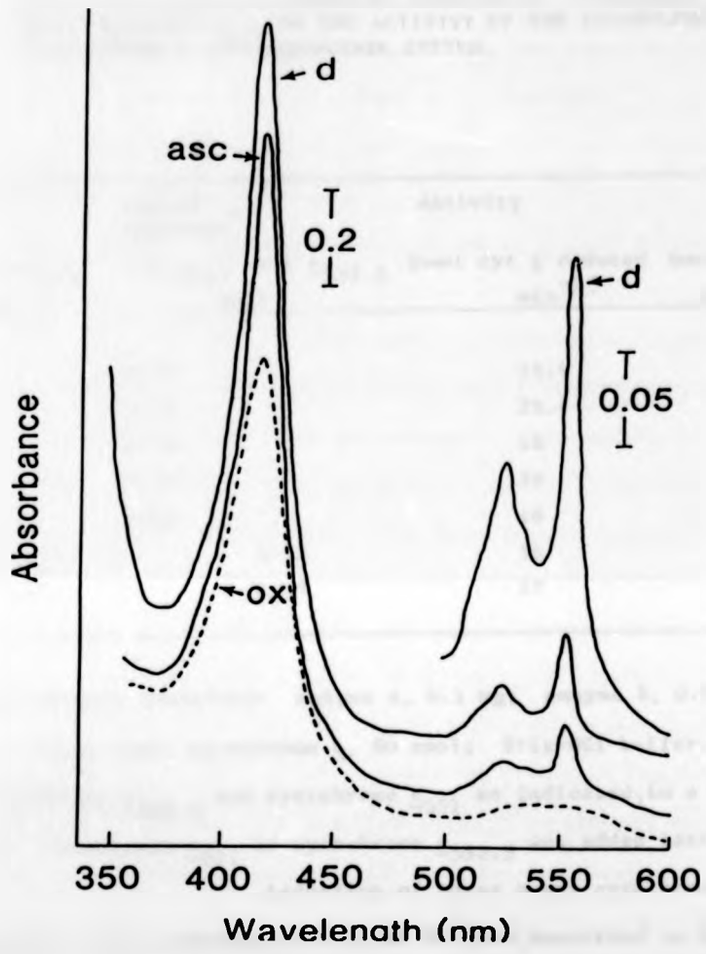


Fig 6.6 Absorption spectrum of cytochrome $\underline{c}_{552.5}$:(– – – – ox) ,oxidized;(——asc), ascorbate-reduced ; (——d) dithionite-reduced. 1 cm cuvette contained 0.32 mg of highly purified cytochrome $\underline{c}_{552.5}$ in 50 mM Tris-HCl buffer, pH 7.3, in a final volume of 1 ml. The ascorbate-reduction and dithionite reduction were allowed to proceed for 15 min and 2 min respectively before recording the spectra.

TABLE64. EFFECT OF AMOUNT AND ORDER OF ADDITION OF CYTOCHROME $\underline{c}_{552.5}$ AND CYTOCHROME $\underline{c}_{551}$ ON THE ACTIVITY OF THE THIOSULPHATE: CYTOCHROME $\underline{c}$ OXIDOREDUCTASE SYSTEM.

First addition*		Second addition*		Activity	
cyt $\underline{c}_{552.5}$ (mg)	cyt $\underline{c}_{551}$ (mg)	cyt $\underline{c}_{551}$ (mg)	cyt $\underline{c}_{552.5}$ (mg)	(nmol cyt $\underline{c}$ reduced min^{-1})	(nmol cyt $\underline{c}$ reduced min^{-1} mg^{-1})
0.03		0.12		21.4	70
0.08		0.12		21.4	58
0.018		0.12		18	61
0.03		0.17		28	79
0.03		0.08		18	67
	0.12		0.03	16	52
	0.12		0.08	17	46

*Basic reaction mixture contained: enzyme A, 0.1 mg; enzyme B, 0.08 mg; $S_2O_3^{2-}$, 2 µmol; horse heart cytochrome $\underline{c}$, 80 nmol; Tris-HCl buffer, pH 7.3 45 µmol and cytochrome $\underline{c}_{552.5}$ and cytochrome $\underline{c}_{551}$ as indicated, in a final volume of 1 ml. Cytochrome $\underline{c}_{551}$ or cytochrome $\underline{c}_{552.5}$ was added last as indicated. Reduction of horse heart cytochrome $\underline{c}$ at 550 nm was recorded spectrophotometrically at 30°C as described in Methods.

prevented the good interaction between cytochrome $\underline{c}_{552.5}$ and the two enzymes and caused the lower activity if cytochrome $\underline{c}_{551}$ was added before cytochrome $\underline{c}_{552.5}$. Table64 also demonstrates that the amount of cytochrome $\underline{c}_{552.5}$ to promote the activity of the multi-enzyme system was about 5 times less than that of cytochrome $\underline{c}_{551}$.

Although the single addition of cytochrome $\underline{c}_{552.5}$ or cytochrome $\underline{c}_{551}$ with enzyme A and enzyme B catalysed the reduction of mammalian cytochrome by thiosulphate, the oxidation of thiosulphate to sulphate was complete only in the presence of all four compounds (Table65) apart from the presence of cytochrome oxidase and horse heart cytochromes. The complete oxidation of thiosulphate was also confirmed by experiments with ^{35}S-labelled thiosulphate to measure the formation of sulphate. The reaction mixture without cytochrome $\underline{c}_{552.5}$ gave no reaction at all. However, the reaction mixture without cytochrome $\underline{c}_{551}$ did show very low rate of oxygen uptake and an incomplete oxidation of thiosulphate with a ratio of O_2 to $S_2O_3^{2-}$ about 1. This finding was interesting in the sense that it showed a partial reaction, but the low oxidation rate did not enable us to separate and identify the intermediates or the product(s) of the partial reaction using ^{35}S-labelled thiosulphate.

The different observations obtained with the spectrophotometric method and with oxygen electrode methods were probably due to (a) the former method being much more sensitive (over 100 times in the terms of number of electrons transported for a response of one division on the chart paper) than the latter; (b) the reaction conditions were not the same: for example, the intensive stirring used in the oxygen electrode might affect or disrupt interactions between components of the complex which were essential to the reaction. A typical profile of oxygen electrode measurement was shown on Fig. 6.7

Table66 shows that cytochrome $\underline{c}_{552.5}$ and cytochrome $\underline{c}_{551}$ as well as cytochrome $\underline{c}_{550}$ (basic) were gradually reduced by thiosulphate in the presence of enzyme A and enzyme B. The reduction of cytochrome c_{550} (basic) was

TABLE65. OXIDATION OF THIOSULPHATE BY THE PURIFIED COMPONENTS

Reaction mixture	Rate of O_2 uptake (nmol O_2 min^{-1})	$S_2O_3^{2-}$ added (nmol)	O_2 uptake (nmol)
Complete	22	100	204
—cytochrome $\underline{c}_{552.5}$	no reaction		
—cytochrome $\underline{c}_{551}$	1.6	100	88
—Enzyme A	no reaction		
—Enzyme B	no reaction		

The experiment was done in an oxygen electrode as described before [3]. Standard reaction mixture contained: enzyme A, 0.4 mg; enzyme B, 0.2 mg; cytochrome $\underline{c}_{552.5}$, 0.09 mg; cytochrome $\underline{c}_{551}$, 0.25 mg; horse heart cytochrome $\underline{c}$, 0.5 mg; bovine heart cytochrome oxidase, 5 units, and Tris-HCl buffer 50 mM, pH 7.3, to a final volume of 1 ml. $Na_2S_2O_3$ (100 nmol precisely) was added to start the reaction. All of the components were highly purified, and there was no sulphite:cytochrome $\underline{c}$ oxidoreductase activity at all in the enzyme B and the cytochrome $\underline{c}_{551}$ fractions. All of the figures were means of three experimental results. A detailed description of the involvement of sulphite:cytochrome $\underline{c}$ oxidoreductase in the thiosulphate oxidation is in preparation (Lu and Kelly, unpublished).

TABLE 6.6 REDUCTION OF c-TYPE CYTOCHROMES BY ENZYME A AND ENZYME B WITH THIOSULPHATE AND THE EFFECT OF SUBSEQUENT ADDITION OF SULPHITE:CYTOCHROME c OXIDOREDUCTASE, SULPHITE AND DITHIONITE

Cytochrome	µM	Change in absorbance at α-band maximum for each cytochrome					Ratio of B/E[c]
		A	B	C	D	E	
		No addition (background)	After addition of $NO_2S_2O_3$	Rate (maximum) (ΔAα-band per min)	Effect of [a] addition of "sulphite oxidase" to B (ΔAα-band)	Effect of dithionite (ΔAα-band)	
Cytochrome $c_{552.5}$	11.4	0.23	0.394	0.06	0.394 [b]	0.51 [d]	0.59
Cytochrome c_{551}	11.5	0.18	0.38	0.05	0.75	0.8 [d]	0.67
Cytochrome c_{550} (basic)	25		0.45	0.02	-	0.45 [e]	
Cytochrome c_{550} (acidic)	50		0	0			
Horse heart Cytochrome c	13		0.4	0.003	-	0.4 [e]	

The reaction mixture contained: enzyme A, 0.06 mg; enzyme B, 0.05 mg; $S_2O_3^{2-}$, 2 µmol; Tris buffer, 40 µmol, pH 7.3 and cytochrome as indicated in a total volume of 1 ml. The reaction was started by adding enzyme B and the increase in the absorbance at the α-band of the cytochrome being assayed was followed spectrophotometrically. Both enzyme A and enzyme B were necessary for the reduction of the c-type cytochromes.

[a] Sulphite:cytochrome c oxidoreductase (10 µg).

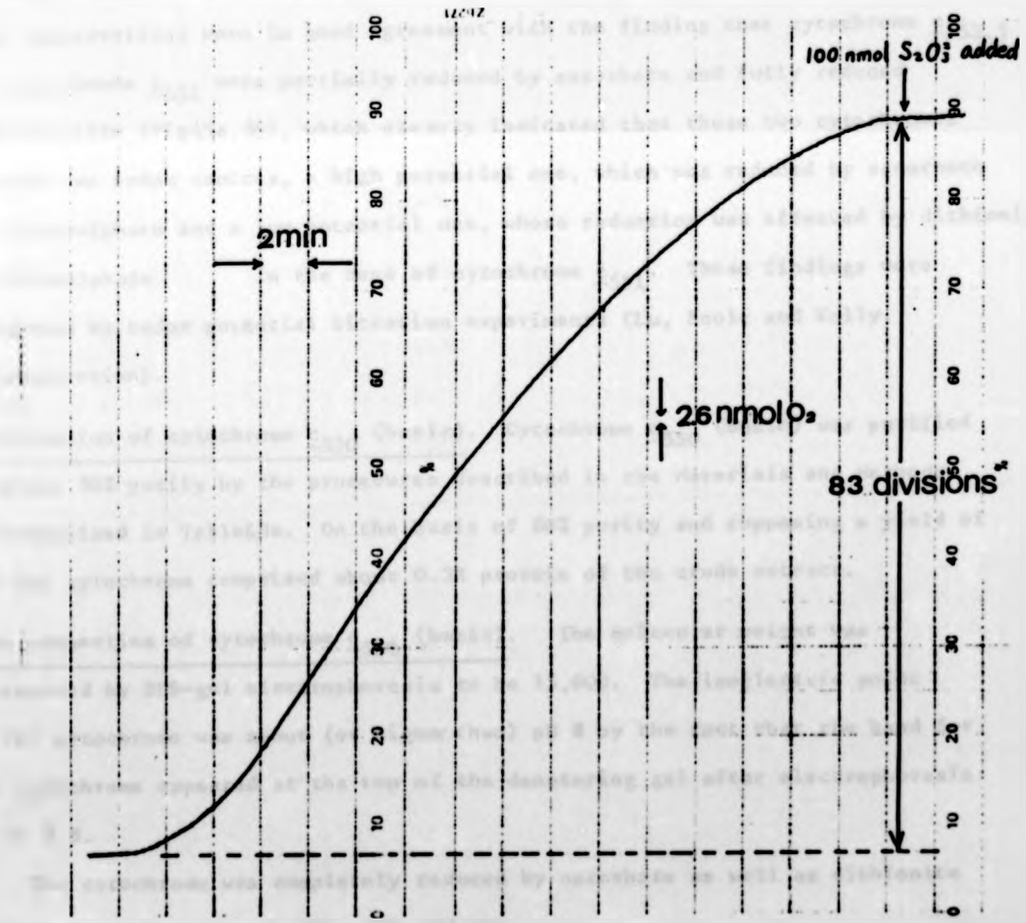
[b] Na_2SO_3 (2 µmol) added with the sulphite oxidase

[c] Background values deducted from B and E.

[d] Dithionite added after treatment D.

[e] Dithionite added after treatment B

Fig. 6.7 A typical profile of the oxygen electrode experiment to determine the stoicheiometry of thiosulphate oxidation by the highly purified components. The experimental conditions were as described in the legend to Table 6.5 except that the final volume was 0.95 ml. Thus, 205 nmol (2.6 x 83 x 0.95) of O_2 was consumed for the oxidation of 100 nmol of thiosulphate.



complete in the sense that the supplementary addition of dithionite did not increase the absorbance any more, whereas reductions of cytochrome $\underline{c}_{552.5}$ and cytochrome $\underline{c}_{551}$ were only partial, about 60% and 70% of fully reduced form respectively. Cytochrome $\underline{c}_{551}$ was nearly fully reduced by supplementary addition of sulphite:cytochrome $\underline{c}$ oxidoreductase. The reduced cytochrome $\underline{c}_{551}$ was slowly and partially re-oxidized. On the other hand adding sulphite and sulphite:cytochrome $\underline{c}$ oxidoreductase did not effect the further reduction of cytochrome $\underline{c}_{552.5}$, which was completely reduced by dithionite. Horse heart cytochrome $\underline{c}$ also was fully reduced by thiosulphate in the presence of enzyme A and enzyme B although the rate was very slow. These observations were in good agreement with the finding that cytochrome $\underline{c}_{552.5}$ and cytochrome $\underline{c}_{551}$ were partially reduced by ascorbate and fully reduced by dithionite (Fig65a,66), which clearly indicated that these two cytochromes contain two redox centres, a high potential one, which was reduced by ascorbate and thiosulphate and a low potential one, whose reduction was effected by dithionite or thiosulphate in the case of cytochrome $\underline{c}_{551}$. These findings were confirmed by redox potential titration experiments (Lu, Poole and Kelly, in preparation).

6.3.11

Purification of cytochrome $\underline{c}_{550}$ (basic). Cytochrome $\underline{c}_{550}$ (basic) was purified to about 80% purity by the procedures described in the Materials and Methods and summarised in Table63a. On the basis of 80% purity and supposing a yield of 50% the cytochrome comprised about 0.3% protein of the crude extract.

Some properties of cytochrome $\underline{c}_{550}$ (basic). The molecular weight was determined by SDS-gel electrophoresis to be 15,000. The isoelectric point of the cytochrome was about (or higher than) pH 8 by the fact that the band for the cytochrome appeared at the top of the denaturing gel after electrophoresis at pH 8.8.

The cytochrome was completely reduced by ascorbate as well as dithionite with absorption maxima at 550, 521 and 415

TABLE 63. PURIFICATION OF CYTOCHROME c_{550}(basic) and c_{550}(acidic)

Purification step	Protein (mg)	Purity index* ($A_{550\ red}/A_{280\ ox}$)
a) c_{550}(basic)		
Crude extract	49,100	-
Ammonium sulphate fraction (A65%)	12,000	-
DEAE-Sepharose CL-6B (OM NaCl(II)fraction)	234	0.25
Sephadex G-75	55	0.83
b) c_{550}(acidic)		
Crude extract	39,500	-
Ammonium sulphate fraction (A65%)	11,400	-
DEAE-Sepharose CL-6B (0.2M NaCl)	660	0.18
Sephadex G-100	125	0.51
Sephadex G-75	50	0.76

*see Table 1

The absorption maxima of the pyridine ferrohaemochromogen of cytochrome $\underline{c}_{550}$ (basic) were at 550, 521, 414 nm. On the basis of the molecular weight of the cytochrome and a purity of 80% the millimolar extinction coefficient was found to be 26 cm^{-1} (Δ_{550}), which was very close to that of mammalian cytochrome c. Therefore, the cytochrome apparently contained one haem group per molecule.

Cytochrome $\underline{c}_{550}$ (basic) was slowly but completely reduced by thiosulphate in the presence of enzyme A and enzyme B (Table 66) as was horse heart cytochrome c. However, the cytochrome did not accelerate the oxidation of thiosulphate by the multi-enzyme system measured either spectrophotometrically or polarographically.

6.3.12
Purification of cytochrome $\underline{c}_{550}$ (acidic). Cytochrome $\underline{c}_{550}$ (acidic) was purified to about 70% purity by the procedures described in the Methods and summarised in Table 63b. The cytochrome did not precipitate in any concentration of ammonium sulphate, so the ultrafiltration procedure was used to concentrate the cytochrome in the purification.

Some properties of cytochrome $\underline{c}_{550}$ (acidic). The molecular weight was estimated by SDS-gel electrophoresis to be 14,000, but was about 29,000 by gel filtration on Sephadex G-75. This presumably indicates the formation of a dimer composed of two 14,000 Mr subunits. The isoelectric point was found at pH 5 ($\pm$ 0.2) by isoelectric focusing on polyacrylamide gel.

The cytochrome was completely reduced by ascorbate or dithionite with absorption maxima at 550, 552 and 415 nm. The absorption maxima of its pyridine ferrohaemochromogen were at 550, 520 and 414 nm. The millimolar extinction coefficient was 36 cm^{-1} (A_{550}), indicating one haem group per mole.

The cytochrome was not reduced by thiosulphate at all in the presence of enzyme A and enzyme B (Table 66), and had no effect on the thiosulphate-oxidising system.

6.4 DISCUSSION

The novel redox carriers or effectors of the thiosulphate-oxidising

system, cytochrome $\underline{c}_{552.5}$ and cytochrome $\underline{c}_{551}$, have been purified from Thiobacillus A2. They are termed c-type cytochromes mainly because of their spectral and haem group properties, although in many aspects such as high molecular weight, presence of several subunits and haem groups and, most of all, the presence of two redox centres, they are quite different from the c-type cytochromes previously found in living organisms. The two cytochromes comprise about 2.5% of the total crude extract protein, which indicates their importance. It is also worth noting that this thiosulphate-oxidizing multi-enzyme system (two enzymes and two cytochromes) represents some 4% of the crude extract protein or about 3.2% of the total protein of the organism.

From the facts that cytochrome $\underline{c}_{552.5}$ and cytochrome $\underline{c}_{551}$ are necessary components for the thiosulphate-oxidizing multi-enzyme system and both of them can easily be reduced by thiosulphate in the presence of enzyme A and enzyme B, the two cytochromes appear to be the primary electron carriers linking oxidation by the two enzymes to the next components of the electron transport chain, which could be membrane-bound or associated c-type cytochrome(s).

Furthermore, since the thiosulphate oxidizing activity of the multi-enzyme system in the presence of both cytochrome $\underline{c}_{552.5}$ and cytochrome $\underline{c}_{551}$ is much higher than that in the presence of only one of them, cytochrome $\underline{c}_{552.5}$ and cytochrome $\underline{c}_{551}$ also appears to function as effectors. The mechanism of this effect might be that (a) molecular interactions between the two cytochromes or between them and enzyme A and enzyme B, or (b) as thiosulphate oxidation involves the transfer of 4 pairs of electrons, one or other cytochrome might accept electron specifically from particular oxidation reactions, such as sulphane-sulphur oxidation steps or from sulphonate sulphur, so that if only one of them is present in the reaction mixture some partial product(s) of the reaction would accumulate which would prevent or retard further reaction(Table 5). There is some evidence to support point (b) showing that cytochrome $\underline{c}_{551}$ might be a redox carrier for electrons from sulphonate-sulphur, as follows: (i) from the purification results, cytochrome $\underline{c}_{551}$ is very closely associated with

sulphite:cytochrome c oxidoreductase, and the total separation of the two causes dramatic loss (around 90%) of the activity of sulphite:cytochrome c oxidoreductase; (ii) the low activity was variably increased by 15-30% in several experiments by the addition of a small amount of cytochrome c_{551} and only about 10% by that of cytochrome $c_{552.5}$ (Lu and Kelly in preparation); (iii) the low redox potential centre of cytochrome c_{551} is partially reduced by sulphite: cytochrome c oxidoreductase . If further evidence shows this to be the true situation, one can expect that cytochrome $c_{552.5}$ probably serves as a direct acceptor of electrons from sulphane-sulphur, for which direct evidence is lacking. The partial oxidation of thiosulphate catalysed by cytochrome $c_{552.5}$, two enzymes and the electron chain (Table 5), could mean that intermediates may accumulate and would be available for detection and identification, provided that the problem of low activity can be overcome by improving the experimental conditions, for example, by increasing the amounts of the components, decreasing or omitting stirring, or finding some further stimulatory cofactor.

Although an Mr of 260,000 was established for cytochrome c_{551} in this paper, the molecular weight of the active unit of the cytochrome remains uncertain. The combination of the six polypeptides of cytochrome c_{551} was indicated to be non-covalent because the subunits were easily dissociated. As about four to five haem groups are present per molecule of cytochrome c_{551} it is unlikely that the single polypeptide unit is the active form of the cytochrome.

The aggregation property of cytochrome c_{551} suggests that the cytochrome contains hydrophobic surfaces for interaction with the lipid membrane, which is in agreement with the fact that the cytochrome is a redox carrier between the membrane system and enzyme A and enzyme B, and may be a property of enzymes normally bound to membranes as was shown, for example, for UDP-glucose-

lipolysaccharide glucosyltransferase I from Salmonella typhimurium [11]. Actually it is not surprising to find this similarity if one bears in mind that not only cytochrome c_{551} but the whole of the multi-enzyme system is a respiratory enzyme complex attaching intimately to the membrane system in some way.

Recently a cytochrome c_{551} was purified from another facultative thiobacillus, T. novellus [12, 13]. This cytochrome was shown to be tightly bound to the sulphite:cytochrome c oxidoreductase, and Mr 23,000 (by SDS-gel electrophoresis) or about half that of the cytochrome c_{551} from Thiobacillus A2. It is interesting to note that the two c_{551} cytochromes show some similarities such as both being involved in sulphite oxidation, having the same PI value (5.2) and more or less the same spectral properties at room or liquid nitrogen temperatures and of their pyridine ferrohaemochromogens. Only two c-type cytochromes (the other being a small cytochrome c_{550}) have, however, been identified and purified from T. novellus so far.

Two distinct c_{551} cytochromes involved in thiosulphate metabolism were also purified and characterized from two thiosulphate-oxidizing Chlorobium strains [14, 15]. One of them had an Mr of 45,000 (by SDS-gel electrophoresis), two haem groups per molecule and was shown to be reduced by thiosulphate in the presence of a thiosulphate-cytochrome c_{551} reductase [16]. Coincidentally, this reductase had an Mr, 80,000 which may be compared to a combined Mr of about 80,000 for enzymes A and B of Thiobacillus A2 [6]. The reduction rate was greatly enhanced on addition of cytochrome c_{555}, a flavin-cytochrome of Mr, 50,000. Although the system in the photosynthetic bacteria may not be directly comparable with that in chemolithotrophs, these findings do suggest an important and general role in the oxidation of thiosulphate for these novel c-type cytochromes, which are characterized by their large molecular weights, low PI values and often containing more than one haem group per protein.

Acknowledgements

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CHAPTER 7

OXIDATION-REDUCTION POTENTIALS OF THE MULTIHAEM CYTOCHROME $\underline{c}_{551}$ AND CYTOCHROME $\underline{c}_{552.5}$ AND THE LOW TEMPERATURE SPECTRA OF THE PURIFIED AND MEMBRANE CYTOCHROMES.

7.1 INTRODUCTION

The thiosulphate-oxidizing multienzyme system (complex) of T. versutus consists of four major components, enzyme A, enzyme B, cytochrome $\underline{c}_{551}$ and cytochrome $\underline{c}_{552.5}$, which can affect the complete oxidation of thiosulphate in the presence of mammalian cytochrome c and cytochrome oxidase or cytochrome c and membrane fraction from T. versutus and these components have been highly purified from the organism (Chapter 5, Chapter 6). Cytochrome $\underline{c}_{551}$ and cytochrome $\underline{c}_{552.5}$ are essential parts of the multienzyme system, and probably function as electron transfer carriers between the membrane-cytochrome $\underline{c}_{552}$ and enzyme A and enzyme B (Chapter 6). It has also been shown that cytochrome $\underline{c}_{551}$ (Mr 260,000) contained five to six haems and cytochrome $\underline{c}_{552.5}$ (Mr 52,000) two haems. Each of them had two redox centres, the high one could be reduced by ascorbate and the low one was only effected by dithionite or by thiosulphate in the presence of sulphite oxidase in the case of cytochrome $\underline{c}_{551}$ (Chapter 6). Cytochrome $\underline{c}_{550}$ (basic) and cytochrome $\underline{c}_{550}$ (acidic) were also partially purified from T. versutus, but their functions were obscure (Chapter 6).

In the present work, midpoint redox potentials of cytochrome $\underline{c}_{551}$ and cytochrome $\underline{c}_{552.5}$ were measured in an initial attempt to assess the sequence of their involvement as electron carriers. The liquid nitrogen temperature difference spectra of the four purified soluble c-type chromosomes, A65% fraction and the membrane fraction, were also studied to characterize further these redox components.

7.2 Methods

Growth of T. versutus (A2) and the preparation of cell-free extract and membrane fraction. These were described previously (see 2.2; 3.2).

Purification of cytochrome c_{551}, cytochrome $c_{552.5}$, cytochrome c_{550} (basic) and cytochrome c_{550} (acidic). These were as stated before (see 6.2).

7.2.1 Oxidation-reduction titration. The method employed was based on that described by Dutton (1978). Absorption spectra were recorded with a Johnson Research Foundation dual-wavelength spectrophotometer, which had two monochromators: one was set at a fixed reference wavelength, (540 nm in the present work) and the other was used to scan the sample. Spectra of samples at different potentials could be automatically subtracted from each other by means of a digital memory computer which stored any selected spectrum and subtracted it from the following spectra. A home-made anaerobic glass cuvette as described by Dutton (1978) with a path length of approximately 1 cm and a working volume of give to nine ml was used. Oxidation-reduction potentials were measured with a Beckman (Model 4500) pH/mV meter using a combination platinum and calomel electrodes. The meter was calibrated by immersing the electrode in 200 ml of a solution of 0.85 g $K_4Fe(CN)6.3H_2O$ and 0.66 g $K_3Fe(CN)_6$ at 25°C to give a reading of 184 mV, which was equivalent to an E_h of 429 mV. The value of +245 mV was used to correct the potentials read from the meter to actual E_h, potential with standard hydrogen electrodes as the reference electrode. The titrations were conducted at 25°C maintained by water circulation to the cuvette base. To achieve mediation between the platinum electrode and the cytochrome tested, the following dyes (Em 7 and μm) were used: Ferricyanide (+430 mV, 20 μm), quinlydrone (+280 mV, 20 μm), N,N,N,'N'-tetramethyl-p-phenylenediamine (+260 mV, 40 μm), 1,2,napthaquinone-4-sulphonate (+215 mV, 40 μm), 1,2,napthaquinone (+143 mV, 20 μm), trimethylhydroquinone (+115, 200 μm),

phenazine methosulphate (+65 mV, 200 μm), 2-methyl-1,4,napthoquinone (+10 mV, 400 μm), tetramethyl-p-benzoquinone (+5 mV. 2400 μm), 2-hydroxy-1,4 nathaquinone (-145 mV, 25 μm), riboflavin-5-(p)(-219 mV, 25 μm), anthroquinone-2-sulphonate (-225 mV, 25 μm), benzyl viohogen (-350 mV, 2 μm), methyl viohogen (-430 mV, 1 μm). Tetramethyl-p-benzoquinine and 2-hydroxy-1,4 nathaquinone were dissolved in ethanol while the others in distilled water. After the addition of buffer (0.1M phosphate, pH 7.0), the mediators and sample into the cuvette argon gas, which had been moistened by bubbling through water, was sparged into the solution for 15 min before start of the titration and gassing was maintained during the titration to create a slight back pressure in the anaerobic system. A fresh solution of sodium dithionite in 1M phosphate buffer, pH 7.5 served as the reductant while potassium ferricyanide (1M in 0.1M phosphate buffer, pH 7.0) was used to supply oxidizing equivalents. Addition of reductant or oxidant were made with a 25 μl Hamilton microsyringe and each addition was terminated when the absorbance became constant following a 20-40 mV oxidation-reduction potential change. When a cytochrome exhibits two midpoint potentials with similar spectral properties a sigmoid curve of the logarithm of the ratio of oxidized to reduced against oxidation-reduction potential resulted.

An alternative method of analyzing the data involved plotting % reduction (i.e. % of the maximum absorbance change seen at the specified wavelength pair) against E_h (mV). From the sigmoid curve thus obtained, estimates of the relative proportions of the two potentiometrically distinct species were made and of their midpoint potentials. Assuming an n-value of 1, Nernstian curves satisfying the above parameters were calculated, using a simple programme for a Hewlett-Packard HP-33E hand-held calculator, and superimposed on the plotted data. E_m values and proportions of the two species were optimised by eye on a trial and error basis.

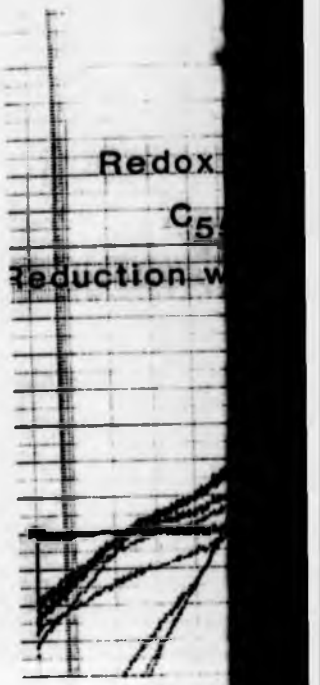
7.2.2 77K difference spectra. Difference spectra at liquid nitrogen temperature were obtained as described by Salmon and Poole (1980) using a Pye Unicam SP1700 spectrophotometer, fitted with an accessory constructed in the Department of Microbiology, Queen Elizabeth College, London. This consisted of a Dewar flask, of which the lower 3 cm was silvered, and which was positioned close to the photomultiplier. A brass cuvette holder, the lower end of which was immersed in liquid nitrogen in the Dewar flask, held two small (0.5 ml capacity, 2 mm path length) Perspex cuvettes, about 5 cm from the photomultiplier. Solutions of cytochromes, either reduced with Na dithionite, or oxidized with NH_4 persulphate, were pipetted into the cuvettes held in the brass holder. The cuvettes were then immediately frozen by immersion of the holder with cuvettes in liquid nitrogen.

7.2.3 Effect of CO on the difference spectra of the purified c- type cytochromes at room temperature. This was done by sparging the sample solution in cuvette with CO gas for 10 min before scanning with the oxidized spectrum as the reference and using the dual-wavelength spectrophotometer described above.

7.2.4 Reagents. All chemicals were of the highest grade commercially available.

7.3 Results

7.3.1 Oxidation-reduction titration of the purified cytochrome c_{551} and cytochrome $c_{552.5}$. Simultaneous measurements of potentials and absorbance changes were carried out for cytochrome c_{551} over a wide potential range from -400 mV to +340 mV, which are shown in Fig. 7.1a (reductive titration) and Fig. 7.1b (oxidative titration). Another set of the titrations but with higher concentrations of some mediators gave the same results. When the logarithm of the ratio of the oxidized form to reduced form (assuming that the increase in absorbance on reduction is proportional to reduced form) is plotted against the redox potential, the titration curve is sigmoid (Fig. 7.2), indicating the presence of two midpoint



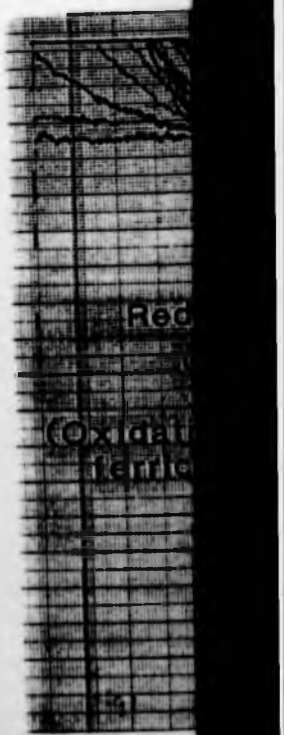


Fig. 7.1 The absorbance changes accompanying reductive (a) and oxidative (b) titration of cytochrome c_{551}. The experimental conditions were as described in Methods. The suspension contained 6 mg protein in a final volume of 7.8 ml. For reductive titration (Fig. 1a) the anerobic suspension was adjusted to 340 mV with ferricyanide and the spectrum recorded as a baseline. The potential was then lowered stepwise with dithionite to a final -400 mV and at each step the spectrum was recorded against spectrum at +340 mV (the baseline). For the oxidative titration (Fig. 1b), spectrum at -400 mV was recorded as a baseline. The final pH of the titrated suspensions was 7.

accompanying reductive (a) and
chrome c_{551}. The experimental
Methods. The suspension contained
of 7.8 ml. For reductive titration
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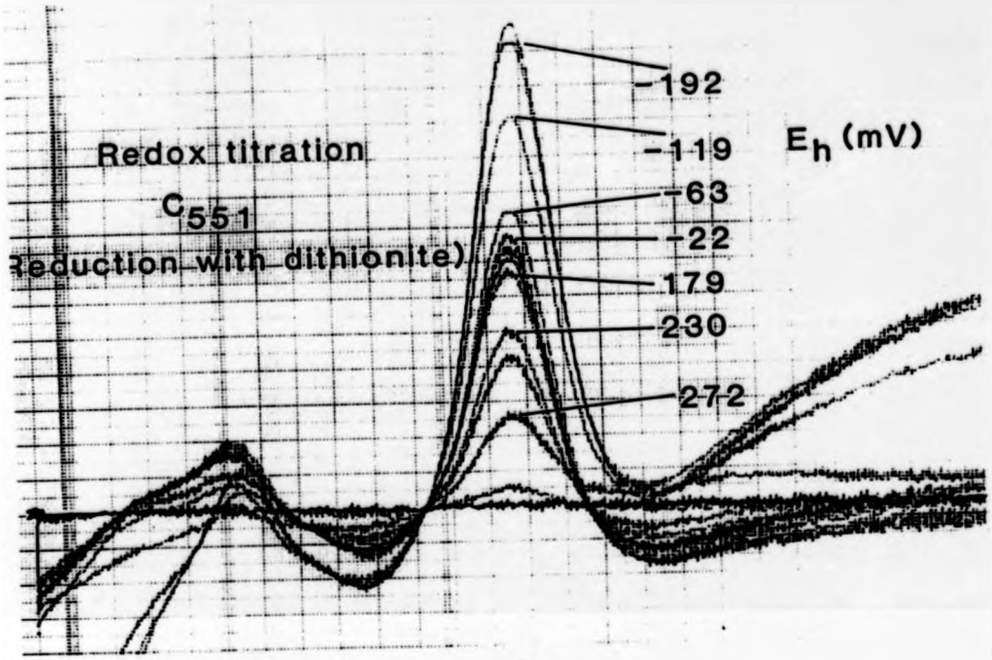
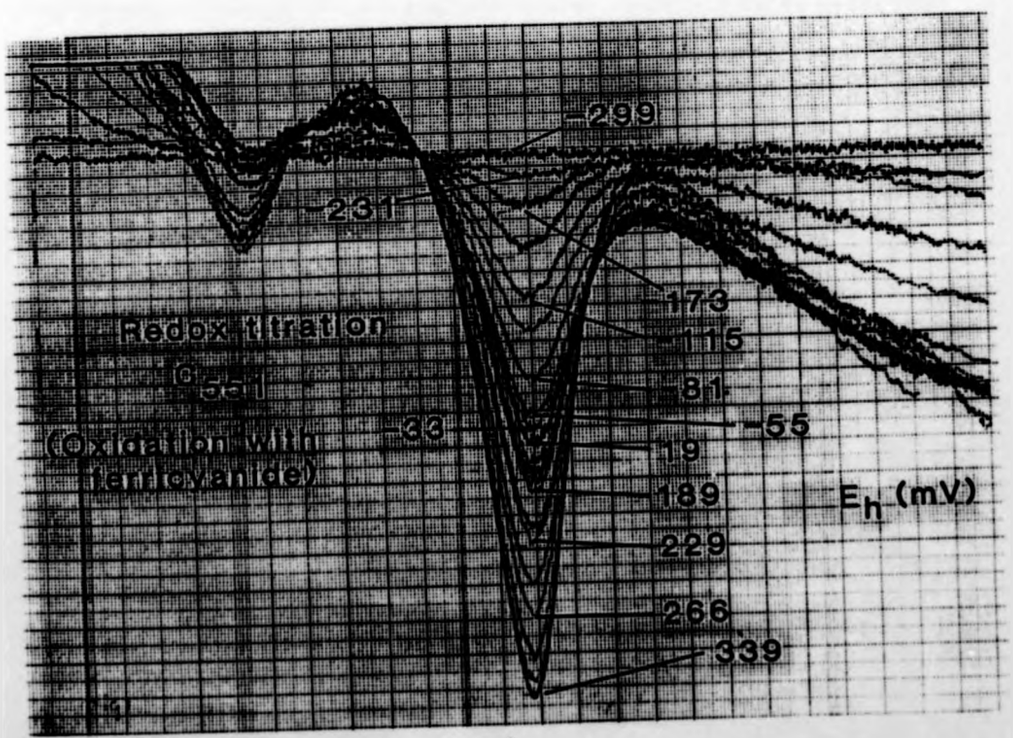


‹a›



‹b›

Fig. 7.2a The dependence of the state of reduction of cytochrome $\underline{c}_{551}$ on the oxidation-reduction potential (E_h). The data come from Fig. 7.1 ●, reductive titration; O, oxidative titration.

Fig. 7.2b The fitting of the experimental data of the dependence of the state of reduction of cytochrome $\underline{c}_{551}$ on the E_h with a theoretical curve based on estimates of E_m and the relative proportions of the two reduced species assuming an n value of one and Em 7 of -115 mV and +240 mV. For detail see the text and Methods.

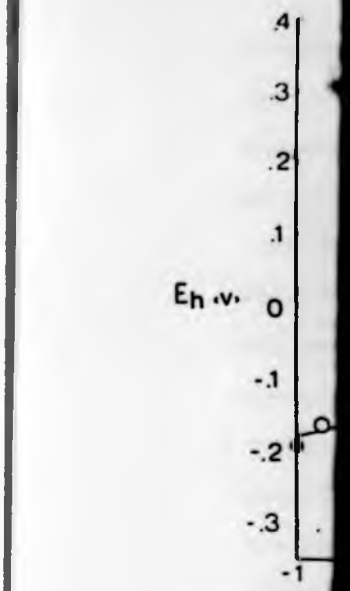


fig 7.2a

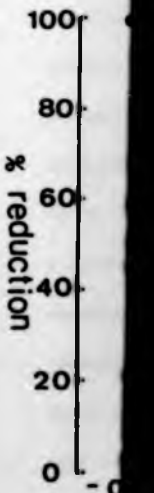


fig 7.2 b

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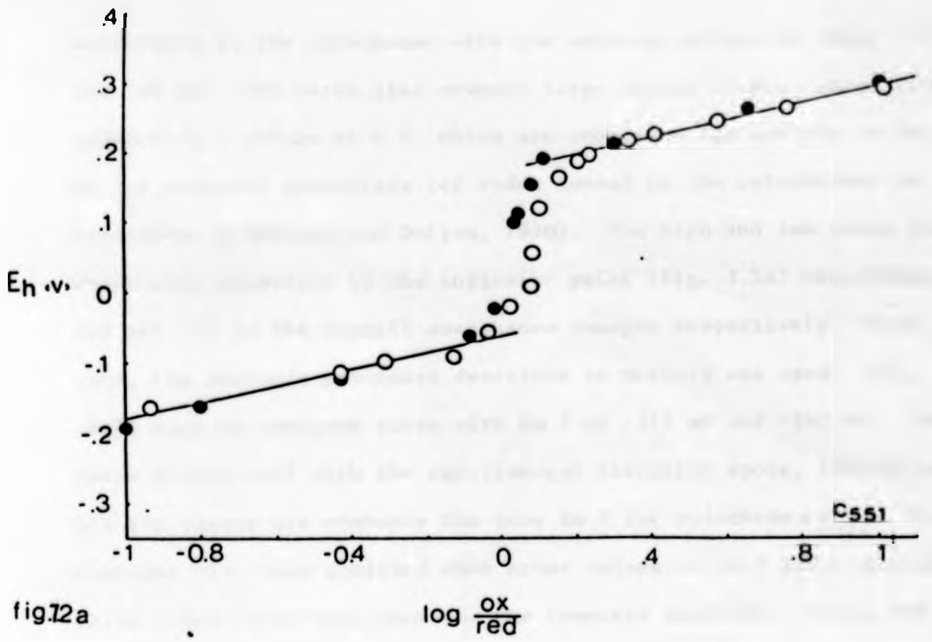


fig.12a

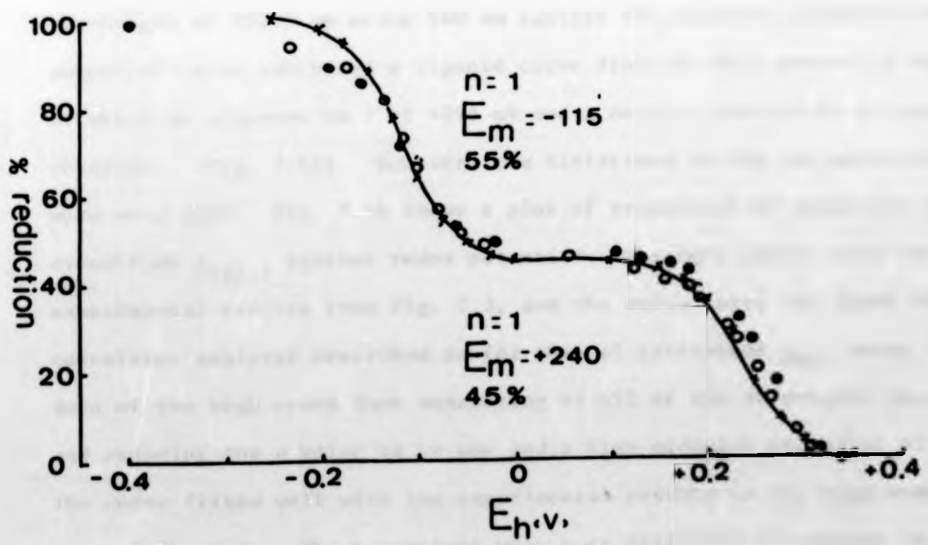


fig 12b

potentials in the cytochrome with the apparent values of about +175 mV and -50 mV. The curve also reveals large Nernst slopes, about 120 mV, suggesting n values of 0.5, which are anomalous and are due to the presence of two midpoint potentials (or redox forms) in the cytochromes as found in cytochrome b (Wilson and Dutton, 1970). The high and low redox forms, which were separated by the inflexion point (Fig. 7.2a) contributed about 45% and 55% to the overall absorbance changes respectively. With these data, the analysis procedure described in Methods was used. Fig. 7.2b shows such an analysed curve with Em 7 of -115 mV and +240 mV. The curve fitted well with the experimental titration spots, indicating that the two values are probably the true Em 7 for cytochrome c_{551}. Poor or nonsense fits were obtained when other values of Em 7 and n and one or three redox forms were used for the computer analysis. Using the same procedure, the other set of titration values for cytochrome c_{551} was also analysed and the apparent values were confirmed.

The difference spectra of cytochrome $c_{552.5}$ obtained during the anaerobic potentiometric titration are shown in Fig. 7.3. The plot of the logarithm of the ratio of the oxidized form to reduced form at fixed wavelength of 552.5 nm minus 540 nm against the measured oxidation-reduction potentials also exhibited a sigmoid curve from the high potential region of which an apparent Em 7 of +195 mV and a Nernst slope of 85 mV could be obtained. (Fig. 7.4a). However, the titrations in the low potential part were very poor. Fig. 7.4b shows a plot of proportion of reduction of cytochrome $c_{552.5}$ against redox potential. The data points were the experimental results from Fig. 7.3, and the solid curve was drawn by the calculator analysis described as for that of cytochrome c_{551} using the data of the high redox form consisting of 65% of the absorbance change and assuming the n value to be one and a high midpoint potential of +220 mV. The curve fitted well with the experimental results in the high redox potential region. The titrations were very difficult to conduct in the low potential region, especially between -200 to -350 mV, due to the instability

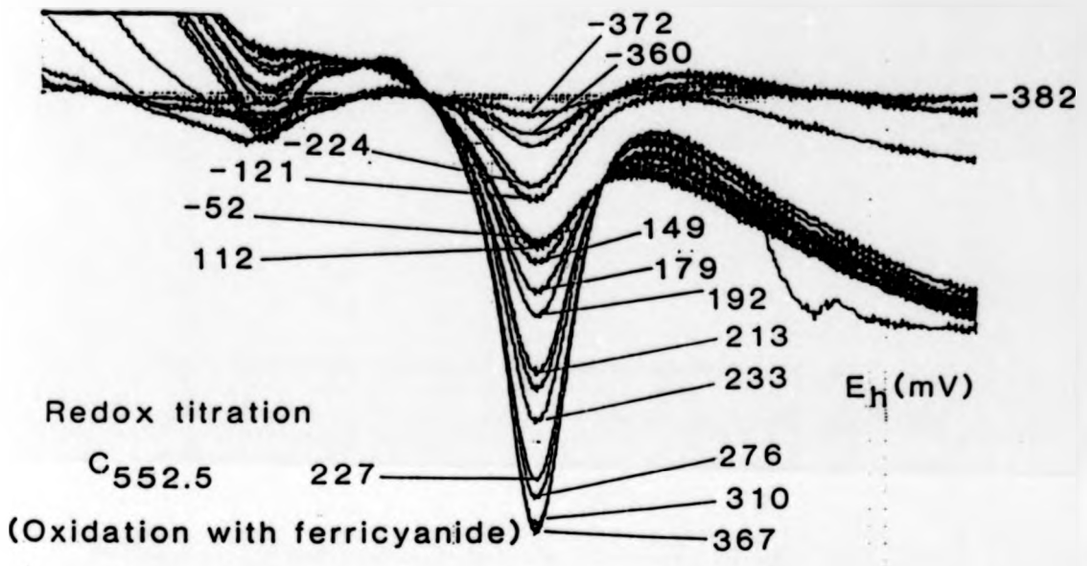


Fig. 7.3 The difference spectra of potentiometric titration of cytochrome $c_{552.5}$. The experimental conditions were as described in the legend to Fig. 1 except that 2.1 mg protein was used. The figure shows an oxidative titration.

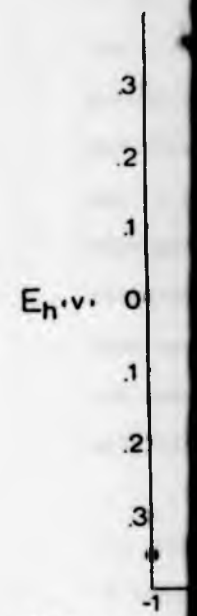
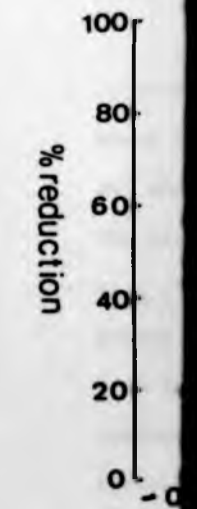


fig. 4a

Fig. 7.4a The oxidation-reduction potential dependence of the absorbance change of cytochrome $c_{552.5}$ at 552.5 minus 540 nm. O Reductive titration; O, oxidative titration (the data from Fig. 3).

Fig. 7.4b Plot of state of reduction of cytochrome $c_{552.5}$ against correspondent oxidation-reduction potentials with the experimental data as Fig. 4a and a theoretical data ( ) for the high potential region, which was produced as described in the legend to Fig. 2b. by assuming an n value of one and the Em 7 of +220 mV which contributed 65% of the overall absorbance change of the α-band.



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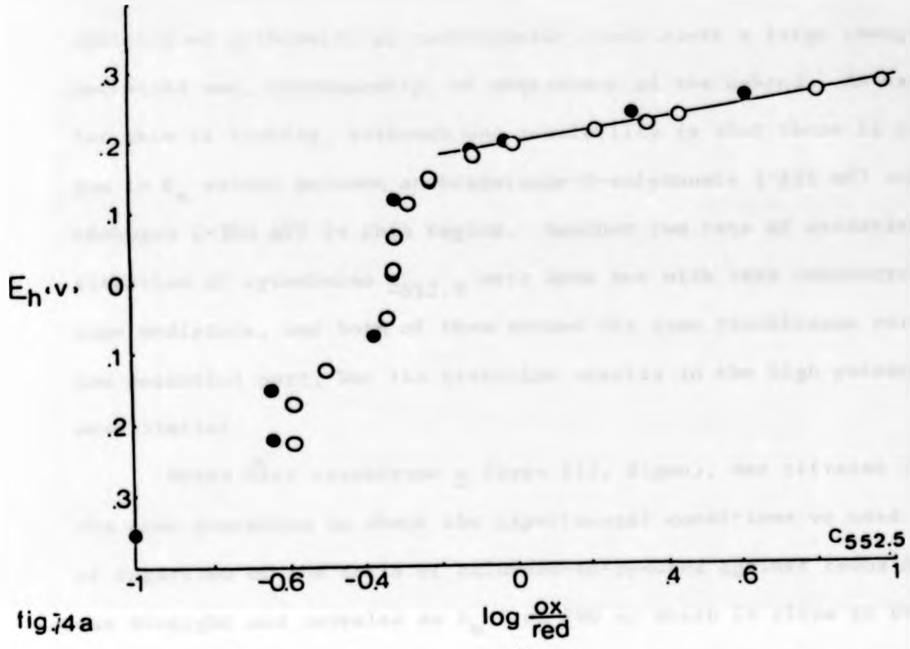
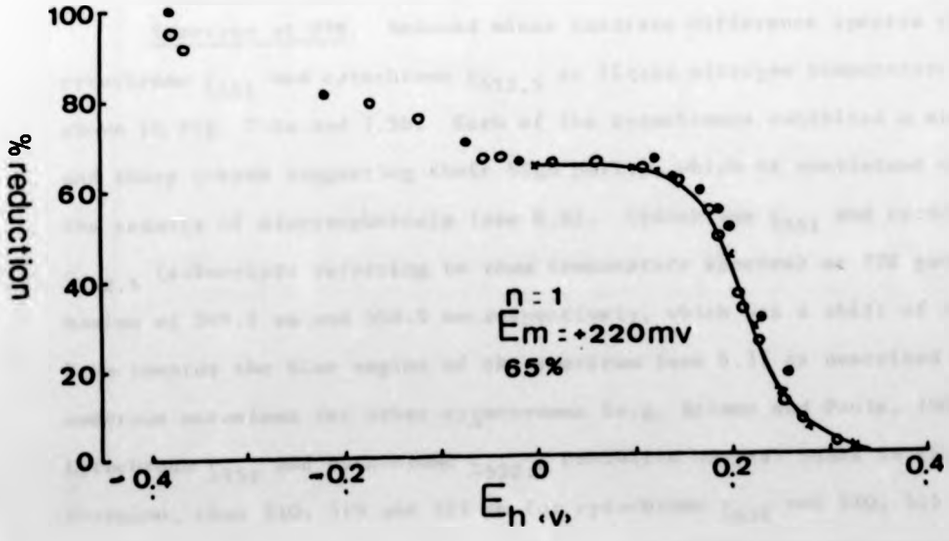


fig 4a



of the absorbance spectra and the redox potentials. Thus, a small addition of dithionite or ferricyanide could cause a large change of potential and, consequently, of absorbance of the α-band. An explanation for this is lacking, although one possibility is that there is an undesirable gap in E_m values between anthraquinone-2-sulphonate (-225 mV) and benzyl viohogen (-350 mV) in this region. Another two sets of oxidation-reduction titration of cytochrome $\underline{c}_{552.5}$ were done but with less concentrations of some mediators, and both of them showed the same troublesome results in the low potential part, but the titration results in the high potential part were similar.

Horse heart cytochrome $\underline{c}$ (type III, Sigma), was titrated using the same procedure to check the experimental conditions we used. The curve of logarithm of the ratio of oxidized-to-reduced against redox potential was straight and revealed an E_m 7 of 280 mV which is close to the published value, and n value of one. But the cytochrome did show some increase of absorbance at the α region on reducing the potential down to +100 mV (in a reductive titration).

7.3.2 Difference spectra of the purified cytochromes and membrane fraction at 77K. Reduced minus oxidized difference spectra of cytochrome $\underline{c}_{551}$ and cytochrome $\underline{c}_{552.5}$ at liquid nitrogen temperature are shown in Fig. 7.5a and 7.5b. Each of the cytochromes exhibited a single and sharp α-band suggesting their high purity, which is consistent with the results of electrophoresis (see 6.3). Cytochrome $\underline{c}_{551}$ and cytochrome $\underline{c}_{552.5}$ (subscripts referring to room temperature spectra) at 77K gave maxima of 548.5 nm and 550.5 nm respectively, which was a shift of about 2 nm towards the blue region of the spectrum (see 6.3) as described on numerous occasions for other cytochromes (e.g. Salmon and Poole, 1980). Cytochrome $\underline{c}_{551}$ and cytochrome $\underline{c}_{552.5}$ exhibited several peaks in the β-region, thus 510, 519 and 527 nm for cytochrome $\underline{c}_{551}$ and 510, 515, 522 and 530 nm for cytochrome $\underline{c}_{552.5}$. The same complexity in this region of the spectra was found in purified cytochrome $\underline{c}$ (Salmon and Poole 1980) and

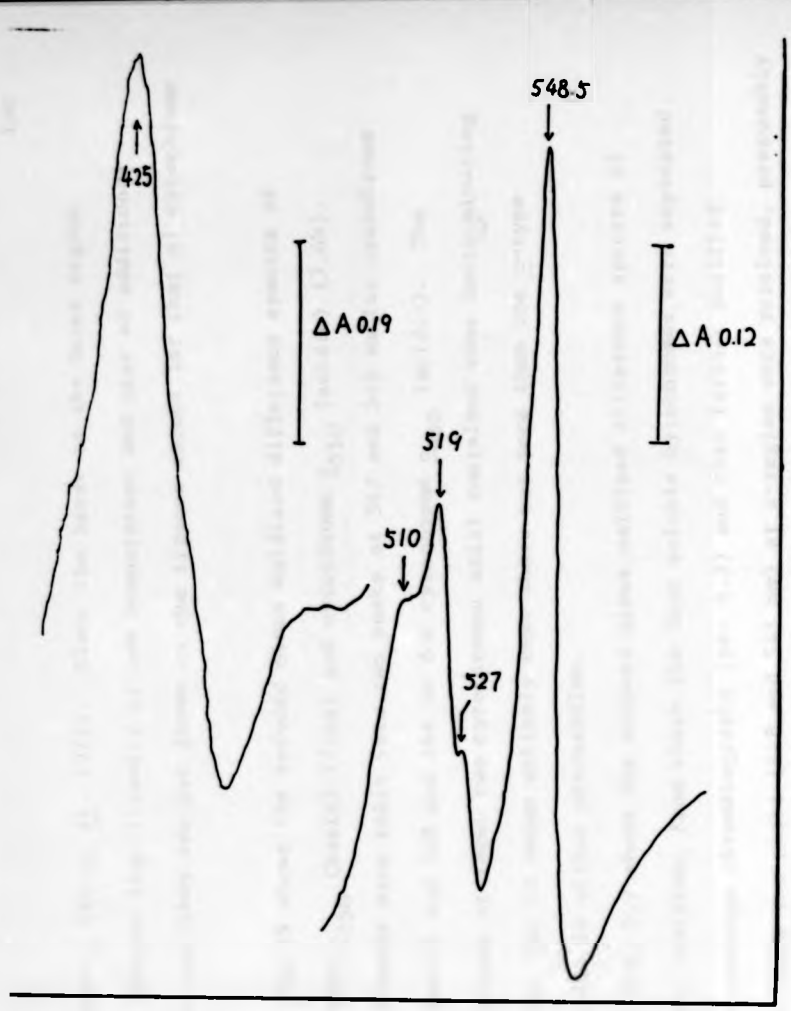


Fig. 7.5a Difference spectrum of cytochrome c_{-551} at 77K. 6 mg proteins in a final volume of 1 ml was used. The spectrum was scanned at 1 nmS^{-1} with a spectral bandwidth of 1 nm.

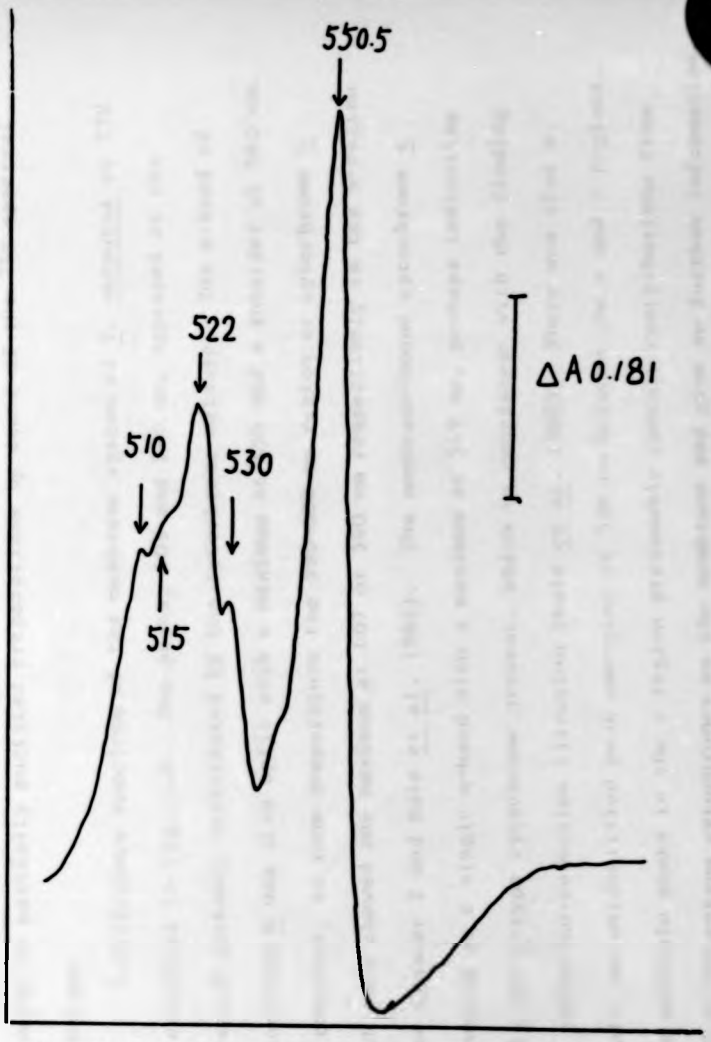


Fig. 7.5b Difference spectrum of cytochrome $c_{-552.5}$ at 77K. 4 mg protein in a final volume of 1.2 ml was used. The spectrum was run at 1 nmS^{-1} with a spectral bandwidth of 1.3 nm.

cytochrome c (Yu et al. 1972). Since the bands in the Soret region do not sharpen significantly at low temperature and give no additional information, they are not shown in the figures except for that of cytochrome c_{551}.

Fig.76 shows the reduced minus oxidized difference spectra of cytochrome c_{550} (basic) (7.6a) and cytochrome c_{550} (acidic) (7.6b). Their α-bands were split into two peaks at 547 and 545 nm for cytochrome c_{550} (basic) and 548 and 546 nm for cytochrome c_{550} (acidic). The preparations of these two cytochromes still contained some contaminating proteins, but it seems unlikely that there was more than one c-type cytochrome in either preparation.

Fig. 7.7 shows the reduced minus oxidized difference spectra of the A65% fraction, from which the four soluble cytochromes were separated by ion exchange chromatography (see 4.3) and then further purified. However only two peaks (548 and 545 nm) at α-region were resolved, presumably due to their broad natural band widths. This suggests that it is impossible to identify unequivocally the composition of c-type cytochromes in crude extracts or partially purified preparations by means of the 77K spectral technique.

A difference spectrum of the membrane system of T. versutus at 77K is exhibited in Fig. 7.8. Two peaks, 603 and 590 nm, appeared at the α-region probably contributed by the cytochrome oxidase. The α-band of cytochrome b was also split with a maximum at 556 and a shoulder at 560 nm. In contrast, at room temperature the 590-608 nm region or cytochrome b each only showed one maximum at 607 or 560 nm respectively in the α-region (see Chapter 2 and Kula et al. 1982). The membrane-bound cytochrome c appeared as a single α-band with a maximum at 549 nm, perhaps indicating only one c-type cytochrome present, which is consistent with the finding of oxidation-reduction titration (Kula et al. 1982). There was also a small and unidentified peak emerging at 536 nm between the α and β regions. The multiple peaks in the β region presumably contain contributions from all b and c-type cytochromes on the membrane and give no further information.

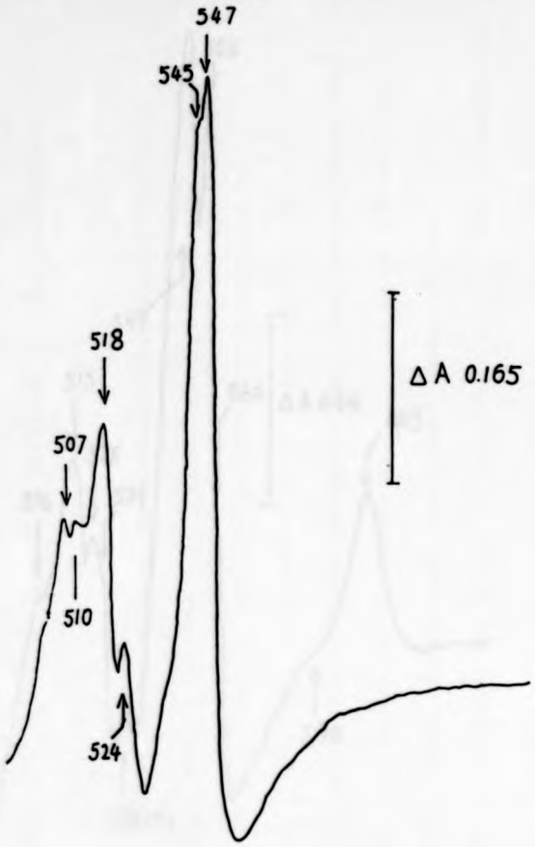


Fig. 7.6a Difference spectrum of cytochrome c_{550} (basic) at 77K. 1.8 mg protein in a final volume of 1.1 ml was used. The spectrum was scanned at 1 nmS^{-1} with a spectral bandwidth of 1.2 nm.

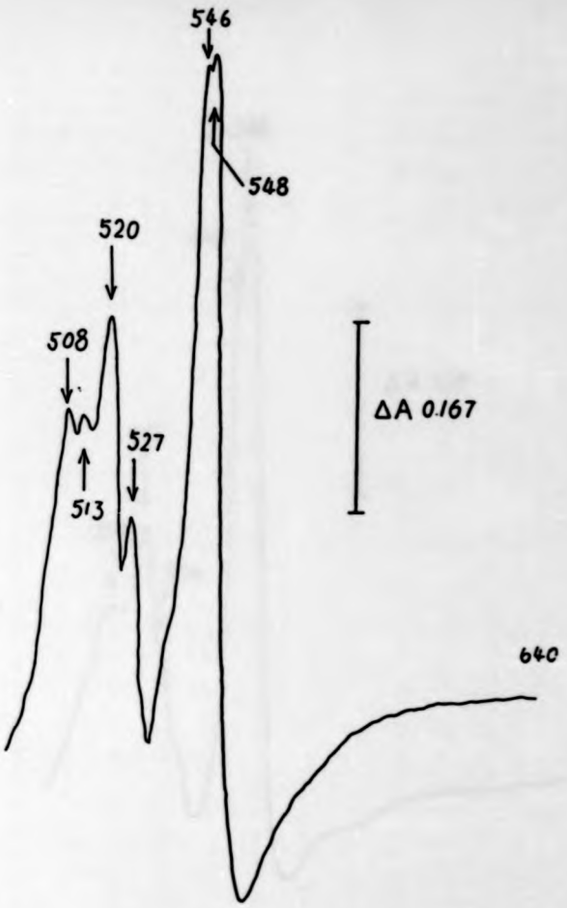


Fig. 7.6b Difference spectrum of cytochrome c_{550} (acidic) at 77K. 3.2 mg protein in a final volume of 1.1 ml was used. The spectrum was scanned at 1 nmS^{-1} with a spectral bandwidth of 1.2 nm.

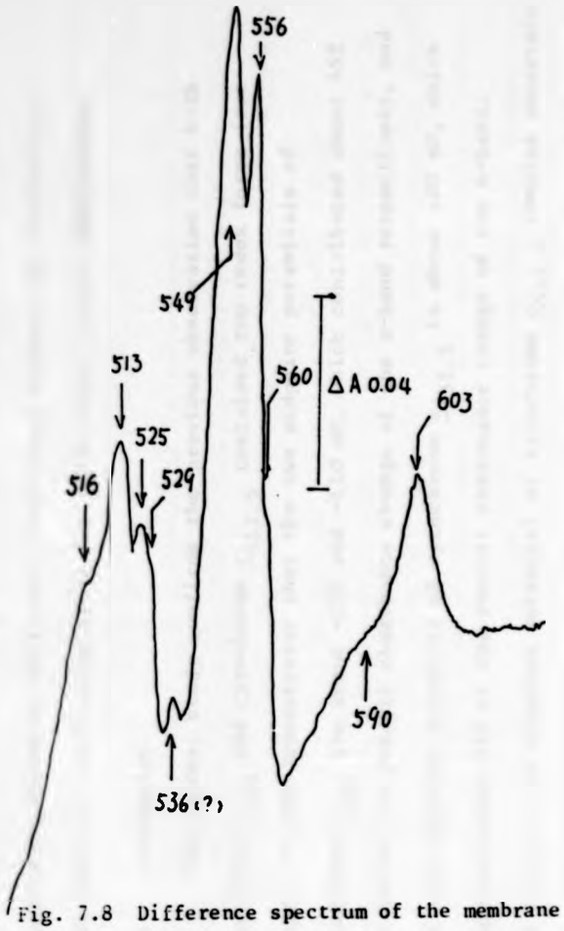


Fig. 7.8 Difference spectrum of the membrane fraction of T. versutus at 77K. 6 mg protein in a final volume of 1.1 ml was used. The spectrum was scanned at 1 nmS^{-1} with a spectral bandwidth of 1.2 nm.

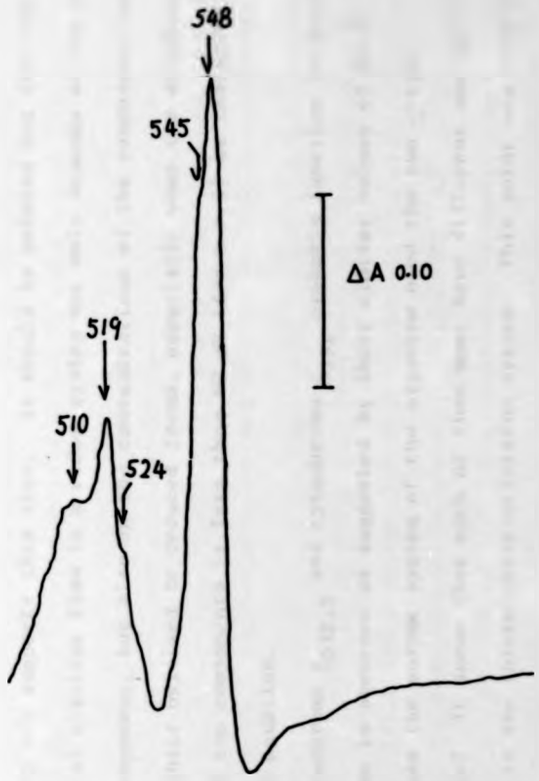


Fig. 7.7 Difference spectrum of A65% fraction at 77K. 7 mg protein in a final volume of 1.1 ml was used. The spectrum was scanned at 1 nmS^{-1} with a spectral bandwidth of 1.2 nm.

The CO difference spectra of the four soluble cytochromes at room temperature showed no difference from those without CO, indicating that there are no binding sites for CO in these c-type cytochromes.

7.6 Discussion

The present study confirms the previous observation that both cytochrome c_{551} and cytochrome $c_{552.5}$ contained two redox forms (see Chapter 6) and demonstrates that the two midpoint potentials of cytochrome c_{551} are about +235 and -110 mV, which contributed about 45% and 55% of the overall absorbance change of the α-band respectively, and the high midpoint potential of cytochrome $c_{552.5}$ is about 220 mV, which comprises about 65% of the overall absorbance change of the α-band. The value of low midpoint potential of cytochrome $c_{552.5}$ remains uncertain, but from the titration results, a value between 0 - -50 mV can be deduced.

Earlier we had shown that cytochrome c_{551} and cytochrome $c_{552.5}$ probably function as electron transfer components between the membrane cytochrome c_{552} and enzyme A and B in the thiosulphate-oxidizing system (Chapters 5 and 6). The finding that the high midpoint potentials of the two soluble cytochromes are very close to that of the membrane cytochrome c_{552} (+ 205 mV) supports this view. It should be pointed out that the direction of electron flow in a redox system not only depends on the Em 7, of each component, but also on the concentrations of the components and the ratio of their oxidized to reduced forms, especially when the difference of Em 7 of two components is less than 60 mV (for n = 1) as indicated by the Nernst equation.

Cytochrome $c_{552.5}$ and cytochrome c_{551} probably function in parallel rather than in sequence as suggested by their similar values of Em 7. But why does the enzyme system of the organism need the two c-type cytochromes? It seems that each of them must have different and specific functions in the thiosulphate-oxidizing system. This point was also suggested by the previous observation that the presence of both of them in the enzyme system gave a much higher activity than with only one of them (Chapter 6).

But we do not have a definite answer at the present time.

Furthermore, what is the role of the low potential forms of the two cytochromes in the thiosulphate-oxidizing system? One possibility is that they might serve as a part of the reversed electron transport process to transfer electrons from thiosulphate (through enzyme A and enzyme B) to cytochrome b and finally NAD^+. This is certainly a more economical way than that using the high potential forms for this purpose. This proposal is supported by some data, such as the E_o' 7 of SO_3^{2-}/SO_4^{2-} couple is sufficiently low (-280 mV, Kelly 1982; or as low as -450 mV to -510 mV. Thauer et al. 1978, Dickerson and Timkovich 1975) and the partial reduction of the low potential form of cytochrome c_{551} occurred when the sulphite oxidase was added to the reaction mixture of cytochrome c_{551}, enzyme A and B and thiosulphate (Chapter 8). According to this proposal (Chapter 9) electrons from thiosulphate enter the respiratory chain either through the low potential forms of cytochrome c_{551} and cytochrome $c_{552.5}$ at cytochrome b level or through the high potential forms of the cytochromes at the membrane cytochrome $c_{552.5}$, depending on the redox potentials of the four electron pairs of thiosulphate and possibly some other factors. The other published and calculated values of the standard redox potentials (E_o' 7) involved in thiosulphate oxidation are -10 mV (Kelly 1982) and +13 mV (Dickerson and Timkovich 1975, or my calculation, see 1.12) for $S_2O_3^{2-}/SO_3^{2-}$ couple and -310 mV for $S_2O_3^{2-}/SO_4^{2-}$ (my calculation, see 1.12). From these values it seems that during the thiosulphate oxidation, the electrons from the SO_3^{2-}/SO_4^{2-} couple (although we are not sure if the enzyme-associated transient intermediate such as SO_3^{2-} has the same redox potential as the free anion since the existence of free SO_3^{2-} has been seriously questioned in this thiosulphate-oxidizing system (see 8.3.5)) are able to reduce the low Em 7 of the cytochromes, while the electrons from $S_2O_3^{2-}/SO_3^{2-}$ couple can only be donated to the high Em 7 of the cytochromes. However, caution should be taken in using these redox values since there are a number of problems in the calculation of them as discussed before in some detail (see 1.12).

A noteable feature of these two cytochromes is that the difference of midpoint potentials between the high potential forms and the low potential forms are extremely large, being about 330 mV in cytochrome c_{551}. This potential gap may present some problems in the explanation of the arrangement of the components, such as how the electrons from thiosulphate were transferred nearly simultaneously to the membrane cytochrome *c* and cytochrome *b* (with a Δ Em 7 of about 250 to 300 mV) through cytochrome c_{551} and $c_{552.5}$. A similar value of Δ Em 7 (350 mV) was found in a dihaem cytochrome $c_{552.5}$ with Em 7 values of +175 and -180 mV from *Pseudomonas perfectomarinus* (Liu *et al*. 1981). But the physiological function of the cytochrome and the significance of the different redox potentials were unclear. An intensively studied multihaem and multi midpoint potential cytochrome is cytochrome c_3 from sulphate-reducing bacteria (Dickerson and Timkovich 1975). Cytochrome c_3 (Mr 13,000) from *Desulfovibrio vulgaris* contained four haems with various potentials from -284 to -319 mV (DerVartanian *et al*. 1978). Though it is generally accepted that the cytochrome functions as an electron carrier between hydrogenase and ferredoxin in these bacteria, an explanation is still lacking for the presence of four *c*-type haems in this molecule and their multiple oxidation-reduction potentials (Peck and LeGall 1982). Cytochrome *b* was resolved into two forms by the oxidation-reduction technique (Kula *et al*. 1982) with Em 7 of 39 and -100 mV, and was reported by the same authors to give two maxima at 557 and 563 nm at liquid nitrogen temperature, which, however, are somewhat different from the present figures of 566 and 560 nm.

Table 7.1 lists the main properties of the four soluble *c*-type cytochromes from *T. versutus*.

TABLE 7.1 Some properties of the soluble c-type cytochromes from T. versutus

Cytochrome	Absorption maximum[a] (nm) At 25°C α	β	γ	At 77K α					Molecular weight Native	Subunit	PI	Em 7 (mV)	Haems	$\frac{A_{a(red)}}{A_{280(ox)}}$	% of crude extract (w/w)
$c_{552.5}$	552.5	523	418	550.5	509	515	521	529	56,000	29,000	4.8	+220 and −50 to 0	2	0.76	1.5
c_{551}	551	522	418	548.5	510	519	527		260,000	43,000	5.2	+240 and −115	4 to 5	0.27	1
c_{550} (basic)	550	521	415	545	547	507	510	518 524	15,000		8		1	0.83	0.3
c_{550} (acidic)	550	522	415	546	548	508	513	520 527	29,000	14,000	5		1	0.75	0.4

[a] Taken from reduced minus oxidized difference spectra with dithionite as reductant.

7.5 REFERENCES

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CHAPTER 8

STUDIES ON SULPHITE:CYTOCHROME c OXIDOREDUCTASE

8.1 INTRODUCTION

We reported previously that the thiosulphate-oxidizing multienzyme system in Thiobacillus A2 consisted of five components (Chapters 5 and 6), namely, 'enzyme A', 'enzyme B', cytochrome c_{551}, cytochrome $c_{552.5}$ and sulphite: cytochrome c oxidoreductase (subsequently referred to as 'sulphite oxidase'). The system could effect the reduction of mammalian cytochrome c with thiosulphate and the complete oxidation of thiosulphate to sulphate in the presence of an electron transfer chain to oxygen, such as mammalian cytochrome c and cytochrome oxidase or membrane particles from Thiobacillus A2. Sulphite oxidase was purified 600 fold (Chapter 5) but the preparation was still heterogeneous, showing more than six bands after SDS-gel electrophoresis. The significance of the enzyme in the thiosulphate-oxidizing system was also still obscure (Chapter 5).

The present chapter describes the further purification and some properties of the sulphite oxidase as well as its essential role in the oxidation of thiosulphate. Some comparison is made with sulphite oxidase previously reported from other species of thiobacilli.

8.2 MATERIALS AND METHODS

8.2.1

Culture of the organism and the preparation of crude extracts. Thiobacillus A2 (T. versutus) was grown autotrophically in continuous chemostat culture and the crude extract prepared as described previously (see 2.2.1 and 3.2.1).

Purification of enzyme A and enzyme B. This was detailed previously see Chapters 4 and5).

Purification of cytochrome c_{551} and cytochrome $c_{552.5}$. This was as described earlier (Chapter 6).

Sulphite: cytochrome c oxidoreductase was measured spectrophotometrically essentially as described before (Chapter 2 ,. The reaction mixture contained, unless otherwise specified, cytochrome c (horse heart), 40 nmol;

enzyme preparation, 0.5-50 µg protein; Na_2SO_3, 2 µmol in 5 mM EDTA; Tris-HCl, pH 7.3, 40-45 µmol in a final volume of 1 ml.

Thiosulphate: cytochrome c oxidoreductase activity was estimated as described previously (see 6.2.2). Sulphite- or thiosulphate-oxidizing activity was assayed in an oxygen electrode essentially as detailed before (see 6.2.2). Variations in procedures are given in the Legends to the Figures or Tables.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis, nondenaturing disc-polyacrylamide gel electrophoresis and preparative polyacrylamide gel electrophoresis were described previously (see 5.2.9).

Determination of molecular weight. This used SDS-polyacrylamide gel electrophoresis as stated previously (see 5.2.9).

Preparative isoelectric focusing in Sephadex IEF. The procedure was the same as described for purification of enzyme A (see 5.2.5).

Protein estimation. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Results

Purification and some properties of sulphite oxidase

As shown previously (Chapter 3 see Table 1) fractionation of the crude extract with ammonium sulphate concentrated most of the sulphite-oxidizing and thiosulphate-oxidizing activities into the A44% to A65% fractions. The total recovery of the sulphite oxidizing activity in the A65% fraction was even higher than in the crude extract. This might be due to the removal of some inhibitors of the enzyme activity from the crude extract by the fractionation procedure.

The A65% fraction was resolved into five major fractions by chromatography on DEAE-Sepharose CL-6B, of which the 0.35 M NaCl (I) fraction

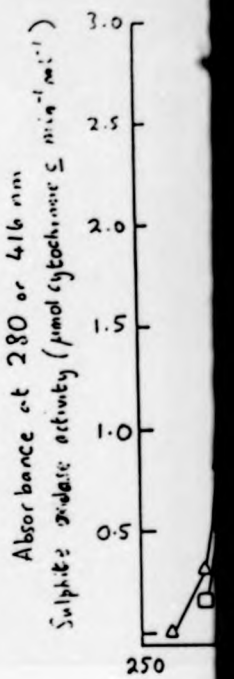
contained sulphite oxidase as well as enzyme B and cytochrome c_{551}. Sulphite oxidase in the 0.35 M CaCl (I) fraction was separated from the bulk of the cytochrome c_{551} and enzyme B by gel filtration on Sephadex G-200 (see 5.2.6 ,) or on Sephadex G-100 (see 6.2.5).

Several batches of sulphite oxidase (400 mg) from the previous step were combined, subjected to gel fitlration on a Sephadex G-100 column of 3.2 x 81 cm, and then eluted with 50 mM Tris buffer, pH 7.3 at 4°C using a flow rate of 30 ml h^{-1} (Fig.8.1a). Active fractions were pooled and concentrated by $(NH_4)_2SO_4$ precipitation. This preparation was called the G-100 fraction.

The G-100 fraction (140 mg) was then loaded on to a column (2.6 x 7 cm) of Phenyl-Sepharose CL-4B equilibrated at 4°C with 50 mM Tris buffer, pH 7.3, containing $(NH_4)_2SO_4$ at 10% of saturation and eluted with a linear gradient of decreasing $(NH_4)_2SO_4$ concentration , which was produced from two 150 ml volumes of 50 mM tris buffer, pH 7.3, one of which contained $(NH_4)_2SO_4$ at 10% of saturation. Fig.8.1b shows the elution profile. The active fractions were combined and concentrated by ultrafiltration under nitrogen pressure through an Amicon PM 10 membrane. This prepartaion was termed the HIC fraction.

Table8.1 summarises these procedures, which resulted in about 2000-fold purification in terms of specific activity with a 39% recovery of the total activity. The purified enzyme was almost free of enzyme B activity. This final preparation (HIC fraction) only comprised about 0.05% of the crude extract protein. Moreover this preparation still showed multiple bands on nondenaturing gel (Fig.8.2a) and a major band with several minor bands on SDS-gel (Fig.8.2b). The major band had an Rf value nearly identical with that of cytochrome c_{551} , so the band seemed not to be homogeneous.

In order to solve the problem the HIC fraction was further purified by preparative isoelectric focusing on Sephadex. After running for six hours, using Pharmalyte pH 4 to 6.5, three to four protein bands appeared at the region 2.5 cm - 4.5 cm from the anode on the gel plate, in which only the fraction at 3.5 cm - 4 cm contained sulphite oxidase activity. The same active band appeared



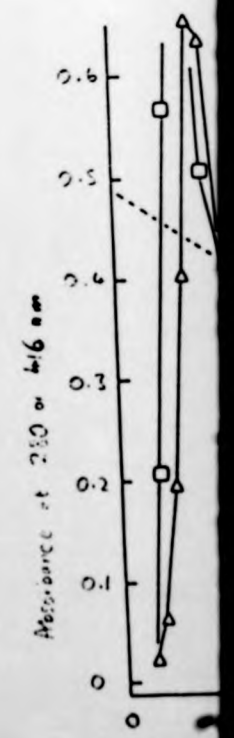


Fig. 1. Purification of sulphite oxidase

For details see Results. (a) Elution pattern on Sephadex G-100 of combined active preparations from Sephadex G-200 and Sephadex G-100 columns. The void volume was 280 ml.

△ , Protein (A_{280}); □ , c-type cytochrome (A_{416}); ● sulphite oxidase; ⊢——⊣ , the pooled active fractions.

Enzyme B activity, eluting from 370 ml to 400 ml, is not shown; (b) Elution profile on Phenyl-Sepharose CL-4B of the G-100 fraction. △ Protein (A_{280}); □ , c-type (A_{416}); ● , sulphite oxidase; ⊢——⊣ , the pooled active fractions. Enzyme B activity eluting after the sulphite oxidase, is not shown.

se

tion pattern on Sephadex G-100 of

dex G-200 and Sephadex G-100 columns.

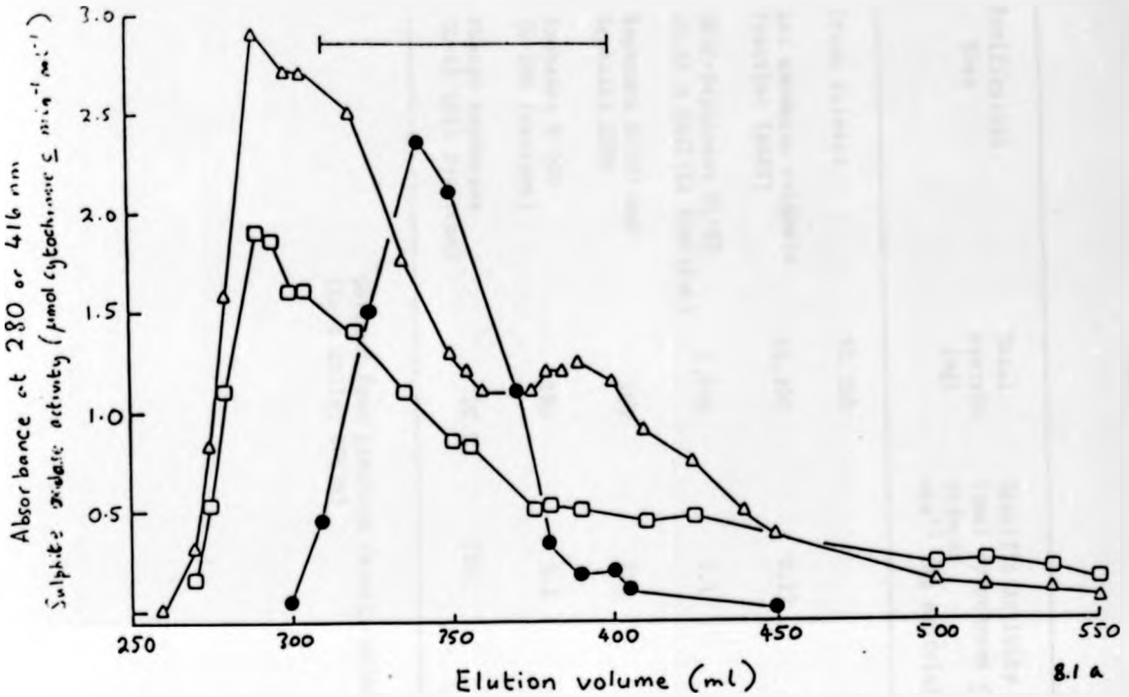
cytochrome (A_{416}); ● sulphite oxidase;

to 400 ml, is not shown; (b) Elution

G-100 fraction. Protein (A_{280});

oxidase; |——| , the pooled active

fter the sulphite oxidase, is not shown.



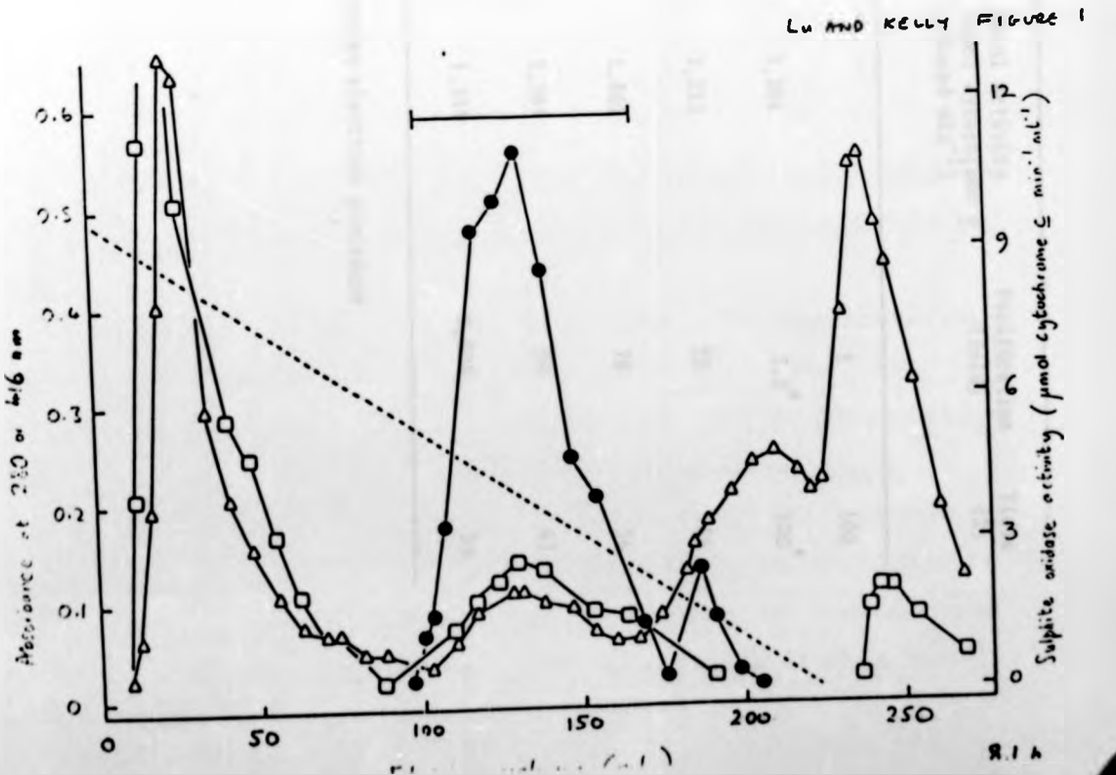


TABLE 1. Purification of sulphite oxidase

Purification Step	Total protein (mg)	Specific activity (μmol cytochrome c reduced min^{-1} (mg protein)$^{-1}$)	Total activity (μmol cytochrome c reduced min^{-1})	Purification (fold)	Yield (%)
Crude extract	52,700			1	100
1st ammonium sulphate fraction (A68%)	15,200	0.22	3,344	3.5*	100*
DEAE-Sepharose CL-6B (0.35 M CaCl(I) fraction)	1,500	2.1	3,213	33	96
Sephadex G-100 and Sephadex G200	380	4.9	1,862	78	56
Sephadex G-100 (G-100 fraction)	220	6.2	1,364	99	41
Phenyl sepharose CL-4B (HIC fraction)	10.3	128	1,318	2,036	39

*Derived from previous results using the oxygen electrode procedure (Lu & Kelly, 1983b)

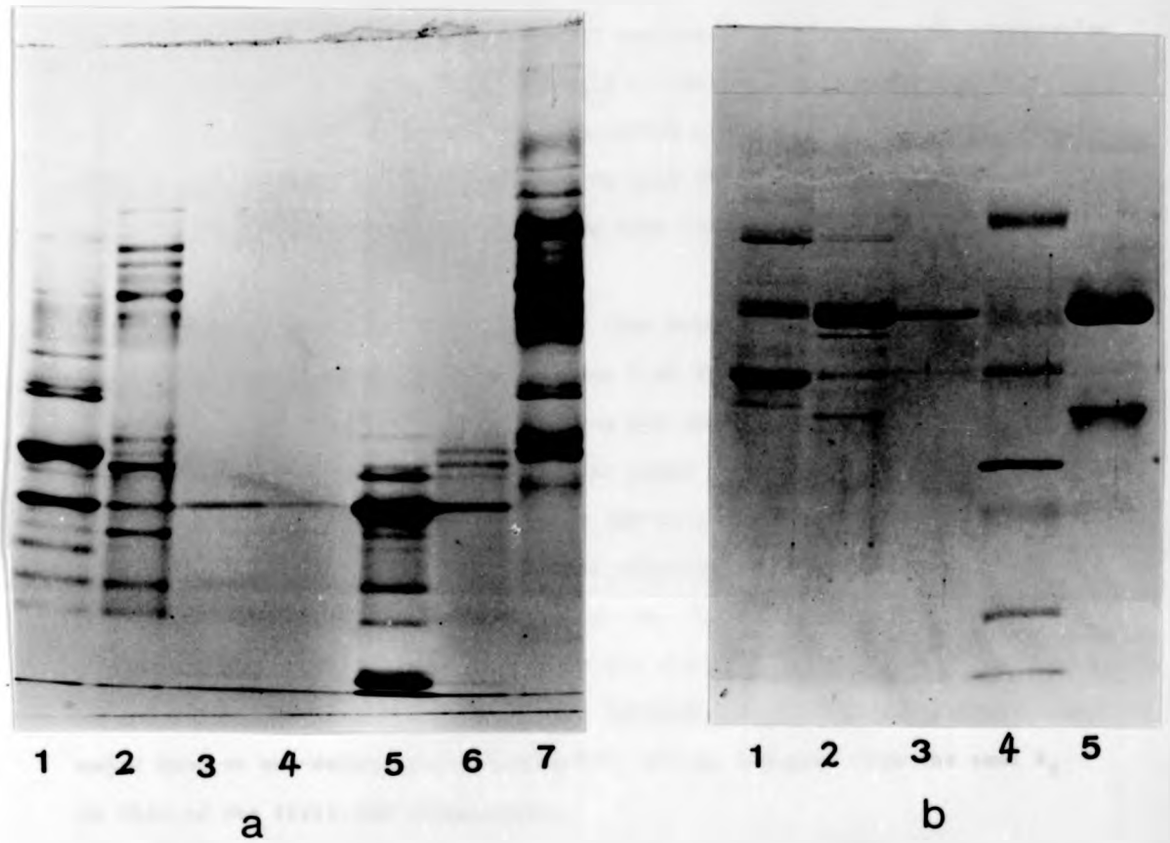


Fig. 2. Non-denaturing polyacrylamide gel electrophoresis (a) and SDS-polyacrylamide gel electrophoresis (b) of the fractions from the purification of sulphite oxidase

(a) PAGE: (1) G-100 fraction; (2) HIC fraction; (3) preparation from IEF (pH 2.5-5); (4) preparation from IEF (pH 4-6.5, running for 6 h); (5) preparation from IEF (pH 4-6.5, running for 3 h); (6) preparation from PAGE (For (3) to (6) see Table 2 for details); (7) cytochrome c_{551}.

(b) SDS-PAGE: (1) G-100 fraction; (2) HIC fraction; (3) preparation from IEF (pH 4-6.5, running for 6 h); (4) marker proteins (M_r 66,000, 45,000, 36,000, 29,000, 24,000, 20,100 and 14,200, from the top to the bottom respectively); (5) purified cytochrome c_{551} (the upper band, M_r 43,000) 20 to 60 µg protein was used in each case.

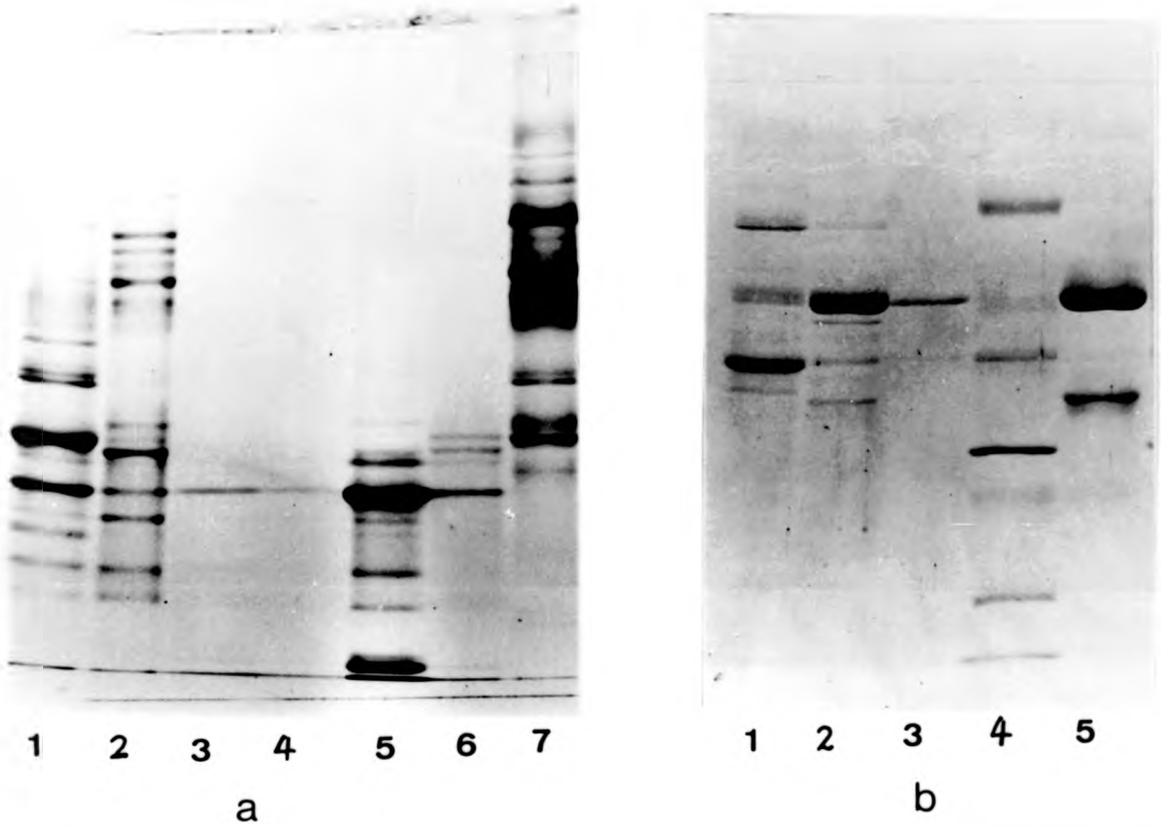


Fig.8.2. Non-denaturing polyacrylamide gel electrophoresis (a) and SDS-polyacrylamide gel electrophoresis (b) of the fractions from the purification of sulphite oxidase

(a) PAGE: (1) G-100 fraction; (2) HIC fraction; (3) preparation from IEF (pH 2.5-5); (4) preparation from IEF (pH 4-6.5, running for 6 h); (5) preparation from IEF (pH 4-6.5, running for 3 h); (6) preparation from PAGE (For (3) to (6) see Table 2 for details); (7) cytochrome c_{551}.

(b) SDS-PAGE: (1) G-100 fraction; (2) HIC fraction; (3) preparation from IEF (pH 4-6.5, running for 6 h); (4) marker proteins (M_r 66,000, 45,000, 36,000, 29,000, 24,000, 20,100 and 14,200, from the top to the bottom respectively); (5) purified cytochrome c_{551} (the upper band, M_r 43,000) 20 to 60 µg protein was used in each case.

at 15 cm to 16 cm from the anode after focusing on Pharmalyte of pH 2.5 to 5. However, as shown Table 2, both specific and total activity in this preparation dropped considerably to about 7% and 1.2% of the initial values respectively (See line 1 of Table 2). Addition of purified cytochrome c_{551} to the reaction mixture enhanced the specific activity by only about 10 to 15%. Nevertheless, the preparation showed one major band on both non-denaturing gel and SDS-gel (Fig. 2 a,b).

Isoelectric focusing for a shorter time gave better recovery in terms of both total and specific activity (line 2 of Table 2), but the preparation exhibited mutiple bands on non-denaturing gel and SDS-gel, although the major band had the same R_f as that of the purer preparation (line 1, Table 2).

Using sulphite oxidase purified by IEF as a marker, the enzyme in the HIC fraction was isolated from a preparative polyacrylamide gel after electrophoresis. The specific activity of this preparation was higher than the IEF preparation, but was still only about 60% of that of the initial sample, and only 7% of the initial activity was recovered (line 3, Table 2). The preparation showed one major band on non-denaturing gel (Fig. 2a) and on SDS-gel, with the same R_f as that of the first IEF preparation.

As shown on Fig. 2b, the molecular weight of the finally purified sulphite oxidase was 44,000. The PI value was 4.5 ($\pm$ 0.3), according to the results of preparative isoelectric focusing.

6.3.2
Involvement of cytochrome c_{551} for sulphite oxidase activity

Cytochrome c_{551} exhibited an R_f almost identical on SDS-gel to that of purified sulphite oxidase (both HIC and further purified fractions), but they seemed not to be the same protein, as shown on the non-denaturing gel (Fig. 2a), where cytochrome c_{551} showed its typical pattern of multiple bands (for details see 6.3.2). However, the same pattern of multiple bands did appear in the HIC fraction on the non-denaturing gel (Fig. 2a), which indicated the presence of a substantial amount of cytochrome c_{551} in the fraction. This was also evidenced by the red colour of the fraction. These observations strongly

TABLE 2. Further purification of sulphite oxidase with preparative isoelectric focusing (IEF) and preparative polyacrylamide gel electrophoresis (PAGE)
The experiments were as described in the Methods. All the samples were from HIC, but several batches were employed. Enzyme activity was measured as stated in the Methods.

	HIC Sample Activity			Further Treatment	Results			Recovery (%) of activity in sample	
periment No.	Protein (mg)	Specific activity (μmol cytochrome c reduced min^{-1} (mg protein)$^{-1}$)	Total activity (μmol cytochrome c reduced min^{-1})	Experimental conditions	Protein (mg)	Specific activity (μmol cytochrome c reduced min^{-1} (mg protein)$^{-1}$)	Total activity (μmol cytochrome c reduced min^{-1})	Specific activity	Total activity
1	2.9	5.6	163	IEF (pH 4-6.5 (6 h)	0.5	3.9	2	7	1.2
2	3.2	60	192	IEF (pH 4-6.5) (3 h)	0.9	29	26	48	13.5
3	2.2	128	281	PAGE	0.25	77	19.5	60	7

suggested that the cytochrome $\underline{c}_{551}$ was an essential requirement for sulphite oxidase activity and possibly a major and integral part of the sulphite oxidase itself, as the removal of the cytochrome from the preparation by the preparative isoelectric focusing and polyacrylamide gel electrophoresis resulted in a dramatic decrease in enzyme activity (Table 8.2), which could not be restored by mixing of the separated cytochrome $\underline{c}_{551}$ and sulphite oxidase.

The polymerization property of the cytochrome $\underline{c}_{551}$ caused great difficulty in the purification of the cytochrome (see 6.3.2.), which might also have accounted for the problems encountered in the purification of sulphite oxidase. The HIC fraction also appeared to contain a certain amount of enzyme B as judged by the R_f values on the SDS-gel (Fig.8.2b). So, enzyme B might also play some role in the activity and structure of the sulphite oxidase, similar to that of cytochrome $\underline{c}_{551}$

8.3.3

Some properties of sulphite oxidase

In the following experiments the HIC fraction was used, unless otherwise specified. The pH optimum for sulphite oxidase activity, measured spectrophotometrically in 50 mM Tris buffer, was around pH 8 and activity was identical at pH 8 in 10 mM or 50 mM Tris, but was about 10% lower at pH 7.3 and 8.5. The enzyme exhibited a high affinity for sulphite, showing an apparent K_m value of 14 µM, calculated from a Lineweaver-Burk plot. From the same plot a V_{max} value of 192 µmol cytochrome $\underline{c}$ reduced/min/mg protein was obtained .

The activity of the purified sulphite oxidase was markedly inhibited (about 95%) by phosphate buffer (55 mM pH 8) as found previously with the crude extract (Chapter 2). The inhibition by phosphate was found to be non-competitive (Fig.8.3a) and the K_i, as determined from the intercept replot, was about 12 mM (Fig.8.3b).

Enzyme activity was also inhibited by p-hydroxymercuribenzoate (pHMB) and cyanide. Inhibition was dependent on the length of incubation with the inhibitors prior to sulphite. Incubation for 1, 5 and 15 min with pHMB (1 mM) gave 30, 40 and 75% inhibition, and incubation for 0.1, 2, 6 and 10 min with CN^- (2 mM) showed 15, 50, 70 and 100% inhibition respectively. 4 mM CN^- gave 35% and 100% inhibition after incubation for 0.1 and 4 min. Incubation for 15 min in the presence of both pHMB (1 mM) and glutathione (1 mM) gave 51% inhibition.

Lu and KELLY Figure 3

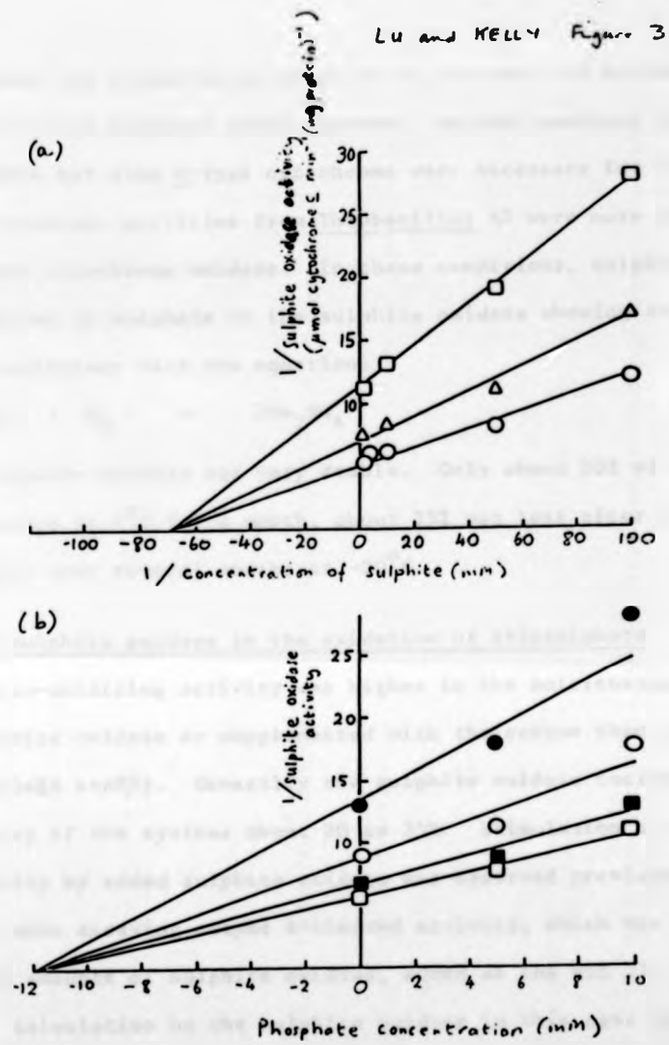


Fig.3. Effect of phosphate on sulphite oxidase activity

(a) Double-reciprocal plot of velocity against sulphite concentration with variable amounts of sodium phosphate (pH 7.8). (○), no phosphate; (△) 5 mM phosphate; (□) 10 mM phosphate.

(b) Reciprocal plot of velocity against phosphate concentration for sulphite at 0.01 mM (●), 0.02 mM (○), 0.1 mM (■) and 1.0 mM (□). The tests were conducted as described in Materials and Methods except that 40 μmol Tris-HCl, pH 8, 80 nmol horse heart cytochrome c and 0.6 μg enzyme were used.

Table 3 shows the oxidation of sulphite by the purified sulphite oxidase in the presence of the electron chain systems. Not only membrane particles or cytochrome oxidase but also c-type cytochrome were necessary for enzyme activity. The membrane particles from Thiobacillus A2 were more effective than bovine heart cytochrome oxidase. In these conditions, sulphite was completely oxidized to sulphate by the sulphite oxidase showing an O_2/SO_3^{2-} ratio of 0.5, consistent with the equation:

$$2Na_2SO_3 + O_2 = 2Na_2SO_4$$

The purified sulphite oxidase was very stable. Only about 20% of the activity was lost on storage at 4°C for a month, about 35% was lost after a week at 20°C and none was lost over several months at -20°C.

8.3.4

Involvement of sulphite oxidase in the oxidation of thiosulphate

Thiosulphate-oxidizing activity was higher in the multienzyme system containing sulphite oxidase or supplemented with the enzyme than that without the enzyme (Table 4 and 5). Generally the sulphite oxidase increased the specific activity of the systems about 20 to 25%. Stimulation of thiosulphate-oxidizing activity by added sulphite oxidase was observed previously (see 5.3.6), when assaying enzyme B-limited activity, which was stimulated 30-40% by small amounts of sulphite oxidase, added as the HIC (I) fraction. The greater stimulation by the sulphite oxidase in this case was obviously related to the low concentration of enzyme B in the reaction mixture.

In Table 4 (Experiments 6 and 7) the oxidation rate declined after incubation for 20-25 min, probably because of the accumulation of some intermediate, which could then be further oxidized following addition of sulphite oxidase resulting in the oxidation rate resuming or exceeding the initial one. The subsequent decrease in oxidation rate (Experiment 7) obviously resulted from loss of activity of components during long term incubation. Table 4 also shows that membrane particles from Thiobacillus A2 were more

TABLE 3. Oxidation of sulphite by the purified sulphite oxidase in the presence of electron carriers. The activity was measured in the oxygen electrode as described in Materials and Methods using the quantities of the components indicated. Membrane particles were prepared as described before (Lu & Kelly, 1983a). Greater amounts of the membrane particles or horse heart cytochrome c produced no further effect on activity.

Composition of reaction mixture (mg protein)				Rate of oxidation
Sulphite oxidase*	Horse heart Cytochrome c	Bovine heart Cytochrome oxidase	Thiobacillus A2 membrane particles	μmol O_2 min^{-1} mg^{-1}
0.1	-	-	0.2	0
0.1	0.5	-	0.2	0.78
0.1	0.5	0.05	-	0.052
0.005	0.5	-	0.2	8.27

*The G-100 fraction was used except the last case, where the HIC fraction was used.

TABLE 4. The effect of added sulphite oxidase on the oxidation of thiosulphate by the reconstituted multienzyme system. The experiments were done in an oxygen electrode cell essentially as stated in the Materials and Methods, except that various amount of the components were used as indicated. The activity was calculated in terms of total protein added as the four components. The components in the different sets came from several separate stocks of organisms and purification batches, so the absolute activities varied among experiments, for example, as in 5 and 7.

Experiment No.	Reaction mixture components (mg protein)							Substrate	Supplementary addition of Sulphite Cytochrome c oxidoreductase	Rate of oxidation (nmol O_2 min^{-1} mg^{-1})
	Enzyme A	Enzyme B	Cytochrome $\underline{c}_{552.5}$	Cytochrome $\underline{c}_{551}$	Horse Heart Cytochrome c	Cytochrome Oxidase (aa_3)	Membrane Particles			
1	0.22	0.15	0.07	0.2	1	1		SO_3^{2-}	-	3
2	0.22	0.15	0.07	0.2	1	1		$S_2O_3^{2-}$	-	12
3	0.22	0.15	0.07	0.25+	1	1		$S_2O_3^{2-}$	-	18
4	0.2	0.1	0.07	0.2	1		0.2	SO_3^{2-}	-	4
5	0.2	0.1	0.07	0.2	1		0.2	$S_2O_3^{2-}$	-	53
									3 µg	68
6	0.4	0.2	0.09	0.3	1	1		$S_2O_3^{2-}$	-	15
										8‡ (after 30 min)
									3 µg	17
7	0.22	0.1	0.07	0.28	0.5		0.2	$S_2O_3^{2-}$	-	23
									-	7‡ (after 30 min)
									4 µg	
										18‡

*The Membrane particles were preapred from Thiobacillus A2 as described before (Lu & Kelly, 1983a)

+The Cytochrome $\underline{c}_{551}$ preparation (G-100 fraction) contained sulphite oxidase.

‡The decrease in rate observed during experiments might be due to general loss of activities of the enzymes and cytochrome preparations.

TABLE 5. The effect of sulphite oxidase on the activity of thiosulphate:cytochrome c oxidoreductase

Experiment No.	Substrate	Supplementary addition of sulphite oxidase	Activity (nmol cytochrome c reduced min^{-1} $(mg\ protein)^{-1}$)
1	$Na_2S_2O_3$	-	60[+]
		5 µg[*]	72[+]
2	Na_2SO_3	-	0
	$Na_2S_2O_3$[‡]	-	10

The activity was measured as described in Materials and Methods.

[*] Sulphite oxidase (HIC fraction) was added after the activity had reached steady state.

[+] More than a dozen of these types of experiments were performed: although the activities varied nearly all of them did show a 15 to 30% rate increase following additions of sulphite oxidase.

[‡] Thiosulphate was added about 5 min after the sulphite.

effective as an electron transfer system than mammalian cytochrome oxidase.

Although the four components (enzyme A, enzyme B, cytochrome $\underline{c}_{551}$ and cytochrome $\underline{c}_{552.5}$) free of sulphite oxidase were not able to reduce horse-heart cytochrome $\underline{c}$ with sulphite (Table 5), they did show a capacity to oxidize sulphite in the oxygen electrode (Table 4). Moreover, the oxidation of thiosulphate by the multienzyme system free of sulphite oxidase was complete, with an $O_2/S_2O_3^{2-}$ ratio of 2.0, as reported before (See 6.3.10). The explanations for these observations might be (a) free sulphite or sulphonate-sulphur were slowly oxidized chemically in the experimental conditions in the oxygen electrode, (note that the reaction mixture containing horse-heart cytochrome $\underline{c}$, cytochrome oxidase or the membrane particles showed no sulphite-oxidizing activity at all); and (b) sulphonate-sulphur was oxidized by the thiosulphate-oxidizing multienzyme system without appearing in the system as free sulphite as shown below.

8.3.5

Demonstration of no formation of free sulphite during the oxidation of thiosulphate by the multienzyme system

2,6-Dichlorophenol-indophenol (DCPIP) was spontaneously reduced by sulphite: thus, addition of 50 nmol Na_2SO_3 to a solution (1 ml) of 60 nmol DCPIP in 50 mM Tris-HCl buffer, pH 7.3 exhibited an initial rate of 30 nmol DCPIP reduced/min (ΔA_{605nm} at 30°C). However, there was no DCPIP reduction by the multienzyme system containing enzyme A, enzyme B, cytochrome $\underline{c}_{551}$, cytochrome $\underline{c}_{552.5}$ and sulphite oxidase supplemented with $Na_2S_2O_3$ instead of Na_2SO_3 under the same experimental conditions. The same reaction mixture showed a rapid reduction of horse heart cytochrome $\underline{c}$ by $Na_2S_2O_3$ (Table 5). This result provides direct evidence that there was no free sulphite formed during the oxidation of thiosulphate by the multienzyme system. It also revealed that the multienzyme system could not use DCPIP as an electron acceptor instead of horse heart cytochrome $\underline{c}$ though the dye had a E_o' of 0.193 v.

3.4 DISCUSSION

AMP-independent and soluble sulphite oxidases have been found and partially purified from several species of thiobacilli (Aminuddin & Nicholas, 1974; Yamanaka et al. 1981). Before 1983 the highest purification achieved was 163-fold and none of them was claimed to be highly purified. It is, therefore, difficult to make a proper comparison with the present study in which a 2000 fold purification was achieved. However, the sulphite oxidase of T. novellus (Yamanaka et al. 1981) did show an interesting similarity to its counterpart in Thiobacillus A2, in that the enzyme was tightly associated with cytochrome c_{551} and the separation of the two resulted in complete loss of the enzyme activity. Furthermore the great difficulty encountered generally in attempts to purify sulphite oxidase reflect a resemblance to the enzyme in the thiosulphate-oxidizing system from Thiobacillus A2. Recently, Toghrol and Southerland (1983) have reported a 206-fold purification to homogeneity of the enzyme from T. novellus. They provided evidence that the enzyme is a molybdohaemoprotein, in contrast to an earlier report that no haem was present (Charles and Suzuki, 1966), but did not indicate if the haem might have been a c-type cytochrome that could be separated from the enzyme. Furthermore according to their purification results the proteins concentration of sulphite oxidase in T. novellus was about 0.45% of the crude extract, the figure is something 10 times greater than we found in Thiobacillus A2. Such a big difference might mean that they are basically different enzymes or components or may do to that (a) the lower (than actual value) of specific activity of sulphite oxidase in the A65% fraction of Thiobacillus A? was obtained. So, the recovery was higher than it should be, and (b) the final purified enzyme from T. novellus, actually consisted of a c-type cytochrome and a sulphite oxidase with the same molecular weight (after SDS-gel electrophoresis) as that which we found in the present study.

There are also other similarities between the sulphite oxidase of Thiobacillus A2, T. novellus and T. thioparus (Charles & Suzuki, 1966; Lyric & Suzuki, 1970). The M_r of our preparation (44,000) is very similar to that reported (41,000) for the homogeneous preparation from T. novellus (Toghrol and Southerland, 1983) The enzymes from the three organisms showed a high affinity for sulphite with K_m values of 14 μM. 20–40 μM and 40 μM respectively. All of them were inhibited by phosphate and the K_i for T. thioparus (10 μM) was similar to that for Thiobacillus A2. Although the latter was non-competitive and the former, uncompetitive. The pH optimum was around pH 8 for the three enzymes. They were also sensitive to sulphydryl inhibitors and could couple to either mammalian cytochrome c or ferricyanide as electron acceptors.

Even following the high degree of purification and characterization achieved, it is still difficult to describe precisely how the enzyme functions in vivo. In contrast to the other four major components of the thiosulphate oxidizing multienzyme system (enzymes A and B, cytochromes c_{551} and $c_{552.5}$), each of which comprises 1-2% of the crude extract protein, sulphite oxidase is less than 0.05% of the protein and its extraordinarily close association with cytochrome c_{551} may indicate the sulphite oxidase protein to be integrated in vivo into the cytochrome itself. This would imply that 'sulphite oxidase'

was an integral component of the complex, being closely associated both with Enzyme B and cytochrome c_{551}, rather than functioning as a free enzyme. An essential role for the sulphite oxidase protein is indicated by the facts that (a) thiosulphate-oxidizing activity by the reconstituted system is stimulated by sulphite oxidase; and (b) the low redox potential haem(s) of cytochrome c_{551} is reduced by the enzyme in the presence of thiosulphate and the multienzyme system (See 6.3.5). The conclusion would thus be drawn that the sulphite oxidase is an essential part of the multienzyme system for the oxidation of thiosulphate and probably functions in the oxidation of the sulphonate-sulphur. The process of thiosulphate oxidation would thus seem to depend on the sequential oxidation of sulphane-sulphur to the sulphonate level, and retention of the original moiety of thiosulphate in this form, all by means of multi-enzyme-complex-bound intermediates. The sulphonate-sulphur-complex is indicated by the data not to be in equilibrium with free sulphite, which is consequently not a substrate for the multi-enzyme complex in the absence of 'free' sulphite oxidase. Although the mechanism is still uncertain, and obviously depends on understanding the general mechanism of oxidation of thiosulphate by the whole multienzyme system, the evidence so far suggests that there are no individually separable steps in the oxidation of thiosulphate in the sense that free intermediates seem not to exist and the individual functions of the enzymes and cytochromes are at present impossible to define. The situation might be similar to the reduction of nitrogen by the nitrogenase system, where no free partially-reduced intermediates are found and the only one detected was an enzyme-bound dinitrogen hydride which could be cleaved from the enzyme by acid treatment to yield hydrazine. Free hydrazine was, however, a very poor substrate for the nitrogenase (Postgate, 1982) in contrast to the Thiobacillus system in which the presence of sulphite oxidase with high activity towards free sulphite occurs (for further discussion see 9.4)

Future work will need to establish whether the true substrate is free sulphite or whether the enzyme acts *in vivo* on enzyme-bound sulphonate-type groups to produce sulphate.

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CHAPTER 9

A PROPOSED MODEL SYSTEM AND GENERAL DISCUSSION

9.1 General aspects of the thiosulphate-oxidizing multienzyme system

An overall purification scheme for the soluble thiosulphate-oxidizing enzyme system is presented in Fig. 9.1. The four major components (enzymes A and B; cytochrome $\underline{c}_{551}$ and cytochrome $\underline{c}_{552.5}$) comprise 4-5% of the cellular protein, whereas the sulphite oxidase alone is less than 0.05% of the cellular protein. Table 9.1 shows some principal properties of these components.

The four major components jointly catalyse the complete oxidation of thiosulphate to sulphate in the presence of an electron transport system, consisting of either the membrane particle fraction of T. versutus and horse heart cytochrome c or mammalian cytochrome oxidase and cytochrome c (Chapers 5 and 6). The membrane particles of T. versutus are more effective than the mammalian system. Horse heart cytochrome c in both cases probably provides a better or more efficient electron coupling between the multienzyme complex and the membrane or cytochrome oxidase systems. The horse heart cytochrome c can be replaced by c-type cytochromes from other sources. Sulphite oxidase is an important part of the enzyme system in the sense that addition of the enzyme to the four components increases the activity by 20-30% (see 8.3.4.). The complete oxidation of thiosulphate was confirmed by demonstrating a 2:1 stoicheiometry of O_2 uptake:thiosulphate oxidized by bacteria in the oxygen electrode cell and by the chromatographic demonstration of the quantitative conversion of $^{35}S_2O_3^{2-}$ to sulphate by the reconstituted system.

The enzyme system has a high affinity for thiosulphate (Km about 2 μM, measured by rate of reduction of horse heart cytochrome c). The Km value for the cytochrome c is about 60 μM. The enzyme system does not

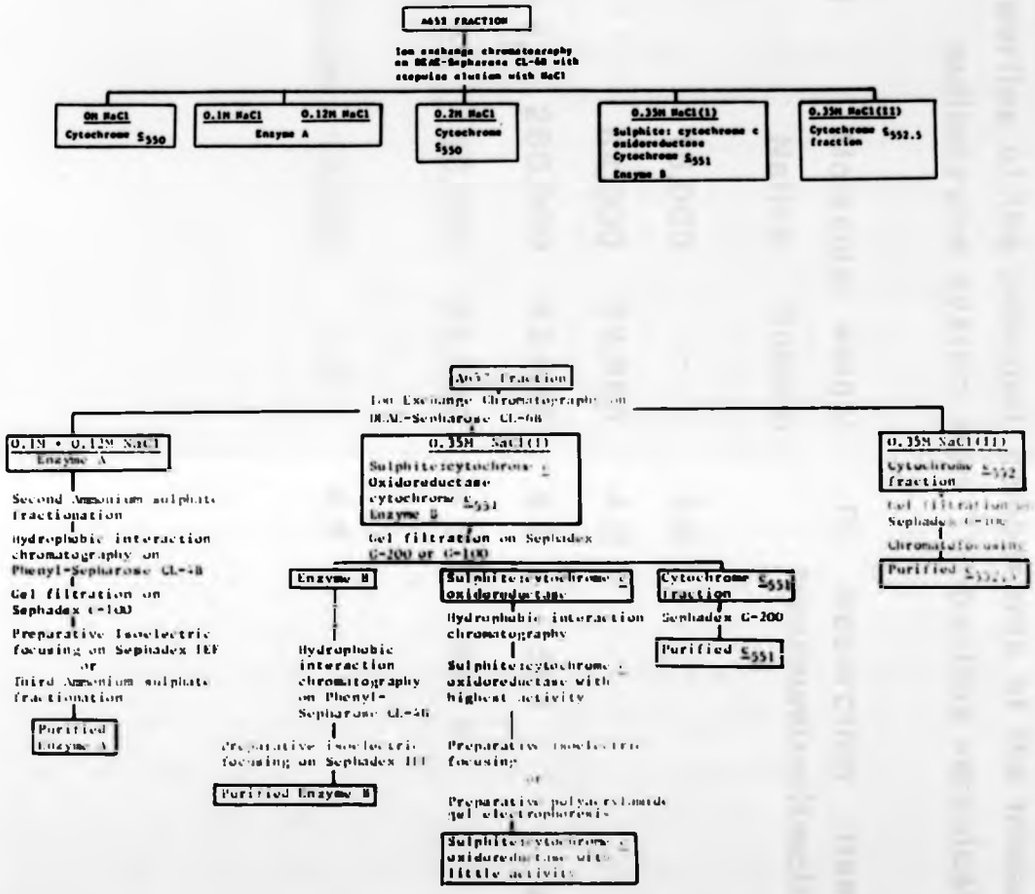

Fig.9.1. Purification of the principal components of the thiosulfate-oxidizing multienzyme system from *Thiobacillus versutus*. Extracts prepared by sonication were centrifuged at 48,000 x g and the proteins precipitated from the supernate by ammonium sulfate between 39-65% saturation fractionated as shown.

table 9.1

Properties of the principal components of the thiosulfate-oxidizing multienzyme system from Thiobacillus versutus(A2)

Component	Molecular weight		PI	Absorption maximum(nm)	Heme content (mol/mol protein)	$E_{m,7}$ (mV)
	Native	Subunit				
Enzyme A	16,000	-	4.2	-		
Enzyme B	64,000	32,000	4.3	-		
Cytochrome c_{551}	260,000	43,000	5.2	551	4-5	240 and -115
Cytochrome $c_{552.5}$	56,000	29,000	4.8	552.5	2	220 and -25 to 25
Sulphite oxidase	43,000	n.d.	4.3			

use ferricyanide or PMS-DCPIP (phenazonium methosulphate + 2,6-dichlorophenol-indophenol) as electron acceptors, but can couple to cytochrome c from different sources. Methane sulphonic acid; sulphonic acid and dithionate are not oxidized by the enzyme system (as measured by reduction of cytochrome c), nor do they affect the oxidation of thiosulphate by the enzyme system. The thiosulphate-oxidizing activity, measured either by reduction of cytochrome c or as oxygen uptake, was inhibited more than 70% when sulphite was added into the reaction mixture containing the four major components completely free of sulphite oxidase activity before the addition of thiosulphate. The mechanism of the inhibition is obscure, and might be a type of competitive inhibition as sulphite can be seen as a part of thiosulphate in chemical structure.

Though we have termed this enzyme system a "thiosulphate-oxidizing multienzyme system (or complex)" or thiosulphate:cytochrome c oxidoreductase when assaying it by the observation of cytochrome c reduction (see 6.2.3), it should be pointed out that the system is quite different from the usual definition, (there is not yet a standard nomenclature for multienzyme systems. Karlson and Dixon (1980) proposed the following definition: Multienzyme proteins include all proteins with multiple catalytic domains, and they be sub-divided into those which are non-covalently linked (multienzyme complex) and those which are covalently linked (multienzyme polypeptides), for a multienzyme complex such as the systems so far well described for pyruvate dehydrogenase or tryptophan synthetase. The present system seems only to function when all components are present and we failed to find any individual catalytic activities for the four major components as we had expected. Therefore the name "multicomponent enzyme" might be better. However, since our failure so far does not necessarily mean that there is no such individual activity and the multiplicity of electron transfer reactions during thiosulphate oxidation and involvement of four or five components still imply the possibility of a

true multienzyme system (or complex) being involved, I shall therefore, keep this term until the system can be more completely understood.

None of the five purified enzyme/cytochrome components contained rhodanese activity. We therefore conclude that rhodanese is not involved in thiosulphate oxidation (Chapter 4).

Since we also exclude the possibility that direct oxygenation is involved in thiosulphate oxidation by the multienzyme system, we propose that the overall process involves hydration, dehydrogenation and electron transport-dependent oxygen reduction as given by:

$$^{-}SSO_3^{-} + 5H_2O + 2O_2 = 2SO_4^{2-} + 4H_2O + 2H^{+} \quad \ldots\ (1)$$

which may be expressed as being comprised of two component systems:

$$^{-}SSO_3^{-} + 5H_2O = 2SO_4^{2-} + 10H^{+} + 8e^{-} \quad \ldots\ (2)$$

$$8H^{+} + 8e^{-} + 2O_2 = 4H_2O \quad \ldots\ (3)$$

9.2 Enzyme A and enzyme B. The exact functions of enzymes A and B in the system are still obscure. These two colourless proteins have no characteristic spectra apart from their absorbance at 230 and 280 nm, and contain no iron, copper or nickel. Both are essential for thiosulphate oxidation since omission of one prevents activity of the system, whereas omission of either cytochrome $\underline{c}_{551}$ or $\underline{c}_{552.5}$ still allows thiosulphate oxidation albeit at a reduced rate (Table 9.2). Preliminary results suggest that enzyme B might be a component that binds thiosulphate (Chapter 5) but this finding needs to be confirmed by other more classical methods such as gel filtration and micro-equilibrium dialysis.

So far, attempts to demonstrate thiosulphate cleavage by the purified enzyme(s) have been unsuccessful. Incubation (in 55 mM phosphate, pH 7.2) of enzyme A or B or mixtures of A, B, cytochrome $\underline{c}_{551}$ (containing sulphite oxidase) and cytochrome $\underline{c}_{552.5}$ with thiosulphate labelled in the inner or outer sulphur atom with ^{35}S for as long as 90 min at 30°C demonstrated no significant formation of sulphite or sulphate.

TABLE 9.2 Oxidation of thiosulphate by the purified components from *T. versutus*

Additions (mg protein)					Rate of O_2 uptake
Enz A	Enz B	$c_{552.5}$	c_{551}	sulphite oxidase*	(nmol O_2 min^{-1} mg^{-1})
	0.15	0.2	0.07	0.003	0
0.22		0.2	0.07	0.003	0
0.22	0.15		0.07	0.003	0
0.22	0.15	0.2		0.003	5
0.22	0.15	0.2	0.07		40
0.22	0.15	0.2	0.07	0.003	55

The reaction mixture contained: Na_2SO_3, 2 umol; membrane particles of *T. versutus*, 0.2 mg; horse heart cytochrome *c*, 0.2 mg; enzymes as indicated and 50 mM Tris, pH 7.3 to a final volume of 1 ml. The reaction was carried out in an oxygen electrode at 30°C. A typical profile is shown in Fig. 6.7.

*The HIC (I) fraction was used, increasing of the enzyme had no effect on the oxygen uptake.

There is no reaction until the addition of an electron transport system, when the thiosulphate is rapidly, continuously and completely oxidized to sulphate. We also failed to show any substantial exchange reaction between free sulphite and radioactive thiosulphate to be catalysed by enzyme A or B or mixtures of the five components. This observation supports the view that the free sulphite is not present during the oxidation of thiosulphate by the enzyme system which was revealed by the DCPIP experiment (see 9.4). The failure to show a thiosulphate-cleaving function using purified enzyme(s) might be due to (a) a requirement for the continuous removal of reaction product from an enzyme, otherwise no continuous oxidation occurs; (b) the correct experimental conditions not having been achieved; or (c) cleavage occurs, but is too slight to be detected by the procedures employed so far(see 5.3.8).

9.3 Cytochrome $\underline{c}_{551}$ and cytochrome $\underline{c}_{552.5}$. The cytochrome c components of the systems are large and rather unusual proteins. Each has two redox centres (Table 9.1): the high potential centres account for 45 and 65% respectively of the absorbance change seen with $\underline{c}_{551}$ and $\underline{c}_{552.5}$. They are designated as c-type cytochromes largely on the basis of their spectral properties and the covalent binding of the haeme groups to the apoproteins. In several respects (see Table 9.1) they are quite different from the c-type cytochromes from other sources, for example, by virtue, of their high molecular weight, multiple haeme content and dual redox centres (Chapter 6 and 7).

Both cytochromes are essential for efficient thiosulphate oxidation and the high potential redox centres in both are reduced by thiosulphate in the presence of enzymes A and B. They probably act therefore as electron carriers between the enzyme-catalysed oxidations and the membrane system. Judging from the similarity of the mid-point redox potential values, the two cytochromes are probably not involved as sequential carriers but rather are employed to accept electrons from different oxidation steps in

thiosulphate dissimilation: there are clearly a number of electron-removal steps in the conversion of (S) to SO_4^{2-} and presumably several different E_h values apply to them also. This view is supported by the observation that the low redox centre of cytochrome $\underline{c}_{551}$ is reduced by the supplementary addition of sulphite oxidase into the reaction mixture containing enzymes A and B (see 6.3.5). On the other hand, this observation and the findings that cytochrome $\underline{c}_{551}$ and sulphite oxidase are closely associated and that considerable loss (>90%) of sulphite oxidase activity after separation from $\underline{c}_{551}$, as well as the low % protein concentration of sulphite oxidase in the cell, all lead to the postulation that the sulphite oxidase is an integral part of the cytochrome $\underline{c}_{551}$.

9.4 Sulphite oxidase. This enzyme was purified some 2000-fold from T. versutus (Chapter 8) as indicated in Fig. 9.1 The enzyme from T. novellus was also purified by several groups (Charles and Suzuki 1966; Toghrol and Southerland 1983 and Yamanaka et al. 1981) and was found to contain c-type cytochrome. The Yamanaka group also observed complete loss of the enzyme activity after separation of the cytochrome $\underline{c}_{551}$ from the enzyme. Toghrol and Southerland (1983) purified the enzyme to homogeneity and reported a molecular weight of 40,000 and the presence of molybdenum. It is not clear if the c-type cytochrome (or haem c) is an integral part of the enzyme as reported by Yamanaka et al. and found in its counterpart from T. versutus (Chapter 8) but the sulphite oxidase of T. versutus is certainly quite similar to that of T. novellus. Both enzymes are sensitive to cyanide, sulphydryl inhibitors and phosphate ion (Chapter 8).

Sulphite has long been regarded as the penultimate intermediate in the oxidation of thiosulphate, mainly on the basis of chemical considerations and the finding of sulphite:cytochrome c oxidoreductase in several Thiobacillus strains. We have, however, failed to show the existence of any dissociable sulphite during the oxidation of thiosulphate.

For example, the multienzyme system (A + B + $\underline{c}_{551}$ + $\underline{c}_{552.5}$) does not catalyse the reduction of DCPIP by thiosulphate in the presence of a small amount of horse heart cytochrome $\underline{c}$, although reduction of the cytochrome occurred. As a small amount of sulphite spontaneously and rapidly reduces DCPIP (Chapter 8), this observation strongly suggests that no free sulphite is released during the operation of the multienzyme system. If sulphite arises it is presumably in an enzyme-bound form of short half-life that is not available for chemical detection. Regarding this, the seemingly non-essential role of the sulphite oxidase in the complete thiosulphate-oxidizing system could be explained if oxidation of free sulphite is a gratuitous function of the enzyme rather than its true physiological role. An observation made by Gottschal and Kuenen (1980) seemingly supports the view, thus, the specific activity of sulphite oxidase was more or less the same no matter whether Thiobacillus A2 was growing on thiosulphate, thiosulphate and acetate or acetate in the chemostat, whereas thiosulphate-oxidizing activity and the RuBP carboxylase showed high activities during autotrophic growth. In vivo, the sulphite oxidase (possibly intimately complexed into the system with cytochrome $\underline{c}_{551}$) may oxidize enzyme-bound form, without free ionic sulphite mediation) or may conceivably be the catalyst for the oxidation of an enzyme-bound component other than sulphite.

9.5 A proposed scheme of action of the thiosulphate-oxidizing multienzyme system and its links to the membrane-cytochrome system

From the data available so far (Kelly, 1982; Kula et al. 1982 and Chapters 2 to 8) as summarized above, the relationship of the components of the thiosulphate-oxidizing multienzyme system to each other and to the electron transport system are likely to be as represented in Fig. 9.2. Electron transport from the system into the membrane cytochromes must clearly bifurcate into energy-yielding electron flow to oxygen, and energy-dependent flow through b-type cytochromes to NAD^+ (Kelly 1982 and Chapter 2).

For example, the multienzyme system (A + B + c_{551} + $c_{552.5}$) does not catalyse the reduction of DCPIP by thiosulphate in the presence of a small amount of horse heart cytochrome c, although reduction of the cytochrome occurred. As a small amount of sulphite spontaneously and rapidly reduces DCPIP (Chapter 8), this observation strongly suggests that no free sulphite is released during the operation of the multienzyme system. If sulphite arises it is presumably in an enzyme-bound form of short half-life that is not available for chemical detection. Regarding this, the seemingly non-essential role of the sulphite oxidase in the complete thiosulphate-oxidizing system could be explained if oxidation of free sulphite is a gratuitous function of the enzyme rather than its true physiological role. An observation made by Gottschal and Kuenen (1980) seemingly supports the view, thus, the specific activity of sulphite oxidase was more or less the same no matter whether Thiobacillus A2 was growing on thiosulphate, thiosulphate and acetate or acetate in the chemostat, whereas thiosulphate-oxidizing activity and the RuBP carboxylase showed high activities during autotrophic growth. In vivo, the sulphite oxidase (possibly intimately complexed into the system with cytochrome c_{551}) may oxidize enzyme-bound form, without free ionic sulphite mediation) or may conceivably be the catalyst for the oxidation of an enzyme-bound component other than sulphite.

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The location of the multienzyme complex shown in Fig. 9.2 was questioned by the following comments: (a) the oxidoreduction enzyme system, so far described, using c-type cytochromes as direct electron acceptors, were found in the periplasm space, such as the enzyme responsible for the oxidation of Fe^{+} in T. ferrooxidans (Ingledew et al. 1977) and methanol dehydrogenase in M. methylotrophus (Alefounder and Ferguson, 1981); (b) all of soluble c-type cytochromes (i.e. non membrane-bound, normally with M.W. around 15K) are restricted to the periplasmic side of membrane (although in our case cytochrome $c_{552.5}$ and cytochrome c_{551} were two unusual cytochromes and it is not clear whether the electrons from the multienzyme system are accepted directly by membrane cytochrome $c_{552.5}$ or mediated by other two soluble cytochrome c_{550}); (c) if the scheme is right, the evidence for cytochrome a,a3 acting as a proton pump is desirable; (d) the result of EDTA-lysozyme experiment was not conclusive since the cell well might not be completely destroyed and the large proteins could still be trapped in between the well and the membrane. It should be possible to obtain more convincing evidence using the techniques such as radioactive labelling and antibody (e.g. raised against enzyme B). The problem could also be tackled by indirect means to test whether a H^{+} thiosulphate symport exists by measuring the effect of ionophores to the thiosulphate oxidation by whole cells and the pH difference before and after the addition of thiosulphate to an anaerobic cell suspension.

Ingledew, W.J., J.C. Cox and P.J. Halling (1977). FEMS Microbiol. Letts. 2, 193.
Alefounder, P.R. and S.J. Ferguson (1981). Biochem. Biophys. Res. Comm. 98, 778-784.
Cobley, J.G. (1976). Biochemical Journal, 156, 481-492; 493-499.

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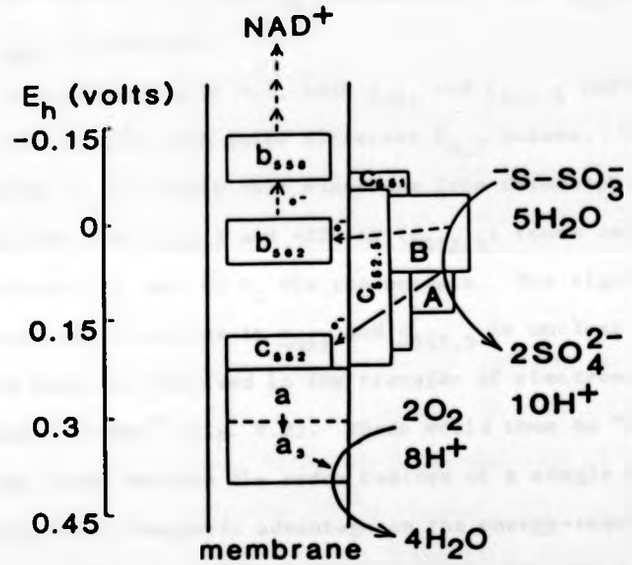

Fig. 2. Probable mechanism of action of the thiosulfate-oxidising multienzyme system and its links to the membrane-cytochrome system. E_o values of the cytochromes are indicated.

Respiratory chain and redox components of T. versutus have recently been the subject of extensive study (Kula et al. 1982; Loya et al.1982; Chapters 2, 6 and 7). Multiple b-type cytochromes were detected using potentiometric methods, giving $E_{M,7}$ values of -90 mV for b_{558} and +30 mV for b_{562} respectively. The components of T. versutus cytochrome oxidase seem very similar to those of mammalian mitochondria in that the oxidase has high absorbance at 608 nm and $E_{M,7}$ values of +210 and +390 mV for a and a_3 respectively. The $E_{M,7}$ of the membrane-bound c_{552} is +205 mV.

As shown on Table 9.1, both c_{551} and $c_{552.5}$ contain two oxidation-reduction centres with quite different $E_{M,7}$ values. From the energetic standpoint it is likely that electrons from thiosulphate oxidation pass through the +240 (c_{551}) and +220 mV ($c_{552.5}$) redox centres, then pass to membrane-c_{552} and to O_2 via the oxidase. The significance of the low potential redox centres in c_{551} and $c_{552.5}$ is unclear. One possibility is that they are involved in the transfer of electrons to cytochrome b and thence to NAD^+ (Fig. 9.2). There would thus be "internal reverse electron flow" between the redox centres of a single cytochrome, possibly bestowing some energetic advantage on the energy-requiring NAD-reduction sequence. Since the redox potential values for the four pairs of electrons generated by thiosulphate oxidation are not known with any certainty, it is of course possible that the low potential centres of c_{551} and $c_{552.5}$ could be direct carriers also. For example, the E'_o for the SO_3^{2}/SO_4^{2-} reaction is about -280 mV (Kelly 1982), consistent with the observed reduction of the low potential redox centres of c_{551} by the T. versutus sulphite oxidase (for the detailed discussion, see Chapter 7).

No redox centres have been found for enzymes A and B, so their position on the scheme of Fig. 2 is not meant to indicate any information on this point: the E_h scale relates only to the cytochromes. Components not shown in Fig. 9.2 include sulphite oxidase (believed to be linked with c_{551}) and two other soluble cytochromes (c_{550}(acidic) and c_{550}(basic)),

which have also been purified from T. versutus (Chapter 6). Their physiological role is unknown: they could mediate between c_{551}, $c_{552.5}$ and the membrane c_{552} or might be located on the outer surface of the membrane to facilitate a protonmotive redox loop mechanism. We have no evidence that the cytochrome oxidase (a, a_3 in Fig. 9.2) is located on the periplasmic or the cytoplasmic side of the membrane. We have not shown any scheme for a prot on gradient in Fig. 9.2, as we have no experimental data, but is is clear that the system generates such systems to drive oxidative phosphorylation.

9.6 Some comparisons with other biological inorganic system on the formation of intermediates

This system is clearly a complex one and is analogous to the better understood enzyme systems involved in the reactions of inorganic compounds such as assimilatory nitrate reduction (Losada et al. 1981), dissimilatory and assimilatory sulphate reduction (Peck and LeGall 1982) and dinitrogen fixation (Postgate 1982). In these cases free intermediates are equally rare, the apparent sequence of reactions seems relatively short and the overall processes involving six or eight electron transfer occur in an integrated manner, making intermediate stage identifications difficult. From these, and the present case, general rules may be formulated for the formation and identification of detectable intermediates: (a) any free intermediate must be chemically stable (e.g. nitrite), otherwise enzyme systems have been evolved to avoid loss of such intermediates; (b) intermediates are detectable when the reaction effected is thermodynamically unfavourable, thus seen in the APS and PAPS pathways for the reduction of sulphate to sulphite (SO_4^{2-}/SO_4^{2-} with E_o' of -440 mV being unable to be effected by NADH, E_o' -320 mV). Similarly, oxidation of ammonium ion to hydroxylamine (E_o' of the NH_3/NH_2OH couple, + 899 mV) generates free NH_2OH, but the subsequent energy-yielding oxidation to NO_2^- by Nitrosomonas does not liberate free intermediates. It is also noteworthy that hydroxylamine is not detected as a dissociable free intermediate of the reduction of nitrate or dinitrogen but is itself rapidly by nitite reductase (Vega and Kanin 1976) and very slowly by nitrogenase (Postgate 1982). This is a situation analogous to our observations with the T. versutus sulphite oxidase, in the sense that free sulphite is oxidized by the enzyme but is not a detectable intermediate. As with these other systems, the $8e^-$ oxidation of sulphur from -2 to +6 does not involve free

quantities, electrons are removed during thiosulphate metabolism. One conclusion that can be drawn from the above comparisons is that the mechanisms or pathways of oxidation or reduction of inorganic compounds seem quite different from those of inorganic compounds in the sense that the reaction steps or intermediates after the transition of each pair of electrons are not normally detectable or separable in the inorganic systems as in the case of the organic systems, thus the oxidation of methane to carbon dioxide involves four distinctive steps and three stable intermediates, for example. Therefore the theories and methods used in the study of organic systems are not necessarily applicable to inorganic systems.

9.7 Some speculation on the nature of the oxidation process and its transient intermediates

Chemically, thiosulphate can be regarded as sulphate in which one of the oxygen atoms has been replaced by sulphur (= the sulphane group). The two sulphur atoms are thus unequal, the outer one having an oxidation number of -2, whereas the central sulphonate-group sulphur atom has an oxidation number of +6 (Roy and Trudinger 1970 and Schmidt and Siebert 1973). Thiosulphate synthesis can be from the condensation of sulphur and sulphite. From these considerations, several postulations can be made regarding the nature of the cleavage of the bond between the sulphane and sulphonate sulphurs in thiosulphate. Hydrolytic cleavage could give rise to sulphur and sulphite (equation 4) or to sulphide and sulphate (equation 5).:

$$^{-}SSO_3^{-} + H_2O = HSO_3^{-} + S^{o} + OH^{-} \quad \ldots\ldots\ldots \quad (4)$$

$$^{-}SSO_3^{-} + H_2O = HSO_4^{-} + H^{+} + S^{2-} \quad \ldots\ldots\ldots \quad (5)$$

Sulphite and sulphide would only be produced by a reductant-requiring thiosulphate reductase (Kelly 1982 and Peck 1960).:

$$^{-}SSO_3^{-} + 2H^{+} + 2e^{-} = SO_3^{2-} + H_2S \quad \ldots\ldots\ldots \quad (6)$$

If thiosulphate metabolism is initiated by a hydrolytic cleavage it would seem likely, therefore, to be consistent with the lack of observation of free sulphite, that either sulphate is produced directly or that sulphite

is not released from the enzyme complex after cleavage. Assuming an initial cleavage, the result of the concerted action of enzymes A, B and the associated cytochromes could be as follows:

$$^{-}SSO_3^{-} + (\text{Enzyme complex, } EC^{-}) + H_2O = EC\text{-}S^{-} + SO_4^{2-} + 2H^{+} + 2e^{-}$$

$$EC\text{-}S^{-} + H_2O = EC\text{-}SO^{-} + 2H^{+} + 2e^{-}$$

$$EC\text{-}SO^{-} + H_2O = EC\text{-}SO_2^{-} + 2H^{+} + 2e^{-}$$

$$EC\text{-}SO_2^{-} + H_2O = EC\text{-}SO_3^{-} + 2H^{+} + 2e^{-}$$

$$EC\text{-}SO_3^{-} + H_2O = EC^{-} + SO_4^{2-} + 2H^{+}$$

Overall (equation 2) $$^{-}SSO_3^{-} + 5H_2O = 2SO_4^{2-} + 10H^{+} + 8e^{-}$$

9.8 Problems in the study and future strategy

While the T. versutus system is proving immensely valuable to the elucidation of the thiosulphate-oxidizing process, further progress has been hampered by a number of factors including (a) low activity: disruption of the organism causes substantial loss of oxidative ability (see 2.3.3 and 5.4 for discussion). Thus intact cells exhibit a Q_{O_2} of about 1000 nmol O_2 uptake/min/mg protein in the oxygen electrode, which falls to about 10 in the cell-free extract. Using the reconstituted system, the rate of thiosulphate oxidation by the five components (including sulphide oxidase) is about 70 nmol cytochrome c reduced/min/mg total protein or about 50 nmol/min/mg total protein (in the presence of the T. versutus membrane particle fraction); (b) a vast amount of work is involved in obtaining sufficient amounts of autotrophically grown cells (Prof. Kuenen once suggested me to continuous mixotrophic culture to improve the yield, as they found that Thiobacillus A2 growing on a medium of 18 mM thiosulphate and 11 mM acetate at the dilution rate of 0.09 h^{-1} showed the same specific thiosulphate-oxidizing activity as one growing on thiosulphate alone and gave a much higher yield since acetate, instead of CO_2, was the carbon source of the organism (Gottschal and Kuenen 1980). This is certainly a way worthwhile to pursue) and to purify enzymes and cytochromes one by one. Enzyme A is oxygen-sensitive and very unstable

as it was found that briefly bubbling the enzyme solution (for example, to dissolve the protein precipitated by $(NH_4)_2SO_4$) and omitting thiosulphate from the elution buffer during the purification caused considerable loss of the activity; (c) as four or five components are necessary, kinetic and inhibitors studies and determination of rate-limiting components and the best ratio of the components for activity are very difficult; (d) the absence of characteristic spectra for enzymes A and B and failure yet to be able to attribute specific functions for them in the system make their further characterization and study of the whole system, for example, the route of electron flow from substrate, a considerable problem.

Future work needs to attempt to establish (a) the initial reaction binding thiosulphate to an enzyme using a micro-equilibrium dialysis or gel filtration method; (b) the presence or absence of separable reaction steps and detectable intermediates; (c) the nature of the hydrolytic or other cleaving reaction if this is the primary step for thiosulphate metabolism. This could be attempted by measuring a time course of formation of sulphate from ^{35}S-thiosulphate labelled on either the inner or outer sulphur atom and using whole cell suspension (see 1.1.5) or even with the purified components; (d) the progress of sulphane-sulphur oxidation: ^{35}S-methods or mass spectrometry may aid in this; (e) the redox levels of the component oxidation steps and their cytochrome acceptors; (I have no other good ideas on how to design experiments to solve these problems (b to e). One experiment in my mind is to incubate enzyme A, B and cytochrome $\underline{c}_{552.5}$ with ^{35}S-thiosulphate for a certain time, then to separate and identify (- which is a considerable technical problem -) the intermediates since we found (see 6.3.10) that in the absence of cytochrome $\underline{c}_{551}$ the oxidation of thiosulphate went on very slowly and incompletely) (f) the role of the dual redox centre cytochromes; (g) amino acid compositions or sequences of cytochrome $\underline{c}_{552.5}$ and cytochrome $\underline{c}_{551}$ to compare with the well defined $\underline{c}$-type cytochromes; (h) the substrate specificity of the multienzyme compounds such as thiosulphonates (RSO_2S^-), thiosulphate esters ($RSSO_3^-$), persulphides (RSSH) and

methyl sulphide are possible substrates, although some preliminary results (see 9.1) suggested that this enzyme system was possibly a very specific one, only using thiosulphate; (i) the effect of these substrate analogues on the thiosulphate-oxidizing activity of the enzymr system; (j) mechanism and energy consumption for thiosulphate transport by the organism, since this could play an important part in the calculation of efficiency of energy conservation as shown in 2.5; (k) ESR spectroscopy of enzyme A on enzyme B and (l) enzyme kinetic studies on the interactions between the four major components during the thiosulphate oxidation.

Among the specific experiments required with T. versutus are (a) tests with ^{18}O-labelled oxygen and water in order to eliminate (or otherwise) a role for an oxygenase; and (b) short-term oxidation experiments with $^{35}S\text{-}SO_3$ and $S\text{-}^{35}SO_3$ to attempt to establish (from the initial rates of $^{35}SO_4^{2-}$ production) the timing of S-S bond cleavage. There have been studies with ^{18}O previously (Kelly 1982), designed to establish the role of APS in sulphite oxidation but it would be desirable to study ^{18}O transfer into sulphate during thiosulphate oxidation by intact bacteria. The following predictions on ^{18}O enrichment can be made.

$$^{-}SSO_3^{-} + 5H_2O + 2\,^{18}O_2 = 2SO_4^{2-} + 4H_2\,^{18}O + 2H^{+} \quad \ldots\ldots\ldots (7)$$

$$^{-}SSO_3^{-} + 5H_2\,^{18}O + 2O_2 = 2SO_4^{2-} (^{18}O{:}\,^{16}O = 5{:}3) + 2H^{+} \quad \ldots\ldots (8)$$

$$^{-}SSO_3^{-} + 5H_2O + 2\,^{18}O_2 = 2SO_4^{2-} (^{18}O{:}\,^{16}O = 1{:}3) + 2H^{+} \quad \ldots\ldots (9)$$

If no oxygenase functions, no ^{18}O should enter SO_4^{2-} from $^{18}O_2$, but $H_2\,^{18}O$ should arise (equation 7) and ^{18}O should be introduced into sulphate from $H_2\,^{18}O$ in the indicated ratio (equation 8). If there is an oxygenase, converting sulphane-sulphur to sulphite, ^{18}O from oxygen should enter sulphate as given in equation (9). Once the mechanisms have been established, it will be possible to deduce more exactly the nature of energy-conserving processes in the thiobacilli (Kelly 1982 and Chapter 2).

9.9 Possibility of the same thiosulphate oxiding system operating in other thiobacilli

The results of growth yields (see 1.1.1) and respiratory chain inhibition (see 1.1.2) indicate that the energy efficiency of thiosulphate oxidation is more or less the same in the thiobacilli examined so far except for T. denitrificans. This implies a possibility of a common pathway for thiosulphate oxidation by these organisms (Chapter 1). There is some other evidence to support the view which I have stated in Chapter 1, such as a similar soluble thiosulphate:cytochrome c oxidoreductase in T. novellus and possibly in T. neapolitanus, high content of c-type cytochromes in thiosulphate-grown T. novellus and T. neapolitanus (about 2 μmol/g protein and more than one-third in soluble fraction found by Sadler and Johnson 1972, also Trudinger 1962). Oxidation of both sulphur atoms of thiosulphate simultaneously by these two thiobacilli etc.

But the evidence against this view also exists such as: (transient) accumulation of elemental sulphur or tetrathionate during the thiosulphate oxidation by whole cells or extracts of T. thioparus, T. neapolitanus and T. thiooxidans, quite different growth rates on thiosulphate, capacity to oxidize elemental sulphur by the three organisms, which is absent from T. versutus etc.

Nevertheless the evidence concerning this subject is still too scanty both qualitatively and quantitatively to make any conclusions, for which, obviously more work is needed.

There are a number of possible approaches towards this matter: (a) establishing the location of the enzyme system and the role of the membrane fraction, by for example, assaying thiosulphate:cytochrome c oxidoreductase in the soluble fraction as a preliminary test; (b) assay the effect of concentration of crude extract on the specific thiosulphate-oxidizing activity to find out if there is a multienzyme complex involved; (c) fractionation of crude extract with $(NH_4)_2SO_4$ or further with DEAE-Sepharose CL-6B to resolve

the components; (d) comparison of the protein patterns of the fractions after SDS-gel electrophoresis with that of purified components from T. versutus. (It is difficult to do this with crude extract as the concentration of the four major components (if as in T. versutus each comprises about 1% of the total proteins) is still too low to show any significance in crude extract) (e) immunological approach using antiserum prepared against highly purified components, for example, enzyme B from T. versutus; (f) high purification of enzyme(s) involved.

9.10 Chemoautotrophic growth on thiosulphate by phototrophic and facultative hydrogen bacteria

It has been long recognised that some heterotrophic bacteria such as species of Pseudomonas and Achromobacter catalyse the oxidation of thiosulphate to tetrathionate (Roy and Trudinger 1970). Recent results, however, show that the complete oxidation of thiosulphate to sulphate to support the chemoautotrophic growth is not limited to thiobacilli and might be a more general phenomenon in the natural environment than we thought.

A survey conducted by Kämpf and Pfennig (1980) revealed that 7 species out of 17 species of purple sulphur bacteria (Chromatiaceae) tested were capable of chemoautotrophic growth in the dark under microaerobic conditions. They also calculated a growth yield of 11.3 (a rather high value compared to that of aerobic thiobacilli) for Thiocystis violacea 2311 on thiosulphate in batch culture with a doubling time of 30h. The easy transition from photolithotrophic growth to chemoautotrophic growth by these organisms and the comparable growth yield suggest that the enzyme system or the metabolic pathway (including electron transport) might bear some similarities to that of thiobacilli, although further evidence is absent and the thiosulphate-oxidizing activities of these organisms were generally rather low.

Friedrich and Mitrenga (1981) demonstrated that Paracoccus denitrificans grew chemoautotrophically in the chemostat giving a growth yield of 4.5 g

dry wt cell per mol thiosulphate with a μ_{max} of 0.14 h^{-1}. They also showed that all strains of Xanthobacter autotrophicus tested, some species of Pseudomonas and a specie of Aquaspirillum were able to oxidize thiosulphate on agar plates to produce acid, indicative of sulphate formation. These observations suggest that complete oxidation of thiosulphate can be carried out by a number of heterotrophic or facultative chemoautotrophic bacteria and at least one of them shows a growth yield comparable to the aerobic thiobacilli, indicating a similar energy efficiency of growth on thiosulphate. These bacteria and facultative chemoautotrophic sulphur bacteria such as T. versutus and T. novellus might play a significant part in the sulphur metabolism in soil, as it was suggested that soil heterotrophs could be more important than autotrophs in the oxidation of sulphur compounds (Vishniac and Santer 1957). Indeed the borders between heterotrophs and autotrophs and between hydrogen bacteria and thiobacilli are becoming less clear considering the versatile capacities of T. versutus, P. denitrificans and X. autotrophicus. Anyway, it will be very interesting to see if the same enzyme system or mechanism of thiosulphate oxidation is operating in these different organisms.

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CHAPTER 10

An enzymatic lysis procedure for the assay of enzymes in *Thiobacillus* A2.

Abstract

A micromethod is described for the production of lysed preparations of *Thiobacillus* A2 following treatment with lysozyme and EDTA. These may be used for the assay of intra-cellular enzymes including rhodanese, hexokinase, glucose 6-phosphate dehydrogenase and phosphoglucoseisomerase. The procedure is useful for assaying enzymes in samples too small to be treated by conventional mechanical methods, but gives comparable recoveries of enzyme activities.

10.1 INTRODUCTION

Lytic procedures have been widely used in the preparation of membrane vesicles from bacteria (Coakley et al., 1977) but have received little attention as a way of assaying individual enzymes. Vandenbergh et al (1979) described a procedure for determining rhodanese in a wide variety of bacteria using extracts prepared by lysis with EDTA and lysozyme, based on the method used by Kaback (1971) for preparing membrane vesicles. We have tested the effectiveness of this procedure on *Thiobacillus* A2 for the release and subsequent assay of several intracellular enzymes, normally detectable only in extracts prepared by mechanical or sonic disruption (Silver and Kelly, 1976; Wood et al., 1977; Lu and Kelly, 1983a). Our aim was to establish a simple procedure enabling assay of enzymes in small samples of organisms taken from chemostat cultures, where removal of large culture volumes disrupts steady state conditions (Wood and Kelly, 1981). A procedure allowing the volume sampled from a 750 ml chemostat to be reduced to 5-10 ml would overcome this problem and enable variation in selected enzymes to be monitored during transition

as well as steady state conditions in the culture.

10.2 MATERIALS AND METHODS

10.2.1
Organisms and Culture Conditions.

Thiobacillus A2 (Taylor and Hoare, 1969) was cultured in flasks shaken at 30°C as described previously (Wood and Kelly, 1977) on a variety of substrates. Anaerobic cultures were grown in bottles completely filled with media containing glucose, formate or acetate and supplemented with 20 mM KNO_3.

Thiobacillus denitrificans (NCIB 9548) was grown anaerobically at 30°C in bottles completely filled with a thiosulphate-nitrate medium as described previously (Justin and Kelly, 1978). *T. neapolitanus* strain C (Kelly, 1969) was grown anaerobically in liquid culture on thiosulphate (Kelly, 1967; Tuovinen and Kelly, 1973).

10.2.2
Preparation of Lysates.

Cultures were harvested by centrifuging, washed with 0.055 M phosphate buffer, pH 7.8 and suspended to a density of 1 mg dry wt ml^{-1} in the same buffer. For lysis, one ml of a solution of 5 mg lysozyme in 0.1 M disodium EDTA was added to either 5 ml or 9 ml of cell suspension and incubated at 30°C for various lengths of time. Mixtures were centrifuged at 2000 g for 5 min. to remove broken cell material. Enzymes were assayed using both uncentrifuged mixtures and the clear supernatant liquids after centrifuging.

10.2.3
Assay of Enzymes.

Rhodanese was assayed in a mixture containing (μmol) in a final volume of 2.2 ml: Tris, 125; $Na_2S_2O_3$, 100; KCN, 100; Lysate, 0.5 ml equivalent to 0.2-0.25 mg original cell protein. Buffer and extract were preincubated at 30°C in small tubes (4 ml) for 5 min before adding $Na_2S_2O_3$, then initiating reaction with KCN. The assay was normally stopped after 10 min by adding 0.1 ml 38% (w/v) formaldehyde solution. Thiocyanate formed was measured by adding 1.1 ml 14.8% (w/v) ferric nitrate in 6% (v/v) HNO_3 and reading

absorbance due to ferric thiocyanate at 470 nm.

Hexokinase, glucose 6-phosphate dehydrogenase ($NADP^+$) and phosphoglucoseisomerase were assayed as described previously (Wood et al., 1977)

Chemicals

Lysozyme (chicken egg white, grade 1), NADP, fructose 6-phosphate, glucose 6-phosphate, ATP and glucose 6-phosphate dehydrogenase were all obtained from Sigma (London) Ltd.

10.3 RESULTS AND DISCUSSION

Rhodanese is apparently formed constitutively in *Thiobacillus* A2 regardless of growth substrate (Table 1; Wood and Kelly, 1981; Lu and Kelly, 1983a,b). While significant activity could be demonstrated using unlysed cells, indicating the surface location of this enzyme, activity was enhanced up to seven-fold following lysozyme treatment. Most of this activity was then recoverable in the supernatant liquids obtained by centrifuging the lysates at 2000 x g. These supernates probably still contained small particles from the membrane material of the cells. Rhodanese could also be demonstrated in anaerobic heterotrophic cultures (Table 1b). Thiosulphate, which is not oxidized by anaerobic batch cultures, had no effect on the amount of enzyme synthesised. These results were in agreement with later results that showed rhodanese not to be involved in the aerobic oxidation of thiosulphate by *Thiobacillus* A2 (Lu and Kelly, 1983c).

Maximum rhodanese activity in thiosulphate-grown bacteria was detectable almost immediately after mixing the organisms with lysozyme but the release of enzyme activity into the supernate took up to two hours and was then similar to activity obtained with French press extracts (Table 2). Similarly about 85% of the rhodanese from maltose-grown organisms was released into the supernate during two hours incubation with lysozyme, about 90% of this being released in the first hour (Table 3). Increasing the lysozyme

TABLE 1. Effect of lysozyme on the assay of rhodanese activity in suspensions of *Thiobacillus* A2.

Growth Conditions (mM)	Rhodanese Activity* (nmol SCN^- min^{-1} mg dry wt^{-1})		
	Intact Cells	Lysozyme-treated suspension	Lysozyme supernate
(a) Aerobic			
Thiosulphate (20)	97	218	141
Glucose (20)	26	342	188
Fructose (20)	31	139	-
Sucrose (10)	-	144	151
Maltose (10)	42	285	247
Acetate (30)	84	200	-
Pyruvate (10)	-	178	166
Succinate (15)	55	166	-
(b) Anaerobic (KNO_3 reduction)			
Glucose (20)	-	144	-
Glucose + $S_2O_3^{2-}$ (20 + 20)	-	153	-
Fructose (20)	-	129	-
Fructose + $S_2O_3^{2-}$ (20 + 20)	-	153	-
Acetate (30)	-	144	-
Acetate + $S_2O_3^{2-}$ (30 + 20)	-	118	-

*5 ml suspension + 1 ml lysozyme-EDTA, 1 hour at 30°C (See Methods).

-, not determined

TABLE 2. Time course of release of rhodanese from thiosulphate-grown *Thiobacillus* A2 (5 ml suspension + 1 ml lysozyme-EDTA).

Time	Rhodanese Activity ($nmol\ SCN^{-}\ min^{-1}\ mg\ dry\ wt^{-1}$)	
	Lysozyme treated suspension	Lysozyme supernate
1 min	141*	35*
1 h	210	110
2 h	218	141
3 h	228	143
4 h	-	140

*Activities in the absence of lysozyme were 97 and 10 respectively.

Rhodanese activity in a crude extract prepared using the French pressure cell was 240.

TABLE 3. Release of rhodanese from maltose-grown *Thiobacillus* A2 (0.5 mg protein ml^{-1}) by lysozyme-EDTA.

Treatment	Rhodanese Activity ($nmol\ SCN^{-}\ min^{-1}\ mg\ protein^{-1}$)	
	Whole suspension	Supernate
Untreated intact cells	42	-
9 ml suspension + ml lysozyme		
1 hour incubation	-	211
2 hour incubation	272	230
5 ml suspension + 1 ml lysozyme		
1 hour incubation	-	217
2 hour incubation	285	247

did not increase the rate of release.

To test the general applicability of the method to other thiobacilli, attempts were made to release rhodanese from *Thiobacillus denitrificans* and *T. neapolitanus*. After one hour's treatment with lysozyme, under the standard conditions, *T. denitrificans* lysates exhibited a specific activity of 179 nmol SCN^- min^{-1} mg dry wt $^{-1}$ (compared to 66 by unlysed cells), of which 75 were in the supernatant. Rhodanese was assayed in *T. neapolitanus* at the apparent optimum of pH 9.0 (Kelly and Tuovinen, 1975). Untreated suspensions had an activity of 90, which increased to 165 after one hour's incubation with lysozyme-EDTA, of which about one-third was in the supernatant solution.

Hexokinase, phosphoglucoseisomerase and glucose 6-phosphate dehydrogenase cannot be assayed with intact cell suspensions. Treatment with lysozyme and EDTA for one to two hours, however, released high levels of enzyme activity (Table 4). Typical specific activities for these enzymes were 240, 790 and 200 respectively when calculated in terms of total cellular protein. Even though this might underestimate activity if compared with extracts made with the French pressure cell, these activities in fact exceed those previously found using conventional cell disintegration procedures: values for maltose-grown *Thiobacillus* A2 being 110, 537 and 164 respectively (Wood and Kelly, 1980; Wood et al., 1977). This procedure has thus been shown to be applicable to the assay of a number of diverse enzymes in *Thiobacillus* A2, and would presumably be suitable for many other enzymes. The experience of Vandenbergh et al. (1979) indicates that this and similar lytic procedures could have applicability to many different bacteria and provides a rapid and convenient micromethod for enzyme analysis.

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TABLE 4. Release of enzymes of carbohydrate metabolism from maltose-grown *Thiobacillus* A2 (0.5 mg protein ml^{-1}) by lysozyme-EDTA.

Treatment	Enzyme Activity* (nmol min^{-1} mg $protein^{-1}$)
(a) Hexokinase	
9 ml suspension + 1 ml lysozyme	
1 h incubation	149
2 h incubation	241
3 h incubation	238
5 ml suspension + 1 ml lysozyme	
1 h incubation	170
(b) Phosphoglucoseisomerase	
9 ml suspension + 1 ml lysozyme (2 h)	765
5 ml suspension + 1 ml lysozyme (2 h)	793
(c) Glucose 6-phosphate dehydrogenase	
9 ml suspension + 1 ml lysozyme (2 h)	200

*Calculated in terms of the protein content of the unlysed suspension.

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