Hydrogen Sulphide as Inhibitor and Substrate
for the Cytochrome c-Cytochrome c Oxidase System

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Abstract

It has previously been recognized that the major biochemical toxicity induced by sulphide is due to an inhibition of cytochrome c oxidase. Inhibition of this enzyme occurs at 30°C and pH 7.4 with a $K_i$ of approximately 0.2 $\mu$M, and a $k_{on}$ of $10^4$ M$^{-1}$ s$^{-1}$, under catalytic conditions. However, the equimolar mixture of sulphide and the enzyme shows identical catalytic behaviour to that of the native enzyme. This cannot readily be attributed to rapid dissociation of sulphide, as both spectroscopic and plot analysis indicate the $k_{off}$ value is low. The addition of stoichiometric sulphide to the resting oxidized enzyme gives rise to the appearance of a low-spin ferric-type spectrum not identical with that seen on the addition of excess sulphide to the enzyme aerobically. Sulphide added to the enzyme anaerobically gives rise to another low-spin, probably largely ferric, form which upon admission of oxygen is then converted into a 607 nm species closely resembling Compound C. The 607 nm form is probably the precursor of oxyferricytochrome $a_3$. The addition of successive aliquots of Na$_2$S solution to the enzyme induces initial uptake of approximately 3 moles of oxygen per mole of the enzyme. Thus, it is concluded that:

1. the initial product of sulphide-cytochrome c oxidase interaction is not an inhibited form of the enzyme, but the low-spin (oxyferri) $a_3^{3+}a_3^{3+}$ species;
2. a subsequent step in which sulphide reduces cytochrome $a$ occurs;
3. the final inhibitory step, in which a further molecule of sulphide binds to the cytochrome $a_3$ iron centre in the cytochrome $a_2^{2+}a_3^{3+}$ species, gives the cytochrome $a_2^{2+}a_3^{3+}$–H$_2$S form which is a half-reduced fully inhibited species;
4. a 607 nm form of the enzyme is produced which may be converted into a catalytically active low-spin (oxyferri) state; and therefore
5. liganded sulphide may be able to reduce the cytochrome a$_3$-Cu centre without securing the prior reduction of the cytochrome a haem group or the Cup centre associated with it.
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Chapter I. Introduction

(A) General Introduction

Cytochrome c oxidase (EC 1.9.3.1) is the principal high affinity oxidase in the aerobic metabolism of animals, plants, algae, yeast and some bacteria. In eukaryotes, it is present in the mitochondria and in bacteria it occurs in the cytoplasmic membrane. This enzyme catalyzes the reduction of molecular oxygen to water. The electrons are provided by reduced cytochrome c according to the overall reaction of Eq. (1):

\[ \text{O}_2 + 4 \text{ cyt. c}^{2+} + 4H^+ \rightarrow 2\text{H}_2\text{O} + 4 \text{ cyt. c}^{3+} \]  

(Eq. 1)

The free energy released in oxygen reduction may be used in oxidative phosphorylation and become available as ATP (Lemberg, 1969; Caughey et al. 1976). Cytochrome c oxidase is a haem-copper-lipoprotein. The two haem groups and the two copper atoms are present in a "molecule" with a size of about 150,000 dalton. The two haem a groups react differently toward oxygen, reducing agents, and inhibitors (e.g. cyanide, azide, carbon monoxide, formate, sulphide). The haem-containing components are known as cytochromes aa (Keilin, 1939, 1966).

(B) Early History of Cytochromes

On 13 December 1884, the Irish physician Charles MacMunn presented his work "On myohaematin, an intrinsic muscle-pigment of vertebrates and invertebrates, on histohaematin, and on the spectrum of the suprarenal bodies" at a meeting of the Physiological Society held in London, England (Keilin, 1966). This was the beginning of research on cytochromes. Later reports appeared in 1886 (MacMunn) and 1887 (MacMunn).
He had discovered respiratory pigments which are widely distributed in plants and animal tissues. These observations were criticized by the prominent German chemist, Hoppe-Seyler and subsequently ignored. (Keilin, 1966). In 1925, Keilin rediscovered and established the modern concepts of cytochromes and Warburg's Atmungsferment (Warburg, 1932) the oxygen-transferring enzyme. This opened a new era in cell respiration and bioenergetic studies. He showed the changes in the absorption spectra of cytochromes, first observed by MacMunn, indicating oxidation and reduction under physiological conditions. In addition, he utilized the inhibitors, carbon monoxide, azide and cyanide. When Keilin carried out these experiments, "his only tools were a bench spectroscope, an immense appreciation of biological principles and a formidable imagination." (Green, 1980) Keilin reported in 1928 that hydrogen sulphide was also an inhibitor of respiratory chain (Keilin, 1928). The formation of a complex between methaemoglobin and sulphide which is distinct from sulphaemoglobin, was first reported five years later (Keilin, 1933).

(C) Hydrogen Sulphide as a Poison

Poisoning by hydrogen sulphide has been recognized as an occupational hazard for at least two centuries (Smith and Gosselin, 1979). In view of the high toxicity of sulphide, it is remarkable that there are so few reports of its effects on enzyme systems. Since Keilin's observations (1928,1930), it has been thought the key biochemical lesion induced by sulphide is the inhibition of the terminal respiratory enzyme. It was subsequently suggested that, as an experimental tool in
studies of inhibition of electron transfer, sulphide might have certain advantages over the other classical inhibitors, cyanide and azide (Chance et al, 1966). Somewhat later revised kinetic and spectroscopic observations on sulphide inhibition of cytochrome oxidase were reported (Nicholls, 1975). In this report, $K_D$ appeared to be $< 0.1\mu M$ and binding of sulphide to cytochrome $a^3_3$ was accompanied by a small blue-shift in the position of $\alpha$-maximum of the reduced enzyme ($a^{2+}a^3_3-H_2S$). It was also suggested that partial electron transfer from sulphide to haem may occur in the cytochrome $aa_3$ - sulphide complex since the reduced sulphide-inhibited enzyme shows a much higher Soret peak at 445 nm than the corresponding cyanide and azide complex. (Nicholls, 1975) The characteristic olfactory response to hydrogen sulphide is an important aspect of its toxicity. Its typical rotten-egg odour is detectable by olfaction at very low concentration (0.035 $\mu g$/litre) in the air. (National Research Council, 1979) 4.2-7$\mu g$ of hydrogen sulphide per litre of air becomes a highly offensive odour. (Yant, 1938) 14$\mu g$ of hydrogen sulphide per litre of air was defined as a threshold limit value for poisoning (U.S.Dept. of Labor, 1974). The Subcommitteee on Hydrogen Sulfide, Committee on Medical and Biologic Effects of Environmental Pollutants, Division of Medical Sciences, Assembly of Life Sciences, National Council, issued the following definitions of hydrogen sulphide intoxication for humans:

Acute intoxication: Effects of a single exposure to massive concentrations of hydrogen sulphide that rapidly produce signs of respiratory distress. Concentrations approximating 1,400$\mu g$/litre are usually required.

Subacute intoxication: Effects of continuous exposure to mid-level (140-1,400$\mu g$/litre) concentrations of hydrogen sulphide. Eye irritation is the
most commonly reported effect, but pulmonary edema has also been noted. Chronic intoxication: Effects of intermittent exposures from low to intermediate concentrations (70-140μg/litre) of hydrogen sulphide, largely subjective manifestations of illness. (National Research Council, 1979)

Animal experiments on acute sulphide poisoning have established conclusively that methaemoglobinemia has both protective and antidotal actions (Smith and Abbanat, 1966; Smith, 1967). It was reported that the methaemoglobin generating agent, sodium nitrite, protects rabbits and dogs against death from exposure to hydrogen sulphide. (Gunter, 1956) Nitrite pretreatment protected mice and armadillos from a subsequent injection of sodium sulphide, and significant delayed death of mice exposed continuously to various ambient concentrations of hydrogen sulphide. (Smith and Gosselin, 1964) Solutions of human methaemoglobin prepared in vitro and washed free of excess sodium nitrite and its reaction products, when injected into the peritoneal cavity, protected mice against death from a subsequent injection of sodium sulphide. (Smith and Gosselin, 1966,1979) Thus, it is clearly methaemoglobin that is responsible for the protective action and not the chemicals used to generate it in vivo. It was also demonstrated nitrite, hydroxylamine and p-aminopropiophenone provided the same degree of protection against sulphide poisoning when given doses that generated equal methaemoglobin concentrations in vivo (Smith, 1967 ; Smith and Gosselin, 1979). When inhaled at higher concentrations hydrogen sulphide exerts its irritant action more or less uniformly throughout the respiratory tract, although
the deeper pulmonary structures suffer the greatest damage. Inflammation of these structures may appear as pulmonary edema (National Research Council, 1979). In its acute form, hydrogen sulphide poisoning is a systematic intoxication, the result of the gas action on the nervous system (National Reasearch Council, 1979). The toxic effects of hydrogen sulphide are believed to be a consequence of reversible inactivation of cellular cytochrome oxidase that results in inhibition of aerobic metabolism (Nicholls, 1975). Only in the form of free, unoxidized gas in the blood stream can hydrogen sulphide exert its systematic effects. Because it is very rapidly oxidized in the blood to harmless and easily eliminated sulphates, hydrogen sulphide may be considered a noncumulative poison (National Research Council, 1979).

(D) Hydrogen Sulphide in Heavy Water Production

A large quantity (tons) of hydrogen sulphide is used in some installations for the production of heavy water, which can serve as a moderator in nuclear power reactors. The advantage of heavy water as a nuclear moderator is that it permits reactor operation with natural uranium instead of the more expensive enriched fuel (National Research Council, 1979). The heavy water is produced in a dual temperature process in which water extracts deuterium from hydrogen sulphide in cold (30°C) towers and hydrogen sulphide extracts deuterium from water in hot (130°C) tower. The exchange is ionic and it occurs in the liquid phase. After a number of stages and distillations, deuterium oxide of 99.8% purity can be produced (Kirshenbaum, 1977).
(E) Natural Occurrence and Industrial production of Hydrogen Sulphide

Hydrogen sulphide occurs naturally in coal, natural gas, oil, volcanic gases, sulphur springs and lakes. It is also a product of the anaerobic decomposition of sulphur-containing organic matter. (Natural Reaearch Council, 1979) Hydrogen sulphide is a by-product of or waste material from a number of industrial operations e.g. production of coke, manufacture of gases from coal, petroleum industry, manufacture of viscose rayon, kraft process (National Research Council, 1979).

In view of its toxicity to higher living organisms, it is binding behaviour towards the enzyme, and the unusual characteristics of its inhibitory reaction, it was decided to study this system further.
Chapter II. Literature Review

(A) Physical-Chemical Properties of Cytochrome c Oxidase

The prosthetic groups of cytochrome c oxidase include two moles of haem a and two of copper atoms per functional unit. (Eq.1) The structure of the haem a moieties has been revealed as the result of the concerted effort of many investigators over nearly three decades. (Lemberg, 1969; Caughey et al, 1975) NMR spectra and other properties show that haem a of bovine heart muscle is probably the iron complex of 8-formyl-6.7-bis(2'-hydroxycarbonylethyl)-2-(1'-hydroxy-5',9',13'-trimethyl-4',8',12',-trans, trans-tetradecatrienyl)-1,3,5-trimethyl-4-vinylporphin (Caughey et al, 1975).

Fig. 1A Structure of haem a.

(Caughey et al, 1975)
It is now widely agreed that both copper and iron are essential components. (Griffiths and Wharton, 1961; Kuboyama et al, 1972). The metal content and the iron to copper ratio (1.0) are well established for the bovine enzyme (Griffiths and Wharton, 1961; Kuboyama et al, 1972). All the iron is present as haem a (Mackler and Tenn, 1957). The coordination environment of copper is less clear, but the reducibility of copper seems to require a ligand environment that stabilizes Cu (I) relative to Cu(II). Only 50% of the copper is e.p.r.-detectable (Beinert et al, 1962), and this species also appears to be responsible for the 830 nm oxidized absorption maximum. (Griffiths and Wharton, 1961) Estimated half-reduction potential values for the four redox components at pH 7.2 are: cytochrome a_3, 0.37 V; cytochrome a, 0.21 V; the e.p.r.-detectable copper, 0.24 V; and e.p.r.-undetectable copper, 0.34 V. (Wilson and Dutton, 1970; Erecinska et al, 1971, Lindsay and Wilson, 1974). One of the more controversial issues in cytochrome c oxidase research centres around the functional characterization of cytochrome a and a_3. Three different concepts have been advanced. Firstly, Chance, Beinert and others believed that the two cytochromes a and a_3 are both physically and chemically distinct components of the oxidase (Chance et al, 1975; Beinert et al, 1976). Secondly, Malmström and Van Gelder have suggested that the two cytochromes are chemically identical but strongly interacting so that the reduction of, or ligand binding to, one of the cytochromes modifies the properties of the other cytochrome (Malmström, 1974; Tiesjema et al, 1973). Thirdly, Nicholls and Wikström favour the so called "neoclassical" concept in which
cytochromes $a$ and $a_3$ are chemically different but have similar and linked oxidation-reduction properties. (Nicholls and Petersen, 1974; Wikström et al, 1976) The relationships between two haems remain unsolved.

Oxidized cytochrome $c$ oxidase as isolated (see methods), is a brownish-green substance with absorption maxima at 420 nm and 600 nm. In its fully reduced state, the enzyme is a green substance with absorption maxima at 445 and 605 nm. (Keilin, 1966; Griffiths and Wharton, 1961) A third absorption maximum, attributed to oxidized copper, occurs at 830nm(Griffiths and Wharton, 1961). It has also been reported that the e.p.r.-undetectable copper atom could be seen at 740-750 nm at low temperatures (Chance et al 1977), but the redox state of this copper atom is not clear, and their interpretation is uncertain.

E.p.r. studies have also provided much information about changes that occur in the functioning enzyme. Early attention was focused upon copper because of its strong signal at $g=2$. (The $g$-values are a measure of the interaction between the applied magnetic field and the unpaired electrons). For a free electron,$g=2.0023$, and departures from this value depend on contributions from the orbital motions (Malmström and Wåhngård, 1960)) is easily visible. Early measurements showed the presence of two types of copper signal: one that exhibits hyperfine structure and one that shows no hyperfine structure. (Van Gelder and Beinert, 1969) E.p.r. spectroscopy allows examination of both high- and low-spin haem signals. In the fully oxidized enzyme a low spin
ferric haem resonance is observed with g value at 3.0, 2.22 and 1.5 (Beinert and Palmer, 1964). This haem resonance corresponds to 50% of the total haem just as the copper signal accounts for 50% of the total copper (Hartzell and Beinert, 1974). Thus only one haem and one copper seem to be detectable under these conditions. One explanation offered for this problem has been an antiferromagnetic coupling between one haem a (cytochrome a₃) and one copper atom (Van Gelder and Beinert, 1969) and a theoretical treatment (Griffith, 1971) has shown that such an interaction is consistent with magnetic data. It is generally agreed that the haem resonance arises from cytochrome a and the copper absorption from that of the 830 nm copper. (Griffiths and Wharton, 1961) In partially reduced oxidase, the low spin ferric haem signal disappears and a high spin ferric resonance at g=6.0 appears whose amount corresponds to up to 25% of the total haem. (Hartzell and Beinert, 1974; Wilson et al, 1976) This signal has been assigned to either cytochrome a or to a₃ by different investigators. (Wilson et al, 1976; Hartzell and Beinert, 1974; Beinert and Shaw, 1977).

Cytochrome c oxidase accepts reducing equivalents from ferricytochrome c and transfers them to molecular oxygen (Eq. 1). The reaction with oxygen occurs with a second order rate constant between $4 \times 10^7 \text{M}^{-1}\text{sec}^{-1}$ and $2 \times 10^8 \text{M}^{-1}\text{sec}^{-1}$ in isolated cytochrome oxidase, in mitochondria and in whole cells. (Chance et al, 1971; Gibson et al, 1963; Petersen et al, 1974). Kinetic studies on the ferrocytochrome c oxidase reaction showed that the oxidation of cytochrome c can occur with a second order rate constant of $4 \times 10^7 \text{M}^{-1}\text{sec}^{-1}$ and that the initial phase of reduction of cytochrome a...
is followed by a slower reaction identified with a transfer of electrons between cytochrome a and the e.p.r.-detectable copper (Gibson et al., 1975). The presence of two binding sites for cytochrome c has been demonstrated on isolated cytochrome c oxidase (Ferguson-Miller et al., 1978; Rieder and Bosshard, 1978).

The distribution and structure of the membranous cytochrome c oxidase molecule have been investigated by X-ray diffraction, optical polarization spectroscopy and e.p.r. spectroscopy using ordered multilayers of purified membranous enzyme (Blasie et al., 1978). These studies reported that the cytochrome c oxidase molecules are oriented asymmetrically in the membrane profile with a significant portion of their mass occurring within the extravesicular surface of the membrane. Electron microscopy was also adapted for this study (Henderson et al., 1977). It revealed that the protein molecules from the two membranes interlock on the inside of the vesicle, and each cytochrome c oxidase molecule sticks far out into solution on the inside surface of the vesicles, but probably less than half as far on the outside surface.

A systematic study has been made of copper and haem a binding to subunits of beef heart cytochrome c oxidase using polyacrylamide gel electrophoresis (Winter et al., 1980). It appeared probable that in the native enzyme, subunit I contains haem a and subunit II contains copper and haem a. These results imply that a relationship of mammalian cytochrome c oxidase to the two-subunit microbial cytochrome oxidase systems appear to exist.

After more than five decades of research, extensive knowledge has been accumulated but the following questions remain unanswered:
1. What is the functional relationship between the two species of haem-containing centre, cytochromes $a$ and $a_3$, in one catalytic unit of cytochrome $c$ oxidase?

2. How many peptide subunits are there and how are they arranged in the membrane? (see Methods)

3. Are one copper and one iron atom coupled antiferromagnetically?

4. Is the first step in reduction of molecular oxygen a one-electron or a two-electron process?

5. What is the significance of the two binding sites for cytochrome $c$?

6. How many redox centers are in the minimal catalytic unit?

7. Where are two haems and two copper atoms located in the subunits?
B. Physico-Chemical Properties of Cytochrome c

Cytochrome c, first seen by MacMunn (1885), was named by Keilin (1925), who established its wide occurrence in cells from mammals to invertebrates and yeast. Its relative stability led to its early isolation in nearly pure state by Keilin (1930) and Theorell (1936). The biological role of cytochrome c in cellular respiration was established by Keilin (1925). A similar cytochrome plays an important role in photosynthetic processes (Hill et al., 1953). The mitochondrial cytochrome c of eukaryotes is a protein with one haem group (haem c, Fig. 1B) and 103-113 amino acids in a single polypeptide chain with no disulphide crosslinks (Margoliash et al., 1961; Margoliash, 1962; Ferguson-Miller et al., 1979). Its molecular weight is 12,400.

Figure 1B. Structure of cytochrome c:

Dickerson and Timkovich (1975)
B. Physico-Chemical Properties of Cytochrome c

Cytochrome c, first seen by MacMunn (1885), was named by Keilin (1925), who established its wide occurrence in cells from mammals to invertebrates and yeast. Its relative stability led to its early isolation in nearly pure state by Keilin (1930) and Theorell (1936). The biological role of cytochrome c in cellular respiration was established by Keilin (1925). A similar cytochrome plays an important role in photosynthetic processes (Hill et al., 1953). The mitochondrial cytochrome c of eukaryotes is a protein with one haem group (haem c, Fig. 1B) and 103-113 amino acids in a single polypeptide chain with no disulphide crosslinks (Margoliash et al., 1961; Margoliash, 1962; Ferguson-Miller et al., 1979). Its molecular weight is 12,400.

Figure 1B. Structure of cytochrome c:

\[ \text{Diagram of cytochrome c structure} \]

Dickerson and Timkovich (1975)
This protein was first crystallized by Bodo (1955) from King Penguin muscle and shortly thereafter from a wide variety of sources: numerous mammals, birds, algae, fish and yeast (Lemberg and Bassett, 1973).

Mitochondrial cytochromes \( c \) form a homogeneous group of proteins with basic isoelectric points (pK ~ 10), oxidation-reduction potentials of about 0.25 V, and the ability to react readily with mammalian cytochrome oxidase (Theorell and Åkesson, 1941 a, b, c). The prosthetic group, haem \( c \), is covalently linked to the protein by thioether bonds from two cysteiny1 residues (position 14, 17) to the vinyl side chains of haem (Margoliash, 1962). These bonds provide a stable, relatively inert prosthetic group.

Methionine (position 80) is the sixth ligand of haem (Harbury et al., 1965) and histidine (position 18) is the fifth (Takano et al., 1973). The amino acid sequences of cytochrome \( c \) (Margoliash et al., 1961; Margoliash, 1962) have enabled us to understand some relationship between structure and function. Ferrocytochrome \( c \) is readily reduced in the solution by various reagents, including dithionite, ascorbate, and N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). The native protein has a characteristic three banded absorption spectrum in the reduced state, with a \( \alpha \)-absorption band at 550 nm, a \( \beta \)-band at 520 nm, and a \( \gamma \) (Soret) band at 416 nm (Keilin, 1966; Margoliash and Firohwirt, 1959). In the oxidized state, a Soret band at 410 nm and a broad \( \beta \)-band at 528 nm appear respectively (Butt and Keilin, 1962). Ferrocytochrome \( c \) can be oxidized rapidly by ferricyanide and with cytochrome \( a_3 \) (Smith and Conrad, 1956), forming protein-protein complexes with the latter. Cytochrome \( c \) binds to cytochrome \( c \) oxidase with a 1:1 or 2:1 stoichiometry (Nicholls, 1964;
Kuboyama et al., 1962) and about 100-fold greater affinity \( (K_m \approx 10^{-8} \text{ M}) \) than reported for non-specific phospholipid interactions (Ferguson-Miller et al., 1976; Vanderkooi et al., 1973).

A crystallographic study of horse heart ferricytochrome c has confirmed that the haem group is located in the crevice of the globular protein, and the iron atom lies in the plane of the porphyrin ring (Dickerson et al., 1971). Iron-binding ligands such as azide, cyanide and imidazole can also coordinate to the iron (George et al., 1967; Nicholls and Mochan, 1967; Sutin and Yandell, 1972; Schejter and Aviram, 1969), and the evidence suggests that these ligands bind by displacing the methionine-80 (Harbury et al., 1965; Schejter and Aviram, 1969). The rate of displacement of the methionine-80 sulfur from its coordination site is of interest since it may control the rate of reduction of ferricytochrome c under certain conditions. The rate of reduction of ferricytochrome c by chromium(II) can be markedly increased by addition of certain anions (Kowalsky, 1969). A possible mechanism for this catalysis involves the formation of anion bridges between the iron and chromium (Sutin et al., 1971; Kowalsky, 1969). It has been reported that oxidized glutathione (GSSG) substantially enhances the rate of reduction of cytochrome c by reduced glutathione (GSH). This phenomenon may be ascribed to a molecular complex of GSSG and GS\(^-\) which could react by sequential one electron transfers generating GS\(^-\) and GS\(^+\) species (Massey et al., 1971; Froese and Hunter, 1970; Everse and Kujundzic, 1979). The GSSG-enhanced reduction of cytochrome c is probably due to the formation of glutathione trisulfide (Massey et al., 1971).
The effect of alkylthiols on the properties of cytochrome _c_ has been studied (Wilms _et al._, 1980). The steady-state oxidation of ferrocytochrome _c_ by molecular oxygen, catalyzed by cytochrome _c_ oxidase, is inhibited non-competitively towards cytochrome _c_ by methanethiol (CH₃-SH), ethanethiol (CH₃-CH₂-SH), 1-propanethiol (CH₃CH₂CH₂-SH), and 1-butanethiol (CH₃-CH₂-CH₂-CH₂-SH) (Wilms _et al._, 1980).
C. Chemistry and Biochemistry of Sulphides

The sulphur-containing gases, H$_2$S, SO$_2$ and SO$_3$, are all air pollutants. Of the three, hydrogen sulphide is the most toxic, and it can readily be detected by smell at very low concentrations (National Research Council, 1979; see also p. 3). The chief source of hydrogen sulphide in the atmosphere is industry, although vegetable matter, volcanos and natural springs are also among the contributors (National Research Council, 1979; see also I.C.). The sulphide-generating industries of greatest importance are petroleum refineries, coke-oven plants, viscose rayon plants, and some chemical plants (National Research Council, 1979). Hydrogen sulphide liquifies at -61.8°C under 1 atm and solidifies at -82.9°C (Macaluso, 1969). It dissolves in water to give a mixture of H$_2$S and HS$^-$ (sulphide ion) as shown by Equation (2):

$$\text{H}_2\text{S} \rightleftharpoons \text{HS}^- + \text{H}^+ \quad \text{Eq. (2)}$$

The secondary sulphide ion, S$^{2-}$, does not exist as a significant fraction of the sulphide content except at very high pH (Sillen, 1964; Weast, 1976). The proportions of dissolved hydrogen sulphide (H$_2$S) and SH$^-$ are thus a function of pH, according to the Henderson-Hasselbach equation (Eq. 3):

$$\log \frac{[\text{HS}^-]}{[\text{H}_2\text{S}]} = \text{pH} - \text{pK}' \quad \text{Eq. (3)}$$

where pK' represents the observed ionization constant. The transformations of sulphide in solution are summarized in Equation (4) (Widher and Schwarzenbach, 1964):
Hydrogen sulphide in water acts as a dibasic weak acid, with $pK_1 = 6.88$ and $pK_2 = 14.15$. The hydrogen sulphide molecules have a bent configuration with bond angle varying from $90^\circ$ in the liquid phase to $92^\circ 20'$ in the gas phase. The H-S bond distance in the gas phase is $1.35 \text{ Å}$ (Burton and Machmer, 1968).

![Structure of hydrogen sulphide](image)

Figure 2. Structure of hydrogen sulphide

Sulphide is easily oxidized, to elemental sulphur, and then to sulphur dioxide (Eq. 5) (Schmidt and Siebert, 1973):

$$H_2S + 1.5O_2 \longrightarrow H_2O + SO_2$$  \hspace{1cm} \text{Eq. (5)}

Compounds of hydrogen containing more than one sulphur atom are named sulphanes. All sulphanes can contain only two hydrogen atoms and, therefore, must have the composition $H_2S_x$. Sulphanes may occur as intermediates in the oxidation of $H_2S$ to sulphur ($S_8$) in solution. Sulphanes are thermo-dynamically unstable at room temperature with respect to hydrogen sulphide and sulphur; but they are metastable because decomposition requires high activation energies, \textit{e.g.}, 25 kcal/mole for disulphane (Wierwiorowski, 1972; Meyer and Schmidt, 1975).
Sulphur exists in a variety of modifications in either the solid, liquid or gaseous state. Much of the complication arising from the existence of these materials is due to the presence of molecules of varying complexities. Chief among such species are the molecules corresponding in composition to the formula $S_8$. They are found in all three physical states and represent puckered octagonal rings which may be diagrammed as Figure (4) (Warren and Burwell, 1935):

![Figure 3. Structure of inorganic sulphur polymer.](image)

where the $S-S$ bond distance is 2.12 Å and the bond angle is 105°. Species with compositions $S_6$ and $S_4$ are also found in the solid and liquid states. They appear to be chain structures (Meyer and Schmidt, 1975).

Sulphur is one of the elements essential for the life of all organisms—plant, animals and micro-organisms (Anderson, 1978). Coenzyme A, ferredoxin, glutathione, lipoic acid, biotin, sulpholipid and thiamine pyrophosphate are all sulphur-containing compounds found in organisms and having very important biological roles, steroid hydroxylation, CO$_2$ fixation, N$_2$ fixation, oxidative phosphorylation and photo-phosphorylation (Hall et al., 1974). As an example, ferredoxins have iron and acid-labile sulphide which are covalently
associated in equimolar amounts in the active center (Hall et al., 1974). The organic sulphur of cysteine and methionine originates biologically from various inorganic forms, such as \( \text{SO}_4^{2-} \), \( \text{S}_2\text{O}_3^{2-} \), \( \text{H}_2\text{S} \), and even elemental sulphur (Anderson, 1978).

The sulphhaem proteins represent a class of compounds formed by analogous reactions of various protohaem proteins and \( \text{H}_2\text{S} \). These include sulphhaemoglobin, sulphmyoglobin and sulphcatalase. Sulphhaemoglobin is found in a number of pathological conditions, particularly those in which a sulphide source is present (National Research Council, 1979). Nicholls (1961) synthesized sulphmyoglobin from the higher oxidation state derivative of myoglobin; the product of \( \text{H}_2\text{O}_2 \) and ferric myoglobin. He showed that the addition of 1 mole of \( \text{H}_2\text{S} \) per haem produced ferric sulphmyoglobin, which could then be reduced to the ferrous compound by excess \( \text{H}_2\text{S} \). It has been also reported that ferrosulphcatalase is formed by the action of hydrogen sulphide on catalase compound II which is the secondary peroxide compound of catalase (Nicholls, 1961). Probably, the catalase inhibition induced by sulphide should be studied for the further understanding of sulphide toxicity.

In sulphur biochemistry, rhodanese (thiosulphate:cyanide sulphur transferase, EC 2.8.1.1) is an important enzyme, since it transfers sulphur from thiosulphate to cyanide, as follows (Villarego and Westley, 1963(a)):

\[
\text{SSO}_3^- + \text{CN}^- \rightleftharpoons \text{SCN}^- + \text{SO}_3^-
\]

Eq. (6)
The biological role of this reaction is not clear, but its possible involvement in CN⁻ detoxication has been suggested (Villarejo and Westly, 1963(b)). Rhodanese was found to catalyze the reduction of $SSO_3^-$ to $HS^-$ and $SO_3^-$ when lipoic acid and/or lipoamide was used as the reducing agent. During the course of the reaction, sulphur is transferred from $SSO_3^-$ to rhodanese, forming a rhodanese-sulphur compound with elimination of $SO_3^-$. The rhodanese-sulphur compound then reacts with dihydrolipoate, transferring the sulphur to the lipoate to form an intermediate which has been called lipoate persulphide. Lipoate persulphide has been suggested as an intermediate in biosynthetic reactions requiring reduced sulphur (Villarejo and Westley, 1963 (a, b)).

When cytochrome $c$ is reduced by reduced glutathione, (Froede and Hunter, 1970) the stimulatory effect of GSSG (oxidized glutathione) is due to $S^0$-containing impurities, and the chief impurity is probably glutathione trisulphide (GSSSG). Consequently the following equations were suggested for the catalytic effect on reduction of cytochrome $c$ by $GS^-$ (Massey et al., 1971):

\[
\begin{align*}
GSSSG + GS^- & \rightleftharpoons GSS^- + GSSG & \text{Eq. (7)} \\
2GSS^- + 2\text{Cyt.}c^3+ & \rightarrow 2\text{GSS}^+ + 2\text{Cyt.}c^2+ & \text{Eq. (8)} \\
2\text{GSS}^+ & \rightleftharpoons \text{GSSSG} & \text{Eq. (9)} \\
\text{GSSSG} + \text{GS}^- & \rightleftharpoons \text{GSSSG} + \text{GSS}^- & \text{Eq. (10)}
\end{align*}
\]
It has been also reported that active rabbit liver aldehyde oxidase has a polysulphide group at the active site (Brazoli and Massey, 1974). Thus, the polysulphide appears to have a very important role in biochemical metabolism of sulphur.

Recently, Beck and Donini (1981) reported that the sulphide site of action might be neutral and on the cellular membrane, contrary to Smith and Gosselin (1966) and Nicholls (1975). Both oxyhaemoglobin and methamoglobin are effective catalytic agents for sulphide detoxification (Beck and Donini, 1981). Nitrite as an antidote for acute H\textsubscript{2}S intoxication can be effective only within the first few minutes after the exposure, at which time resuscitation and ventilation of the victim is likely to produce conditions in which the nitrite actually slows sulphide removal (Beck and Donini, 1981). This point of view contradicts the conventional recommendation in which a large dose (50 mg/kg for rats) of intraperitoneal nitrite injection is required (Smith et al., 1976).
D. Inhibition of Cytochrome c Oxidase by Sulphide

Sulphide was first introduced as a respiratory chain inhibitor by Keilin in 1928. Sulphide inhibits cytochrome c oxidase (Keilin and Hartree, 1939) by forming an oxidized cytochrome $a_3$-sulphide compound (Wilson and Gilmour, 1967). Extensive spectrophotometric and kinetic studies on cytochrome c oxidase inhibition induced by sulphide has been reported (Nicholls, 1975; see p. 3). Sulphide induces a blue shift of $\alpha$-peak of the cytochrome $a^2+_3^3$-H$_2$S species (Nicholls et al., 1976) and this phenomenon, it was suggested, reflects a possible high- to low-spin state change in the $a_3$ haem (Nicholls et al., 1976; Nicholls and Hildebrandt, 1978). The apparent $K_i$ for sulphide at different oxygen concentration is 0.1-0.2 $\mu$M (Petersen, 1977) and sulphide is a non-competitive inhibitor towards oxygen and cytochrome c; but H$_2$S competes with azide, cyanide and formate for the oxidized form of cytochrome oxidase (Wever et al., 1975). Sulphide itself is also a reducing agent (Eq. 4) capable of reducing cytochrome a. With an excess of sulphide added to cytochrome c oxidase, the major product is $a^2+_3^3$-H$_2$S which is a fully inhibited stable species (Nicholls, 1975; Nicholls et al., 1976).

Addition of sulphide to cytochrome c oxidase gives rise to low-spin haem e.p.r. signals at $g = 2.54$, $g = 2.23$, and $g = 1.87$ (Wever et al., 1975). Concomitantly with the formation of these signals, the low-spin haem signal at $g = 3$ and the copper signal near $g = 2$ decrease in intensity, suggesting that partial reduction of the enzyme by sulphide has occurred (Wever et al., 1975). The e.p.r. data obtained from the interaction between cytochrome c oxidase and sulphide are tabulated in Table 1:
Table 1. The interaction between cytochrome c oxidase and sulphide

<table>
<thead>
<tr>
<th>Condition for formation</th>
<th>e.p.r. spectrum (g values)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂S + aa₃</td>
<td>2.54, 2.23, 1.87</td>
<td>Van Gelder et al., 1975</td>
</tr>
<tr>
<td>H₂S + SMP reduced with succinate</td>
<td>2.57-2.54, 2.26-2.22, 1.9-1.8</td>
<td>Wilson et al., 1976</td>
</tr>
<tr>
<td>H₂S + aa₃ partially reoxidized with porphyrexide</td>
<td>2.77, 2.23, 1.71 (intermediate)</td>
<td>Shaw et al., 1978</td>
</tr>
<tr>
<td>H₂S + aa₃ reoxidized with ferricenium</td>
<td>3.03, 2.54</td>
<td>Seiter and Angelos, 1980</td>
</tr>
<tr>
<td>Methanethiol + aa₃ cyt. c. ascorbate</td>
<td>2.39, 2.23, 1.93</td>
<td>Wilms et al., 1976</td>
</tr>
<tr>
<td>Ethanethiol + aa₃ cyt. c. ascorbate</td>
<td>2.43, 2.24, 1.91</td>
<td>Wilms et al., 1976</td>
</tr>
</tbody>
</table>
Chapter III. Materials and Methods

(A) Materials

Reagents for sulphide measurement included sodium sulphide (Na₂S•9H₂O, BDH, Assured Grade), ammonium molybdate ((NH₄)₆Mo₇O₂₄•4H₂O, BDH, AnalAR Grade), ammonium sulphide ((NH₄)₂S, BDH, AnalAR Grade), sulphuric acid (H₂SO₄, J. T. Baker, Technical Grade), urea (NH₂•CO•NH₂, BDH, AnalAR Grade), iodine indicator (C₆H₁₀O₅)n, soluble starch, BDH), and iodine (I₂, BDH).

Reagents used for enzyme isolation included potassium hydroxide (KOH, BDH, AnalAR Grade), ethylenediaminetetraacetic acid (EDTA, (CHOOC-CH₂)₂-N(CH₂)₂N(CH₂-COOH)₂, Sigma, sodium salt), Trizma base (H₂N-C(CH₂OH)₃, Sigma, Reagent Grade), hydrochloric acid (HCl, Fisher, Reagent A. S. C.), boric acid (H₃BO₃, BDH, AnalAR Grade), potassium phosphate, dibasic (K₂HPO₄, J. T. Baker, Analyzed Reagent), potassium phosphate, monobasic, (KH₂PO₄, J. T. Baker, Analyzed Reagent), sucrose (C₁₂H₂₂O₁₁, Sigma, Grade I), mannitol (C₆H₁₄O₆, Sigma, Grade I), ammonium sulphate ((NH₄)₂SO₄, BDH, AnalAR Grade), and sodium hydroxide (NaOH, BDH, Aristar Grade).

Reagents used for SDS gel chromatography included sodium dodecyl sulphate (SDS, CH₃(CH₂)₁₁OSO₃Na, Sigma, 95%), N,N,N',N'-tetramethyl-ethylenediamine (TEMED, (CH₃)₂NCH₂CH₂N(CH₃)₂, Eastman, Electrophoresis Grade), ammonium persulphate ((NH₄)₂S₂O₈, BDH, AnalAR Grade), urea (NH₂•CO•NH₂, BDH, AnalAR Grade), N,N'-methylenebisacrylamide((CH₂:CHCONH₂)₂CH₂, Eastman), phosphoric acid (H₃PO₄, Fisher, Certified A. S. C.), acrylamide (CH₂:CH CO NH₂, BDH, Electrophoresis Grade), coomassie brilliant blue R-250
(C₄H₄N₃NaO₇S, Sigma, Electrophoresis Grade), and glacial acetic acid (CH₃COOH, BDH, 99.8%).

Reagents for kinetic and spectrophotometric studies included sodium ascorbate (C₆H₇NaO₆, Sigma), N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD, C₆H₄[N(CH₃)₂]₂·2HCl, Sigma, 95-97%), Tween-80 (polyoxyethylene (20) sorbitan monooleate, J. T. Baker, Practical Grade), cytochrome c (horse heart, type VI, Sigma, 95-100%), carbon monoxide (CO, Linde Specialty Gas, Union Carbide, C. P. Grade), potassium cyanide (KCN, BDH, AnalaR Grade), sodium azide (NaN₃, Sigma), sodium sulphide (Na₂S·9H₂O, BDH, Assured Grade), sodium dithionite (Na₂S₂O₄, BDH, 85%), potassium ferricyanide (K₃[Fe(CN)₆], Sigma, Grade I), catalase (Sigma, C-100), dithiothreitol (Cleland's reagent, (CHOHCH·CH₂·SH)₂, Sigma) and N-ethylmaleimide (NEM, C₆H₇NO₂, Sigma).

Sephadex G-25 and G-100 were obtained from Sigma Chemical Company and Pharmacia.

Reagents for the protein estimation included cupric sulphate (CuSO₄·5H₂O, BDH, AnalaR Grade), potassium tartrate ((CHOH·COOK)₂·½H₂O, BDH, 98%) sodium hydroxide (NaOH, BDH, AnalaR Grade), and bovine serum albumin (Sigma, Fraction V).

Glass distilled water was used throughout.
B. Methods

(i) Isolation and Purification of Cytochrome c Oxidase

Bovine heart cytochrome c oxidase was prepared by the method of Kuboyama et al. (1972) with minor modifications.

(a) Preparation of Submitochondrial Particles:

The usual practice in the laboratory to isolate cytochrome c oxidase was to start with 6 bovine hearts (about 10 kg). The hearts were purchased immediately after removal from the animals and kept in the ice-buckets during transport back to the laboratory. All subsequent procedures were performed at 4°C.

Fat and connective tissue were removed thoroughly from heart muscle and muscle pieces were passed three times through a meat grinder (Westinghouse, type FZ, Model H). The mince (about 4 kg) was weighed on a top-loading balance and then divided into three equal parts and stirred in three buckets of sodium phosphate buffer (5 mM NaPi, pH 7.4) to remove the haemoglobin. The capacity of each bucket was about 12 litres. The buffer solution was changed more frequently (about every 30 min) at the start of the wash. In later washes, the buffer solution was changed every hour. At each change, muscle-buffer mixture was filtered through two layers of cheesecloth, with the mince being hand-pressed to remove the wash medium as much as possible. The washes were continued until no haemoglobin was detected by a bench-microspectroscope. Usually six washes were enough. At this stage, the mince should look decolorized and can be left overnight in the cold room.
The mince was dispersed in 10 L of 5 mM sodium phosphate buffer solution, pH 7.4 and homogenized in a large-capacity Waring blender (model 91-215) for 10 seconds at slow, 10 seconds at medium and 40 seconds at high speed. The portions of homogenate were pooled and adjusted in volume so that when centrifuged, the supernatant formed about 50% of the total volume. The homogenate was centrifuged in an IEC PR-6000 (6 x 1 L head) at 3700 rpm (3000 x g) for 15 minutes. Supernatants were collected and precipitates which contained unbroken tissue were discarded. Then the pH was adjusted to 5.6 with 1 N cold acetic acid. The now cloudy suspension was immediately centrifuged at 3700 rpm for 10 minutes in the IEC PR-6000 centrifuge. The clear reddish supernatant was discarded and the precipitate was suspended in an equal volume of cold distilled water. The combined resuspended precipitates were centrifuged in the GSA head of a Sorvall RC-5 centrifuge for 10 min at 10,000 rpm (16,000 x g). The supernatant was discarded and precipitate was resuspended either in the Keilin-Hartree Medium (0.55 M sucrose, 62.5 mM borate, 125 mM Na₂HPO₄, 10 mM EDTA, pH 7.5) (if submitochondrial particles only were required), or in 0.1 M borate-phosphate buffer (0.1 M borate, 0.03 M Na₂HPO₄, pH 7.6) (if further purification was to be attempted) using a Potter-Elvehjem homogenizer. At this stage, a rapid protein determination was carried out using the Biuret method (Cleland and Slater, 1953; Gornall et al., 1949) and heating the sample tubes at 80°C for 5 minutes. The submitochondrial particles were then diluted in 0.1 M borate-phosphate, pH 7.6 plus 1% sodium cholate and the protein concentration was adjusted to between 30 and 40 mg/ml. The total final volume was usually 900 mL.
(b) Initial Ammonium Sulphate Fractionation:

Solid ammonium sulphate (13.4 g (NH₄)₂SO₄/100 mL) was then slowly added to give 25% saturation at 4°C and the pH was adjusted to 8.0 with 1 N NaOH. This mixture was allowed to stand for an hour with stirring and then solid ammonium sulphate was added to yield 35% saturation. After the ammonium sulphate dissolved completely, the protein solution was allowed to stand for 10 min with stirring. Then the suspension was centrifuged at 25,000 x g for 20 min (GSA head, 13,000 rpm) in the Sorvall RC-5 centrifuge. The clear orange-red supernatant was discarded. The precipitate was suspended in 0.1 M sodium phosphate buffer, pH 7.4 to a final volume of 675 mL using a Potter-Elvehjem homogenizer. To this solution, 10% sodium cholate was added to a final concentration of 2%. Solid ammonium sulphate was added slowly to give 25% saturation and the pH was adjusted to between 7.4 and 7.8. This mixture was incubated overnight (10 or 12 hours), then centrifuged at 25,000 g (GSA rotor, Sorvall RC-5, 13,000 rpm) for 30 min. The precipitate was discarded. Solid ammonium sulphate was added to the clear greenish brown supernatant solution to give 40° saturation. The mixture was slowly stirred for 30 min and then centrifuged. The light orange supernatant solution was discarded, and the greenish-brown thick precipitate was dissolved in 220 mL 0.1 M sodium phosphate buffer, pH 7.4 containing 1.5% sodium cholate.

(c) Final Ammonium Sulphate-Cholate Fractionation

To this clear greenish solution, saturated, neutralized ammonium sulphate solution was added slowly until a slight turbidity appeared.
Usually, the ammonium sulphate concentration required to reach this stage was 25% saturation. The mixture was allowed to stand for 30 min with stirring and then centrifuged (GSA rotor, Sorvall RC-5 centrifuge, 13,000 rpm) at 25,000 x g for 30 min. A small amount of reddish precipitate was discarded. Saturated ammonium sulphate was added to the clear supernatant to obtain 30% saturation. This mixture was incubated for 30 min with slow stirring, then centrifuged as before. The green pellet was retained.

Neutral saturated ammonium sulphate solution was added to the supernatant to 35% saturation. Again, this mixture was centrifuged. This greenish, thick precipitate was combined with the pellet of 25-30% fraction. Sometimes, almost all protein was precipitated by 25-30% fractionation. Thus, 35% fractionation was not necessary. The combined precipitates were dissolved in 180 mL of 0.1 M sodium phosphate, pH 7.4 containing 1.5% sodium cholate. To the resulting clear greenish solution, neutral saturated ammonium sulphate solution was added to obtain 23% saturation. The mixture was centrifuged at 39,000 x g (SS-34 rotor, Sorvall RC-5 centrifuge, 18,000 rpm for 15 min), and the pellet was discarded. The supernatant was brought to 32% saturation with saturated ammonium sulphate solution, centrifuged and the resulting supernatant discarded. The pellet was dissolved in 90 mL 0.1 M sodium phosphate, pH 7.4 containing 1.5% sodium cholate. This fractionation was repeated twice. Following the last cholate fractionation, the precipitate was dissolved in 0.1 M sodium phosphate, pH 7.4 containing 1% Tween-80, and an ammonium sulphate fractionation was carried out in this cholate-free medium. The final precipitate, which is cytochrome c oxidase,
Figure 4. Procedure for purification of cytochrome c oxidase
was dissolved in 0.1 M sodium phosphate, pH 7.4 plus 0.25% Tween-80, and stored in an ultra-cold freezer (Kelvinator, Series 100) at -70°C. The properties of the final product are tabulated as follows:

Table 2. Yield and characteristics of purified cytochrome c oxidase

<table>
<thead>
<tr>
<th>Total volume</th>
<th>Protein Conc.</th>
<th>aa3 Conc.</th>
<th>Haem a/protein ratio</th>
<th>Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 mL</td>
<td>82 mg/mL</td>
<td>435 µM</td>
<td>10.7 nmole/mg</td>
<td>230 sec⁻¹</td>
</tr>
</tbody>
</table>

* electrons/second/mL aa3 determined polarographically (below)

(ii) Preparation of Reduced Cytochrome c (Smith, 1954)

40 mg of cytochrome c (horse heart, type VI) was dissolved in 1 mL of 67 mM sodium phosphate, pH 7.5, 0.25% Tween-80, to give a 3 mM solution. 1 mg of sodium dithionite was added (dithionite concentration about 4.8 mM), and the mixture allowed to stand at 25°C for 5 min. The mixture was then gel filtered to eliminate excess dithionite, on a Sephadex G-25 column (1.8 x 20 cm) equilibrated with 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80. The eluted cytochrome c was 98% reduced and its concentration was between 0.6 and 1.0 mM.

(iii) Calculation of Cytochrome c and Cytochrome c Oxidase Concentration

The concentration of cytochrome c was calculated according to Eq. 11

\[
[\text{cyt. c}] = \frac{(A_{550}^{\text{red}} - A_{540}^{\text{red}}) - (A_{550}^{\text{ox}} - A_{540}^{\text{ox}})}{E}
\]

Eq. (11)
where $E = 21.2 \text{ mM}^{-1} \text{ cm}^{-1}$ (Margoliash and Frohwirt, 1959). In order to obtain the percentage reduction after gel filtration, Equation 12 was employed.

$$\% \text{ c reduced} = \frac{(A_{550}^{\text{dithio}} - A_{540}^{\text{dithio}}) - (A_{550}^{\text{ox}} - A_{540}^{\text{ox}})}{(A_{550}^{\text{red}} - A_{540}^{\text{red}}) - (A_{550}^{\text{ox}} - A_{540}^{\text{ox}})} \times 100 \quad \text{Eq. (12)}$$

To ensure fully reduced and fully oxidized states, appropriate amounts of dithionite and ferricyanide were added to the sample solution and measured spectroscopically.

The calculation of the cytochrome c oxidase concentration from the absorbance spectra is shown below:

$$[\text{cyt aa}_3] = \frac{(A_{605}^{\text{red}} - A_{630}^{\text{red}}) - (A_{605}^{\text{ox}} - A_{630}^{\text{ox}})}{E} \quad \text{Eq. (13)}$$

where $E = 27 \text{ mM}^{-1} \text{ cm}^{-1}$ (Nicholls, 1978).

(iv) Polarographic Measurement of Cytochrome c Oxidase Activity

The measurement of oxygen reduction by cytochrome c oxidase was carried out with a Clark oxygen electrode (Yellow Springs Instruments Co.) in a closed glass vessel and attached to an appropriate polarizing box (Brock Technical Services) to a Perkin-Elmer (Coleman) 165 recorder (1-10 mV). The capacity of the reaction vessel was 4.2 mL. Samples were added with microsyringes (Hamilton) through a small groove on a plastic mount of the reaction vessel; the electrodes were installed in the middle of this plastic mount. The reaction vessel was continuously stirred by a small stirring bar.
controlled by a magnetic stirrer (Thomas, Model 14). Air bubbles in the system were avoided. Constant temperature was maintained by a water bath (Haake Instruments Inc., Type FE) connected to a water jacket into which the reaction vessel was fitted. The air-saturated reaction medium contains 235 μM O₂ at 30°C (Estabrook, 1967). The rates obtained at different cytochrome c concentrations may be plotted according to Lineweaver and Burk (1934) or Hanes (1932) to obtain Kₘ and Vₘₐₓ values for cytochrome c oxidase.

(v) Spectrophotometric Measurement of Cytochrome c Oxidase Activity

For spectrophotometric experiments, either an Aminco DW-2 spectrophotometer (dual wavelength or split beam) or a Gilford 2400 model spectrophotometer (for single wavelength) was employed.

(a) Smith-Conrad Assay (Smith and Conrad, 1956)

This method uses reduced cytochrome c as a substrate for cytochrome c oxidase activity (see Eq. 1). The rate of oxidation of reduced cytochrome c was monitored either at 550-540 nm or at 550 nm. In a typical experiment, the decrease in absorbance after the addition of 1 nM cytochrome c oxidase to 2.7 mL of 67 mM sodium phosphate, pH 7.4 plus 0.25% Tween-80 containing 16 μM ferrocytochrome c, was recorded at 30°C. A second order rate constant (dimensions, M⁻¹ s⁻¹) can be calculated by dividing the observed first order rate constant (dimensions, s⁻¹) by the concentration of cytochrome c oxidase in the reaction mixture according to Equation (14):
\[
\frac{-d(\text{ferrocytochrome c})}{dt} = k_{\text{obs}}(\text{ferrocytochrome c})(\text{cytochrome aa}_3)
\]

Eq. (14)

where \(k_{\text{obs}} = k/(K_m + [\text{cyt. c}]_{\text{total}})\) (Minnaert, 1961). Even though an inhibitory effect of oxidized cytochrome c is observed in this assay, it remains the simplest method to measure cytochrome c oxidase activity.

(b) Steady State Spectrophotometric Assay (Estabrook, 1961; Nicholls et al., 1972; Mochan and Nicholls, 1972)

By monitoring spectroscopically the steady state reduction of cytochrome c by ascorbate in the presence or absence of the mediator TMPD during oxygen uptake by cytochrome c oxidase together with the time taken to anaerobiosis, it is possible to calculate the enzymatic activity. The reduction of cytochrome c was monitored at 550-540 nm or 550 nm. A typical experiment is as follows: 3.6 mM ascorbate was added to 2.8 mL of 67 mM potassium phosphate, pH 7.4, 0.1% Tween-80, plus 18 \(\mu\)M cytochrome c and 0.1 \(\mu\)M cytochrome c oxidase at 30°C. Cytochrome c was 47% reduced, and remained in this state until the oxygen present in the system was totally depleted, at which time cytochrome c rapidly became 100% reduced (anaerobiosis). The time taken to full reduction is the anaerobiosis time \(t(\text{an})\) and the velocity is calculated according to Equation (15):

\[
v(\mu\text{eq s}^{-1}) = \frac{[O_2] \times 4}{t(\text{an})}
\]

Eq. (15)

Using an oxygen concentration of 235 \(\mu\)M at 30°C in air saturated medium.
(c) Spectrophotometry of Cytochrome c Oxidase Intermediates

In order to detect the spectrophotometric intermediates of cytochrome c oxidase induced by the addition of sulphide, an Aminco DW-2 spectrophotometer, a Hewlett-Packard 8450 A spectrophotometer and Thunberg cuvettes (anaerobic studies) were employed.

(1) Aminco DW-2 Spectrophotometer

For the absolute spectra of cytochrome c oxidase intermediates induced by sulphide, after the addition of sulphide to the enzyme solution, spectra were recorded in the split beam mode against a reference buffer solution. The spectra were obtained at periodic intervals. For the difference spectra, the same procedure as above was followed, but the reference solution was an enzyme solution. The Aminco spectrophotometer was used for either aerobic or anaerobic studies.

(2) Hewlett-Packard Spectrophotometer

Rapidly scanned spectra were obtained using a Hewlett-Packard 8450 A spectrophotometer and plotted on a 7225A graphic plotter. In the place of the usual monochromator system used in the Aminco spectrophotometer, this spectrophotometer has an array of photodiodes. Thus, it is possible to collect and record spectra within a second. Since this instrument is highly computerized, periodic full wavelength spectra may be acquired, stored and plotted.
(3) Anaerobic Experiments

Thunberg cuvettes were employed for all anaerobic experiments. The cuvettes, containing oxidase in the main vessel, a sulphide solution (alkalized with 1 mM KOH to about pH 10) in the side arm, were thoroughly evacuated by a vacuum pump (Edwards high vacuum pump, ES50, attached to Leeson motor CAC 17DH7A) (see Fig. 5). The spectrum of the initial resting oxidized enzyme was taken, then the cuvette was tipped (reactants mixed) and the spectra were recorded at periodic intervals. Thus, it was possible to obtain the anaerobic spectral intermediates of the enzyme induced by the addition of sulphide.
Figure 5. Vacuum system in Dr. Nicholls' laboratory:

A: vacuum pump

B: 6-litre flask open to the atmosphere containing an oxygen free solution (2N NaOH, 2 mM dithionite and 10 mM anthraquinone) with a liquid paraffin layer on top.

C: 6-litre flask filled with nitrogen gas over the oxygen free solution

D: liquid nitrogen cooled moisture trap

E: de-gassing tip

F: mercury column

(Courtesy of Mr. S. Brooks)
(vi) Gelfiltration of Cytochrome c Oxidase (Van Buuren et al., 1972)

Cytochrome c oxidase and sulphide were incubated for 72 hours at 4°C. Usually, 10 μM cytochrome c oxidase was incubated with 30 μM sulphide in 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80. To eliminate excess sulphide from the reaction mixture after incubation, gelfiltration was employed. 3 mL sample was applied at 4°C to a Sephadex G-100 column (20 x 1.5 cm) previously equilibrated with the same buffer mixture. Then the column was eluted with the same buffer at 4°C, and the eluate was collected in 1-2 mL fractions. Elution times were controlled and varied from 30 minutes to 3 hours.

(vii) SDS-Polyacrylamide Gel Electrophoroesis

To obtain a clean separation of cytochrome c oxidase subunits, the method developed by Swank and Munkres (1971) was adapted, involving SDS-urea-gel electrophoresis in highly cross-linked gels. This method reveals up to eight subunits of beef heart cytochrome c oxidase.

Cytochrome c oxidase samples were dissolved at 1 mg protein/mL in 10% sodium dodecyl sulphate, containing 1 mM dithiothreitol and the solutions were heated at 100°C for 1 minute. Usually 10-50 μg of protein solutions were applied to each gel tube. The acrylamide gels contained 0.1% SDS (w/v), 0.075% TEMED (v/v), 0.07% ammonium persulphate (w/v), 0.1 M H₃PO₄, 8 M urea, and were adjusted with Trizma base to final pH of 6.8. Dilutions were made from a 100 mL stock solution containing 38.7 g acrylamide and 1.33 g bisacrylamide. The stock solution was preserved at 4°C in an amber
bottle. To alter cross-linkage levels, appropriate amounts of bisacrylamide were added to 10 mL aliquots of the above stock solution. Ammonium persulphate and urea solutions were freshly prepared for each experiment. Dilutions were also made from 100 mL of a second stock solution, also maintained at 4°C, containing 0.6 mL TEMED, 0.8 g SDS and 0.8 M H₃PO₄ adjusted to pH 5.0 with Trizma base. The properly mixed solution was adjusted to pH 6.8 with Trizma base prior to ammonium persulphate addition. Then, this mixture was partially deaerated using an aspirator pump. Gels were formed in Pyrex tubing 5.7 mm in diameter and 100 mM in length, and covered with water. Polymerization was completed in about 30 minutes. The tops of the gels were rinsed twice with reservoir buffer. The reservoir solutions contained 0.1% SDS, plus 0.1 M H₃PO₄, adjusted to pH 6.8 with Trizma base. A Buchler polyanalyst apparatus and a power supply (Brock Technical Services) were used for electrophoresis with 12 tubes, with the lower reservoir maintained at a constant temperature of 20°C. From 10 to 50 µl of protein sample solutions were layered on the top of the gels beneath the upper reservoir solution. An internal marker of 5 µl 0.02% bromophenol blue in 40% sucrose, 0.1% SDS and 0.1 M H₃PO₄ (adjusted to pH 6.8 with trizma base) was added to each sample.

Samples were subjected to electrophoresis toward the anode at about 2 mA/gel for about 20 hours. Staining and destaining were effected by a modification of the method described by Weber and Osborn (1969). Gels were immersed for 2 hours in a solution containing 1.25 g Coomassie Blue, 454 mL 50% methanol and 46 mL glacial acetic acid. The staining solution was
decanted and gels were rinsed once with water before immersion in a destaining solution containing 75 mL acetic acid, 250 mL methanol and 675 mL water. Destaining was completed either by changing the solution twice or three times over a 24 hour period, or by traverse electrophoresis for 1.5 hours (Buse and Steffens, 1978; Downer et al., 1976; Chan and Tracy, 1978). Destained gels were scanned at 550 nm using a densitometer attached to the Gilford 2400 spectrophotometer and recorder.

(viii) Sulphide Assays

(a) Titration with Iodine Solution

Hydrogen sulphide in aqueous solution can be estimated by titration against iodine solution, according to Equation (16):

\[ \text{H}_2\text{S} + \text{I}_2 \rightleftharpoons 2\text{HI} + \text{S} \]  

Eq. (16)

As a routine practice, approximately 50 mM (0.1 N) of sulphide stock solution was titrated with 0.1 N standard iodine solution (BDH). The indicator was solubilized starch.

(b) Molybdenum Blue Method (Buck and Stratmann, 1964)

This method was developed by Buck and Stratmann for the estimation of sulphide content in the air. Since aqueous sulphide solutions were used in this study, only the colour developing procedures were adopted. 0, 0.15, 0.25, 0.50, 0.75 and 1.00 μmole aliquots of sulphide were added to 10 mL
samples of ammonium molybdate reagent made by mixing three parts by volume of 3.33% aqueous ammonium molybdate solution with two parts by volume of a solution containing 0.59 g urea in 1 litre 1 N sulphuric acid. The ammonium molybdate reagent was made freshly for every experiment. The mixture with sulphide was incubated for 20 minutes to complete the colour reaction. The absorbance of the fully reacted samples was measured at 570 nm using a 1-cm path cuvette against a blank reagent solution. The sample solutions which gave absorbance values above 1.0 were diluted. Using this method, the extinction coefficient obtained for the sulphide product is 7700 M\(^{-1}\) cm\(^{-1}\) which compares with the value of 10,000 M\(^{-1}\) cm\(^{-1}\) reported by Buck and Stratmann (1964).

footnote: \([\text{SH}^-]\) refers to the total concentration of sulphide \(([\text{SH}^-] + [\text{H}_2\text{S}])\) used in the experiments. 'nm' and 'nmol' refer to nanometres and nanomoles respectively, throughout this thesis.
Figure 6. Standard curve for the ammonium molybdenum method of hydrogen sulphide estimation. Sulphide colour was developed in 10 mL of ammonium molybdate solution made up of three parts by volume of a 3.33% aqueous ammonium molybdate solution plus two parts by volume of a solution of 0.5 g of urea in 1 litre of 1 N H2SO4.

$\varepsilon_{550\text{nm}} = 7700 \text{ M}^{-1} \text{ cm}^{-1}$ at 25°C
H$_2$S measurement by Am. mol. method
Chapter IV

Results

(A) Sulphide Inhibition of Cytochrome c Oxidase

(i) Effect of Sulphide upon the Steady State Reaction of Cytochrome c in the Ascorbate-Cytochrome c Oxidase-O2 System.

Figure 7A illustrates the inhibition of cytochrome c oxidase activity induced by the addition of sulphide during an aerobic steady state in a cuvette containing ascorbate, cytochrome c, cytochrome aa3 and O2. The progressive inhibition is monitored by the increasing level of cytochrome c reduction, as described previously (Nicholls, 1975; Nicholls et al., 1972). The steady state was induced on addition of ascorbate to the cyt. c-cyt. aa3-O2 system. Then, during the steady state, the sulphide solution was added to the system to produce the sulphide inhibited state. Data from such experiments were analyzed according to the following equation (Nicholls et al., 1972):

$$\alpha = \frac{[\text{aa}_3]_t}{[\text{aa}_3]_0} = \frac{[\text{C}^{2+}]_0[\text{C}^{3+}]_t}{[\text{C}^{2+}]_t[\text{C}^{3+}]_0}$$

Eq. (17)

where $\alpha$ = the proportion of active enzyme at any time $t$ (subscripts 0 and $t$ refer to time $= 0$ and time $= t$, respectively).

Figure 7B plots the rates of inhibition obtained from such experiments against the sulphide concentration; the first order constant $k$ ($s^{-1}$)
represents a measure of the rate of inhibition. The slope of the plot corresponds to an "on" constant, $k_{\text{on}} = 7.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$, and the intercept on the vertical axis is so close to the origin that it is impossible to calculate an accurate $K_i$ value under these conditions kinetically. But the amount of active enzyme remaining in the system at any given time can be plotted against sulphide concentration as in Figure 7C. This figure reveals that the inhibition increases with increasing sulphide concentrations, but is proportionally more effective at higher concentrations, above 3.5 $\mu$M, and less effective at lower sulphide concentrations. At the low concentration the estimated $K_i$ value is close to 1 $\mu$M; at the higher sulphide concentration it approaches 0.1 $\mu$M.

If sulphide inhibition is a function of $H_2S$ (undissociated acid, see Equation (2)) concentration, it may be expected to increase as pH decreases ($pK = 7.00$). Figure 8 shows the rate constants of inhibition obtained from experiments carried out as in Figure 7A, but at a pH of 6.65. At this pH, the $k_{\text{on}}$ value, second order rate constant, is a little higher, about $2.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$. The calculated $K_i$ is less than 0.1 $\mu$M. These values are qualitatively consistent with the presumed inhibitory role of undissociated $H_2S$ molecules.

Inhibition of submitochondrial particle activity by sulphide is shown in Figure 9. In this system, the development of inhibition was biphasic. Two distinct phases, "fast" and "slow" are observed, with apparent $k_{\text{on}}$ values of $5.3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $1.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ respectively at pH 7.5 and 30°C. Although such biphasicity was not observed with sulphide and
Figure 7. Effect of sulphide on the activity of cytochrome c oxidase

(A) Steady state reduction changes of cytochrome c in the presence of enzyme, ascorbate and inhibitor. 1.1 mM ascorbate and 0.18 μM cytochrome aa₃ were added to 18.5 μM cytochrome c in 67 mM sodium phosphate, 0.5% Tween-80, pH 7.4 as indicated. Subsequently 9 μM Na₂S was added to perturb the steady state. Reaction monitored at 550 nm and 30°C.

(B) Rates of inhibition obtained from experiments as in Figure 7A, plotted against sulphide concentration. $k_{\text{sec}^{-1}}$ represents the first order constant for the transition from uninhibited to inhibited steady state calculated in terms of $\alpha$.

$$\alpha = \frac{[\text{aa}_3]_t}{[\text{aa}_3]_0} = \frac{[c^{2+}]_0[c^{3+}]_t}{[c^{2+}][c^{3+}]_0}$$

where $[\text{aa}_3]_0$ represents the amount of active enzyme in the uninhibited steady state while $[\text{aa}_3]_t$ represents the amount of active enzyme in the steady state perturbed by sulphide.

(C) The proportion of active enzyme ($\alpha$) in the inhibited steady state, plotted against sulphide concentration. Conditions as in Figure 7A, with the $\alpha$ values calculated according to

$$\alpha = \frac{[\text{aa}_3]_t}{[\text{aa}_3]_0} = \frac{[c^{2+}]_0[c^{3+}]_t}{[c^{2+}][c^{3+}]_0} ;$$

theoretical curves are plotted for $K_I = 1.0$ μM (---) and 0.1 μM (...) according to the simple mass action law.
Fig. 7B.

\[ k = 7.5 \times 10^3 \text{ M}^{-1}\text{s}^{-1} \]
Fig. 7C.
Figure 8. Rates of inhibition of cytochrome aa$_3$ at acid pH.

Experiments carried out as in Figure 7A, except that pH was 6.65; rates are plotted against sulphide concentration. Medium was 67 mM sodium phosphate, pH 6.65, 0.5% Tween-80, monitored at 550 nm, 30°C.
Figure 9. Rates of inhibition of submitochondrial particles (SMP) by sulphide
1 mM of SMP was mixed with 0.4 mL of 10% deoxycholate to solubilize, and then 2.6 mL of Keilin-Hartree medium (see Methods (i)) was added to the mixture. Reaction was monitored on addition of sulphide to a mixture of 63 nM cytochrome aa3, 18.5 μM cytochrome c and 1.1 mM ascorbate in 67 mM sodium phosphate, pH 7.4, 0.5% Tween-80, at 550 nm and 30°C.
Figure 10. Rates of inhibition of cytochrome $a_{a3}$ activity by cyanide.

1.1 mM ascorbate and 0.18 μM cytochrome $a_{a3}$ were added to 18.5 μM cytochrome $c$ in 67 mM sodium phosphate, 0.5% Tween-80, pH 7.4. Cyanide was added to disturb the steady state, monitored at 550 nm and 30°C.
isolated enzyme, it does resemble the effect of cyanide seen both with isolated enzyme and submitochondrial particles (Nicholls et al., 1972). Figure 10 shows the rate constants for inhibition of cytochrome c oxidase by cyanide under similar catalytic conditions. The two phases, "fast" and "slow", have apparent $k_{on}$ values of $6.3 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $1.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ respectively, essentially identical with the values reported previously (Nicholls et al., 1972). It may be noted that spectroscopic examination of cyanide binding to cytochrome c oxidase previously gave a second order rate constant of $1.8 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.4 and 21°C (Van Buuren et al., 1972).

These workers concluded that a form of the enzyme present under catalytic conditions had a greater reactivity with cyanide than did the resting enzyme. In order to determine whether this is also the case with sulphide, experiments on the spectroscopic changes in the enzyme induced by this inhibitor were therefore carried out.

(ii) Spectroscopic Changes in Cytochrome c Oxidase Preparations Induced by Sulphide

Figure 11A illustrates the changes occurring in the Soret region of the spectra of submitochondrial particles on addition of sulphide under aerobic conditions in the absence of substrate. The initial phase of the titration is completed upon the addition of 1 mole sulphide:mole $\text{aa}_3$, but it is necessary to introduce about 3 moles of sulphide per mole of cytochrome c oxidase in order to complete the second phase of the titration. The corresponding rates of reaction of submitochondrial particles with sulphide
Figure 11. Effects of sulphide on submitochondrial particles (SMP).

(A) Spectroscopic titration of SMP with sulphide.

1 mL of SMP (aa$_3$ conc, 14.8 µM) was solubilized with 0.4 mL of 10% deoxycholate, then suspended in 2.6 mL of Keilin–Hartree medium, pH 7.5 (total volume 4 mL). Varying amounts of sulphide were then added to the SMP suspension (3.7 µM aa$_3$), and the reaction monitored at 438-417 nm, 30°C.

(B) Rate constants for sulphide-SMP interaction plotted against sulphide concentration. Conditions as in Figure 11A. First order rate constants (k) from the reactions in Figure 11A with the following equation:

\[ k = \frac{0.693}{t_{1/2}} \]
\[ \Delta A \]

\[ \mu M \text{ SH}^- \]

\[ a_3 3.7 \mu M \]
$k = 10^4 \text{ M}^{-1} \text{s}^{-1}$

3.7 μM TITRE
may be plotted as in Figure 11B. Below 3.7 μM, the effective concentration of cytochrome _aa_3, the velocity is independent of sulphide concentration. Above 3.7 μM, the velocity is proportional to [H₂S], with a rate constant of 10⁴ M⁻¹ s⁻¹. Such behaviour is characteristic of a reaction that is effectively irreversible.

Figure 12A shows similar results obtained in a direct titration of isolated cytochrome _c_ oxidase in its "resting" aerobic state with sulphide. As the concentration of sulphide increases, more absorption change occurs and the reaction velocity increases (Figs. 12B and 12C). If the total absorbance changes from Figure 12A are plotted against sulphide concentration as in Figure 12B, the evidence is also obtained for two phases of spectroscopic changes. The initial phase is stoichiometric with added sulphide. If the rate constants from the reactions in Figure 12A are plotted as in Figure 12C, a plot of first order rate constant against sulphide concentration, analogous to that in Figure 11B, is obtained. For the isolated enzyme, the k_{on} value is approximately 1.4 x 10⁴ M⁻¹ s⁻¹.

Difference spectra obtained in the reactions of Figures 11B-12C are shown in Figure 13. In the Soret region, a peak appears at 433 nm and a trough at 414 nm. In the visible region, two distinct peaks occur at 565 and 607 nm, respectively.

Both the catalytic and spectroscopic data presented so far lead us to conclude that sulphide interacts with the enzyme, reacting with a rate constant of about 10⁴ M⁻¹ s⁻¹ to form a complex with a dissociation constant between 0.1 and 1.0 x 10⁻⁶ M at pH 7.4 and 30°C. These results are
Figure 12. Spectroscopic changes in cytochrome $\text{aa}_3$ induced by sulphide.

(A) Absorbance changes at 433-417 nm induced by addition of sulphide to the enzyme. The indicated concentrations of sulphide were added to 3.5 $\mu$M cytochrome $\text{aa}_3$ in 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80 at 30°C, and the reaction monitored aerobically using the dual wavelength spectrophotometer.

(B) Total absorbance changes at 433-417 nm from experiments as in Figure 12A, plotted against sulphide concentration.

(C) Rate constants for the sulphide reaction with cytochrome $\text{aa}_3$ plotted against sulphide concentration. The $k$ ($s^{-1}$) axis represents the first order constants for the transition from free to modified enzyme. Conditions as in Figure 12A.
Fig. 12A.
Fig. 12B.
$k = 1.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$

Fig. 12C.
Figure 13. Difference spectra obtained upon addition of varying amounts of sulphide to an aerobic solution of cytochrome c oxidase.

3.5 μM cytochrome $aa_3$ in 67 mM sodium phosphate, 0.25% Tween-80, pH 7.4, was titrated with (1) 0, (2) 0.83 μM, (3) 3.3 μM, (4) 6.7 μM, (5) 10 μM and (6) 13 μM sodium sulphide at 30°C. The reference cuvette contained oxidized (resting) enzyme.

Note: different scales on left and right hand sides of figure.
consistent with the known high sensitivity of this enzyme towards hydrogen sulphide. Further experiments, however, show that the situation is more complicated than this (sections (iii) and (iv) following).

(iii) Titration of Cytochrome c Oxidase with Sulphide under Catalytic Conditions

Experiments were carried out, monitored at 550 nm, in which the redox state of cytochrome c was kept low in the presence of high levels of the enzyme. Addition of sulphide increases the reduction of cytochrome c in the steady state (Fig. 14A). These data are plotted as in Figure 14B, in the form of $\alpha$ against sulphide concentration, where $\alpha$ values were calculated as in Figure 7C and Equation (17). More than one mole of sulphide was needed to inhibit one mole of cytochrome $\text{aa}_3$.

The cytochrome c oxidase system was also titrated with sulphide under similar conditions, following the reaction at 606 nm where the redox state of cytochrome $a$ is monitored (Figure 15). This experiment shows that the 606 nm species (Figure 13) which appears on addition of sulphide decays, and at low sulphide concentrations does not contribute to the steady state absorbance increase induced by ascorbate (traces 2-4). At higher sulphide concentrations, a more stable species is formed and the ascorbate-induced steady state shows inhibition (traces 5-7).

In a different type of steady state experiment, cytochrome c oxidase was directly titrated with sulphide in the presence of ascorbate and TMPD, but in the absence of cytochrome c (Fig. 16A). The reaction was monitored
Figure 14. Titration of cytochrome $aa_3$ with sulphide under catalytic conditions.

(A) Time course of cytochrome $c$ absorbance changes. 18.2 mM ascorbate was added to 30 μM cytochrome $c$ and 15 μM cytochrome $aa_3$. After anaerobiosis, sulphide and oxygen were introduced into the system and the steady state reduction level was measured at 550 nm. Medium was 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80, 30°C.

(B) A plot of the proportion of active enzyme in the inhibited state ($a$), against sulphide concentration. Conditions as in Figure 14A.
Fig. 14A.
\[ \alpha = \frac{C_{\text{O}}^{2+}}{C_{\text{O}}^{3+}} \times \frac{C_{\text{I}}^{3+}}{C_{\text{I}}^{2+}} \]

$550 \text{ nm}$

**Fig. 14B.**
Figure 15. Titration of cytochrome aa₃ with sulphide under catalytic conditions, monitored at 606 nm. Varying amounts of sulphide were added to a mixture containing 33 μM cytochrome c and 15.2 μM cytochrome aa₃ in 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80, at 30°C. The indicated steady states were induced upon addition of 18 mM ascorbate to the system.

1. control (no sulphide)
2. + 3.7 μM Na₂S
3. + 7.4 μM Na₂S
4. + 11.1 μM Na₂S
5. + 18.5 μM Na₂S
6. + 55.5 μM Na₂S
7. + 74.1 μM Na₂S
Fig. 15.
Figure 16. Titration of cytochrome $\text{aa}_3$ with sulphide under catalytic conditions in the absence of cytochrome $c$.

(A) Time course monitored at 445-470 nm. The reduction was initiated by addition of 270 μM TMPD and 5.5 mM ascorbate to 3 μM cytochrome $\text{aa}_3$ in 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80. After anaerobiosis, 3.7 μM aliquots of sulphide were added stepwise and oxygen was simultaneously introduced. Then the steady state reduction level was measured. As sulphide is added to the system, cytochrome $a_3$ remains oxidized and cytochrome $a$ is gradually reduced. When about 3 moles of sulphide per mole of cytochrome $c$ oxidase is added, no more changes at 445-470 nm resulted. This means that cytochrome $a$ is fully reduced and cytochrome $a_3$ is liganded and remains in the oxidized state.

(B) Absorbance changes at 445-470 nm from experiments as in Figure 16A, plotted against sulphide concentration. □...□, represents cytochrome $a$ steady state and △...△, represents the ligation of sulphide to cytochrome $a_3$. 
in the Soret region using the wavelength pair 445-470 sensitive to the
reduction of both cytochromes $a$ and $a_3$. After 3 moles of sulphide per mole
of cytochrome $aa_3$ were added to the system, the introduction of oxygen to
the cytochrome $c$ oxidase system did not induce the steady state and
absorbance change did not occur. At this stage, the enzyme is fully
inhibited as the species $a^2+aa_3^3+\text{H}_2\text{S}$. The absorbance changes from
Figure 16A obtained before and after anaerobiosis may be plotted against
sulphide concentration (Fig. 16B). This figure reveals that the amounts of
sulphide required for inducing the steady state reduction of cytochrome $a$ and
for binding to cytochrome $a_3$ are identical spectrophotometrically.

(iv) Effect of Sulphide on Oxygen Uptake by the Cytochrome $c$ Oxidase System

Cytochrome $c$ oxidase inhibition by sulphide was examined with the oxygen
electrode system (Fig. 17) (Estabrook, 1961; Nicholls, 1971). Addition of
varying amounts of sulphide to the enzymatic system causes a progressive
decrease in the rate of oxygen uptake, as shown in Figure 17 (cf. the
dashed curves obtained in the presence of sulphide with the uninhibited
reaction indicated by the continuous line). However, the product obtained
by incubating sulphide with enzyme in a 1:1 ratio did not show any
inhibition (Fig. 18). This cannot readily be attributed to rapid dissociation
of sulphide, because both spectroscopic (Figs. 7 -12) and kinetic analysis
(Figs. 7-9) clearly demonstrate that the dissociation rate constant is very
slow. Thus, the initial product of reaction of enzyme with sulphide is an
active species and not identical with the inhibited sulphide complex. The
catalytically competent sulphide-induced species was therefore subjected to
a further investigation in order to examine this apparent discrepancy.
Figure 17. Inhibition of cytochrome aa₃ by sulphide monitored polarographically.

26 nM of cytochrome aa₃ (previously incubated with a mixture of 10 mg/mL phosphatidyl choline plus 20 µM ASC in 67 mM sodium phosphate, pH 7.4, 0.5% Tween-80) and varying amounts of sulphide, 0.2 µM, 0.5 µM, 1 µM, 2 µM, 5 µM, and 10 µM, were added as indicated to 7 mM ascorbate, 178 µM TMPD, 24 µM cytochrome c in 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80, at 30°C. Solid line represents uninhibited control experiment and dashed lines indicate inhibited states by sulphide.
Fig. 17.
Figure 18. Polarographic assay of oxygen uptake by native cytochrome aa$_3$ and its sulphide-modified form.

26 nM cytochrome aa$_3$ was added to 7 mM ascorbate, 178 µM TMPD and 24 µM cytochrome c in 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80 at 30°C (control experiment, ____). In one experiment (---), 21.75 µM cytochrome aa$_3$ was preincubated with 21.75 µM sulphide in 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80, at 30°C for 3 minutes, before addition to the enzymatic system. 26 nM cytochrome aa$_3$ treated with sulphide was added to the system.
ENZYME  

ASC  

5°C  

TMPD  

H2O  

50 μM  

(2 μM  

(SH + βββ)  

(aαα  ALONE)  

2 MIN

Fig. 18.
(B) Action of Sulphide as a Cytochrome c Oxidase Substrate

(i) Spectrophotometric Analysis of the Changes in Cytochrome c Oxidase Induced by Sulphide

The absolute spectra of cytochrome c oxidase in the presence and absence of sulphide are shown in Figure 19A. The solid line indicates the resting ferric enzyme \( \text{a}^3+\text{a}_3^3+ \), the dashed line the enzyme fully reduced with dithionite \( \text{a}^2+\text{a}_3^2+ \). The spectrum shown by the dotted line is that obtained when 1.8 mM sulphide is added to the "resting" (ferric) cytochrome c oxidase (Eq. 18):

\[
\text{a}^3+\text{a}_3^3+ + \text{H}_2\text{S} \rightleftharpoons \text{a}^3+\text{a}_3^3+-\text{H}_2\text{S} \quad \text{Eq. (18)}
\]

On standing, a partially reduced form of the sulphide-complexed enzyme is obtained (Eq. 19):

\[
\text{a}^3+\text{a}_3^3+\text{H}_2\text{S} + \text{H}_2\text{S} \rightarrow \text{a}^2+\text{a}_3^2+-\text{H}_2\text{S} (+ \text{oxidized H}_2\text{S}) \quad \text{Eq. (19)}
\]

Sulphide binding to cytochrome \( \text{a}_3^3+ \) causes a blue-shift in the \( \alpha \) peak of cytochrome \( \text{a} \), as shown in the 60 minute (dot-dash) spectrum (Nicholls, 1975). The corresponding difference spectra are shown in Figure 19B, which may be compared with the spectral changes seen at lower sulphide concentration (Fig. 13).

The addition of near-stoichiometric amounts of sulphide to cytochrome c oxidase produces intermediates which are not the same as the cytochrome c...
oxidase-sulphide complexes produced by excess of inhibitor (Fig. 20A). The spectrum (...) produced by the initial addition of sulphide with a Soret maximum at 435 nm and a visible peak at 607 nm is unstable. This 607 nm species disappears with a half-life at room temperature of 1-2 minutes. Another distinct low spin spectrum (---) is produced from this initial species aerobically. On addition of excess sulphide (-----), a third distinct species is obtained. The final spectrum with a sulphide concentration of 0.65 mM seems to be the inhibited half-reduced form of the enzyme, \( a^{2+}a_3^{3+}-H_2S \).

Sulphide thus induces formation of several cytochrome c oxidase species. In order to distinguish at least two such species, the following experiment was carried out: a six-fold excess (29 \( \mu \)M) of sulphide was added to a sample cuvette which contained 4.6 \( \mu \)M cytochrome c oxidase (Fig. 20B). The product showed the spectrum of the initial sulphide-enzyme interaction (dashed curve, Fig. 20B). When 29 \( \mu \)M of sulphide was added to the reference cuvette and a total of 600 \( \mu \)M sulphide to the sample cuvette, then a difference spectrum which represents the inhibited enzyme-sulphide complex was obtained (...). This shows that the initial product and the inhibited sulphide complex are different.

The cytochrome c oxidase-cyanide complex will also react with sulphide (Fig. 21). In this case, sulphide reduces cytochrome a while competing with cyanide for the cytochrome \( a_3 \) ligand binding site. The relatively small \( \gamma \)-peak obtained after addition of ascorbate and TMPD indicates that cytochrome \( a_3 \) is liganded by either cyanide or sulphide.
Figure 19. Spectral changes in cytochrome aa₃ induced by sulphide.

(A) Absolute spectra

1.8 mM sulphide was added to 3.2 μM cytochrome aa₃ in 67 mM sodium phosphate, pH 7.4, 0.5% Tween-80 at 30°C.

\[
\begin{align*}
\text{\textbullet}, & \quad \text{a}^{3+} \text{a}_3^{3+} \\
\text{\ldots}, & \quad \text{a}^{3+} \text{a}_3^{3+} - \text{H}_2\text{S} \\
\text{\ldots}, & \quad \text{a}^{2+} \text{a}_3^{3+} - \text{H}_2\text{S} \\
\text{\ldots}, & \quad \text{a}^{2+} \text{a}_3^{2+}
\end{align*}
\]

(B) Difference spectra

Conditions are the same as in Figure 18A, except the reference cuvette contained the oxidized enzyme (see Methods (v)c.1 for details)

\[
\begin{align*}
\text{\textbullet}, & \quad \text{a}^{3+} \text{a}_3^{3+} \\
\text{\ldots}, & \quad \text{a}^{3+} \text{a}_3^{3+} - \text{H}_2\text{S} \\
\text{\ldots}, & \quad \text{a}^{2+} \text{a}_3^{3+} - \text{H}_2\text{S} \\
\text{\textbullet}, & \quad \text{a}^{2+} \text{a}_3^{2+}
\end{align*}
\]
Fig. 19A.
Fig. 19B.

\[
\text{RED/OX} \quad \text{OX/OX} \quad \text{60 min later} \quad \text{H}_2\text{O}^+ + \text{SH}^- / \text{OX}
\]
Figure 20. Difference spectra of sulphide-induced complexes of cytochrome $a_{a_3}$

(A) Spectra at low and high sulphide concentrations formed aerobically.

4.6 μM cytochrome $a_{a_3}$ in an aerobic cuvette in 0.1 M sodium phosphate, pH 7.4, 0.25% Tween-80, at 30°C was treated with 14 μM Na$_2$S to give the initial (10 s) spectrum (....) and a spectrum after 5 minutes (____). 43 μM Na$_2$S gave rise to the spectrum (-----) and 0.64 mM Na$_2$S to a product in which cytochrome $a$ is partially reduced sulphide complex peaks are also indicated (-----...).

(B) Effect of ascorbate and high sulphide levels on the aerobic spectra of low sulphide-induced complexes of cytochrome $a_{a_3}$.

Varying amounts of sulphide, ascorbate and TMPD were added as indicated to 4.6 μM cytochrome $a_{a_3}$ in 0.1 M sodium phosphate, pH 7.4, 1% Tween-80, at 30°C. When 29 μM Na$_2$S was added to the reference cuvette and 600 μM Na$_2$S to the sample cuvette, a partially reduced cytochrome $c$ oxidase α-peaks appears.
Fig. 20A.
Fig. 20B.
Figure 21. Spectral changes induced aerobically by sulphide in the cytochrome $a_3$-CN$^-$ complex. The reaction was monitored on addition of 0.35 mM Na$_2$S to 5.4 μM cytochrome $a_3$-CN$^-$ in 0.1 mM KCN, 0.1 M sodium phosphate, pH 7.4, 1% Tween-80, at 30°C. The initial cytochrome $a_3$-CN$^-$ complex was prepared by incubating 110 μM cytochrome $a_3$ with 2 mM KCN.
Fig. 21.
Figure 22. Spectra of sulphide-induced complexes of cytochrome $aa_3$ under anaerobic conditions.

3.7 µM cytochrome $aa_3$ in 67 mM sodium phosphate, 0.25% Tween-80, pH 7.4 at 30°C in a Thunberg cuvette under vacuum was treated with 6.7 µM Na$_2$S to give an immediate (12 s, ____ ) and a 20-minute spectrum (....). Admission of air produced the 607 nm form (----). Further addition of 41 µM Na$_2$S gave the new spectrum (-----....) and finally dithionite reduction produced the spectrum of reduced cytochrome $a^{2+}_{a3}^{3+}$-$H_2$S (-----.......).
Fig. 22.
To determine the role of oxygen in the formation of the early species obtained with sulphide, experiments were carried out under anaerobic conditions. Figure 22 shows the spectroscopic changes taking place on addition of sulphide to cytochrome _aa_3 under vacuum in a Thunberg cuvette (see Methods). The initial low-spin spectrum (—) obtained is much more stable than the initial aerobic species, as shown by the 20 minute spectrum (...). Admission of air gives rise to the initial aerobic form with α-peak at 607 nm (---). A higher concentration of sulphide is needed for the formation of the (presumably) inhibited species (—…—). In the presence of dithionite, the half-reduced inhibited form is obtained (—…—…—).

Rapidly scanned spectra (Hewlett-Packard 8450A spectrophotometer, see Methods III.B.(v)c) clearly distinguish three phases of sulphide interaction with cytochrome _c_ oxidase under aerobic conditions. At 3-6 seconds, the product shows a 606 nm peak, which decays (12-24 seconds) to give the "oxyferri" species. The final spectrum (—…—…—, 2700 s) presumably represents the fully inhibited enzyme species (Fig. 23).

Figure 24 presents an analysis using the slow Aminco instrument (see Methods III.B.(v).c) of the transition between the "oxyferri" form (12 s species in Fig. 23) and the final species. The initial spectrum (—, approx. 12 s), showing two peaks is slowly transformed into a partially-reduced, fully inhibited species (—, ...). Ferricyanide addition oxidizes the ferrocytochrome _a_ present to reveal the final enzyme-sulphide complex with peak at 590 nm (——…—). This final inhibited form is much more stable than the initial 607 nm and "oxyferri" species, as proposed by Nicholls (1975).
The question then arises as to whether the half-reduced enzyme species \((a^{3+}a^{2+}_3)\) can be produced by sulphide. In order to test this question, further experiments were carried out under anaerobic conditions. Figure 25, like Figure 22, illustrates some spectroscopic changes which occur on addition of sulphide to cytochrome \(aa_3\) under anaerobic conditions (see Methods III.B.v.c). Addition of sulphide to an anaerobic enzyme solution initially produces a low-spin spectrum (Figs. 22 and 25, solid line). This low-spin species transformed to give a final stable species slowly (Fig. 25, dotted line). Carbon monoxide was added at this point, anaerobically, and a 590 nm peak characteristic of the half-reduced enzyme-CO complex \((a^{3+}a^{2+}_3-CO)\) was obtained (Fig. 25, dotted-dashed line). Thus, the 3000 s species may, in fact, be a half-reduced enzyme species \((a^{3+}a^{2+}_3)\) which can be produced by sulphide under anaerobic conditions. For comparison, the oxidized enzyme-CO complex was modified by addition of CO to the system aerobically. This gave the 607 nm peak (Fig. 25, dashed curve). Fully reduced enzyme-CO species were obtained by addition of dithionite.
Catalytic activity can be restored by gel-filtration or by dilution (Fig. 26A) of sulphide-inhibited enzyme, which is a measure of the dissociation rate constant ($k_{\text{off}}$) for the cytochrome $c$ oxidase-sulphide complex. For the dilution method, enzymatic activity was initiated by adding ascorbate to a mixture of cytochrome $c$ and cytochrome $aa_3$. Once a steady state level of cytochrome $c$ reduction was achieved, sulphide was added to the reaction mixture; as can be seen in Figure 26A, the absorbance at 550 nm increased. This is due to an inhibition of cytochrome $c$ oxidase by sulphide as previously shown (see Fig. 7A). Once the sulphide inhibited enzyme was formed, a 0.1 mL aliquot was removed and added to a solution of cytochrome $c$. Once again, the absorbance at 550 nm increased, showing the enzyme was still inhibited. This increase in absorbance reached a maximal level (plateau). After about one to two minutes, the absorbance decreased, demonstrating catalytic activity was slowly being restored. The decrease in cytochrome $c$ absorbance is therefore proportional to the dissociation rate of sulphide from the sulphide-inhibited enzyme complex.

For the column method, the system is essentially the same as described above except the sulphide inhibited sample was gel-filtered on a Sephadex G-100 column (15 x 1.5 cm) in 150 mM potassium phosphate, pH 6.8, 0.25% Tween-80. Fractions of the sample were collected in 1.0 mL volumes and 0.2 mL aliquot of the eluate, plus 1 mM ascorbate was added to the cytochrome $c$ solution (See legend of Fig. 26A for details.) The reaction was again followed at 550 nm. The resulting reaction was essentially identical to that described earlier. The logarithm of activity is plotted against time (Fig. 26B).

The dilution method gives a value for $k_{\text{off}}$ of $5.5 \times 10^{-4}$ s$^{-1}$ and the column
Figure 23. Rapid scan traces of the spectral changes in cytochrome $a_{a_3}$ induced by sulphide under aerobic conditions.

15 μM of sulphide was added to 3 μM cytochrome $a_{a_3}$ in 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80, at 25°C. The Hewlett-Packard 8450A spectrophotometer and 7225 plotter were used for this experiment.
Fig. 23.
Figure 24. Spectral changes in cytochrome \textit{aa}$_3$ complexes induced by sulphide and monitored in the visible region.

16 μM sulphide was introduced into a 5 μM solution of cytochrome \textit{aa}$_3$ in 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80, at 30°C. This experiment was carried out with the Aminco DW-2 spectrophotometer.
Fig. 24.
6.6 μM sulphide was added to 5.2 μM cytochrome c oxidase in 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80, at 30°C, in an evacuated Thunberg cuvette (____). After 3000 seconds (....), CO was introduced into the system anaerobically, where as the \( \text{a}^3\text{a}_3^2\text{CO} \) species shows up at 590 nm (-----). An analogous experiment gave the 607 nm species on admission of O₂ (-----). Full reduction of the CO-treated system is shown as (-----...).
Fig. 25.
Figure 26. Measurement of the dissociation rate constant \( k_{\text{off}} \) of the cytochrome \( \text{aa}_3 \)-H\(_2\)S complex.

(A) Experimental methods

(i) Dilution method: Enzymatic activity was initiated upon addition of 60 mM ascorbate to 1 \( \mu \)M cytochrome \( \text{aa}_3 \), 20 \( \mu \)M cytochrome \( \text{c} \) in 150 mM potassium phosphate, pH 6.8, 0.25% Tween-80, at 30°C. In the steady state, 5 \( \mu \)M sulphide was added to the system to induce an inhibited state. During inhibited state, 0.1 mL of sample was removed and added to 20 \( \mu \)M cytochrome \( \text{c} \) in 150 mM potassium phosphate, pH 6.8, 0.25% Tween-80. The reaction monitored at 550 nm. The initial phase was an inhibited steady state, later on the absorbance was slowly decreased. At this stage, it is evident that the sulphide molecules dissociate from the enzyme molecules.

(ii) Column method: After the enzymatic system was reacted with sulphide, whole sample was removed and gel-filtered (Sephadex G-100 column 15 \( \times \) 1.5 cm in 150 mM potassium phosphate, pH 6.8, 0.25% Tween-80). Cytochrome \( \text{aa}_3 \) fraction was collected in 1 mL fractions. 0.2 mL of eluate plus 2.1 mM ascorbate was added to 20 \( \mu \)M cytochrome \( \text{c} \) in 150 mM potassium phosphate, pH 6.8, 0.25% Tween-80, to produce inhibited state as in the "dilution method". This inhibited state diminished faster than the dilution method and it is clear that sulphide dissociates from the enzyme molecules.

(B) Logarithmic plots of the dissociation rate constants obtained as in Figure 26A, against time. The "dilution method" gives \( k_{\text{off}} = 5.5 \times 10^{-4} \) s\(^{-1}\) and the "column method" gives \( k_{\text{off}} = 1.1 \times 10^{-3} \) s\(^{-1}\).
Experimental Diagram of Measuring Koff

Fig. 26A.
method a value for $k_{\text{off}}$ of $1.1 \times 10^{-3} \text{ s}^{-1}$. These values are close to the spectrophotometric value of $6 \times 10^{-4} \text{ s}^{-1}$ reported previously (Nicholls, 1975).

So far, in this section (i), the spectroscopic changes in cytochrome $a_{a3}$ induced by sulphide have been discussed. The initial reaction between sulphide and enzyme under aerobic conditions does not produce an inhibited species, but a low-spin catalytically active form, probably the oxyferri intermediate (Figs. 13, 18, 21 and 22). At the same time, oxygen is taken up. To understand the behaviour of sulphide, direct titrations of the enzyme, ferricyanide and cytochrome $c$ with sulphide were carried out spectrophotometrically as well as polarographically.

(ii) Reactions of Ferricyanide, Cytochrome $c$ and Cytochrome $a_{a3}$ with Sulphide and Oxygen.

A titration of ferricyanide with sulphide carried out aerobically is shown in Figure 27A, and the resulting absorbance changes are plotted as in Figure 27B, against sulphide concentration. 425 $\mu$M sulphide titrated with 450 $\mu$M Fe(CN)$_6^{3-}$ giving a ratio between ferricyanide reduced and sulphide added of 1.1:1. If both reducing equivalents in sulphide were available, a stoichiometry of 2 would be anticipated.

Cytochrome $c$ was similarly titrated with sulphide aerobically, with its reduction monitored at 550 minus 540 nm (Fig. 28A). Sigmoidal reaction curves are produced, indicating that the interaction between sulphide and cytochrome $c$ may be an indirect one. The total absorbance changes are plotted against sulphide concentration in Figure 28B. 12-13 $\mu$M $H_2S$ are
required to reduce 23 µM cytochrome c, close to the expected stoichiometry. But to probe the shortfall in the ferricyanide case polarographic experiments were carried out, as shown in Figure 29A. As seen in this figure, each aliquot of sulphide oxidized by ferricyanide produces the uptake of a quantity of oxygen. These oxygen uptakes are plotted against added sulphide as in Figure 29B. The results show that the oxidation of one mole of sulphide and reduction of one equivalent of ferricyanide is accompanied by the uptake of one half mole of oxygen.

Cytochrome c can also be titrated with sulphide polarographically (Fig. 30A). Bursts of oxygen uptake induced by successive additions of sulphide are seen until the cytochrome c is fully reduced. Figure 30B shows that oxidation of one mole of sulphide is accompanied by the uptake of 0.05 mole of oxygen in this system. As the sulphide additions are continued beyond the titration point with cytochrome c, the bursts of oxygen uptake disappear, and a slow oxygen uptake due to sulphide autoxidation is seen (Fig. 30A).

To obtain an oxygen-independent stoichiometry for the ferricyanide-sulphide reaction, a titration was carried out under anaerobic conditions using the Thunberg cuvette (Fig. 31A). The resulting absorbance changes are plotted against sulphide concentration in Figure 31B. The titration ratio between sulphide and ferricyanide appears to be 1:1.7, indicating that most of the shortfall under aerobic conditions was associated with the catalyzed oxygen uptake.

To compare the ferricyanide and cytochrome c reactions with that of cytochrome aa₃, polarographic experiments were undertaken with high
concentrations of cytochrome c oxidase (20 μM); an oxygen uptake occurs in the absence of substrates immediately upon the addition of sulphide as shown in Figure 32B. In this case, the stoichiometry between sulphide and oxygen is 1:2, indicating that the reaction of one mole of sulphide with one mole of enzyme is accompanied by the uptake of two moles of oxygen. Plotting the logarithm of oxygen concentration against time indicates that two phases exist for this enzymatic oxygen uptake (Figure 32B). Figure 33A shows the oxygen uptake which occurs when cytochrome c oxidase is reacted with small aliquots of added sulphide; the successive sulphide additions produce oxygen uptake in progressively increasing amounts, but at progressively decreasing rates. As shown in Figure 33B, between 2 and 3 μmoles of oxygen are initially taken up per μmole of sulphide added. Then a slow second phase occurs.

Figure 34A, B, C illustrates the effects of various reagents on the sulphide-induced oxygen uptake. N-ethylmaleimide, which is a sulphydryl group specific inhibitor, is not capable of blocking the slow oxygen uptake (Figure 34A), suggesting that the slow phase is not due to accessible sulphydryl groups in the enzyme. Conversely, when dithiothreitol (Clelands reagent) is added to the system (Fig. 34B), it does not stimulate any increase in the slow phase. EDTA, a metal chelator, slightly inhibited the slow phase. The addition of catalase seems to increase the oxygen uptake (Fig. 34C). Cyanide is more effective than EDTA in inhibiting the slow phase. This may mean that this reaction is due to cytochrome aa3.
Figure 27. Aerobic titration of ferricyanide with sulphide.

(A) Time course of absorbance changes.

The reaction was monitored at 420-480 nm by addition of varying amounts of sulphide to 0.5 mM potassium ferricyanide in 67 mM sodium phosphate, pH 7.4, at 30°C.

(B) Data from Figure 27A plotted as total absorbance changes against sulphide concentration. The stoichiometry between ferricyanide and sulphide appears to be 1:1:1.
Fig. 27A.
Fig. 27B.
Figure 28. Aerobic titration of cytochrome c with sulphide

(A) Time courses of reduction

The reaction was monitored at 550-540 nm on addition of the indicated amounts of sulphide to 23.2 μM cytochrome c in 67 mM sodium phosphate, pH 7.4, at 30°C.

(B) Data from Figure 28A plotted as total absorbance changes against sulphide concentration. The ratio between cytochrome c and sulphide was close to 2:1.
Fig. 283.
Figure 29. Oxygen uptake during the aerobic titration of ferricyanide with sulphide.

(A) Polarographic tracing

The reaction was followed by successive additions of sulphide to 0.5 mM ferricyanide in 67 mM aerated sodium phosphate, pH 7.4, at 30°C.

(B) Data from Figure 29A plotted as oxygen uptake against cumulative sulphide concentration.
Fig. 29A.
Figure 30. Oxygen uptake during the aerobic titration of cytochrome c by sulphide.

(A) Polarographic tracing

The reactions were initiated with progressive additions of 100 μM Na₂S to 500 μM cytochrome c in 67 mM sodium phosphate, pH 7.4, at 30°C.

(B) Data from Figure 30A plotted as oxygen uptake against cumulative sulphide concentration.
Fig. 30A.
Fig. 30B.
Figure 31. Anaerobic titration of ferricyanide with varying amounts of Na₂S.

(A) Spectroscopic tracings
0.5 mM ferricyanide in 67 mM sodium phosphate, pH 7.4, at 30°C in a Thunberg cuvette under vacuum, was titrated with varying amounts of sulphide. At the end of each experiment, dithionite was added to the system to check the end point.

(B) Date from Figure 31A plotted as absorbance changes against sulphide concentration. The ratio between ferricyanide reduced and sulphide added is approximately 1.7:1.
Fig. 31A.
Fig. 31B.
Figure 32. Oxygen uptake by cytochrome $a_{33}$ induced by sulphide.

(A) Polarographic tracing

7 mM ascorbate and 178 µM TMPD (___) or 20 µM sulphide

(----) were added to 20 µM cytochrome $a_{33}$ in 67 mM sodium

phosphate, 0.25% Tween-80, pH 7.4, at 30°C.

(B) Data from Figure 32A plotted semilogarithmically.

A biphasic uptake of oxygen is seen.
Fig. 32A.

\[ 20 \mu M \text{SH}^- \leftarrow \text{ASC} \quad \text{TMPD} \]

\[ 20 \mu M \text{AA}_3 \]

\[ 24 \mu M O_2 \quad \rightarrow \quad 2 \text{ min} \rightarrow \]

control
Fig. 32B.
Figure 33. Titration of oxygen with sulphide in the presence of cytochrome \( \text{aa}_3 \)

(A) Polarographic tracing of oxygen uptake upon successive sulphide additions in the presence of the enzyme. 20 \( \mu \)M cytochrome \( \text{aa}_3 \) in 67 mM sodium phosphate, 0.25% Tween-80, pH 7.4, at 30° C, was titrated with successive additions of 4.8 \( \mu \)M Na\(_2\)S as indicated, in an oxygen-electrode chamber.

(B) Data obtained in Figure 33A plotted as incremental oxygen uptake against cumulative concentration of added sulphide.
Fig. 33B.
Figure 34. Activation and inhibition of sulphide-induced oxygen uptake in the presence of cytochrome \textit{aa}_3.

(A) Absence of N-ethylmaleimide effect upon the slow phase of oxygen uptake induced by sulphide. Medium was 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80, at 30°C.

(B) Reactivity of NEM-treated cytochrome \textit{aa}_3 towards oxygen in the presence of sulphide. Oxygen uptake was initiated by addition of 20 \textmu M Na$_2$S to the incubated \textit{aa}_3-NEM mixture in 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80, 30°C. Dithiothreitol and EDTA additions were made as indicated.

(C) Effects of catalase and KCN on the slow oxygen uptake phase induced by sulphide. Medium was 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80, 30°C. Successive additions of sulphide, followed by catalase, cyanide and ascorbate plus TMPD were made as indicated.
Fig. 34A.
Fig. 34B.

- 20 μM H₂S⁻
- 20 μM As³⁻ + 20 μM NEM
- Incubated 24 h, 4°C
- 1 mM Dithiothreitol
- ASC. TMPD
- 1 mM EDTA
- 50 μM O₂ → 2 min →
Fig. 34C.
Chapter V
Discussion

(A) Spectroscopic Effects

Table 3 lists the five species of cytochrome c oxidase formed by reaction with sulphide.

(i) The Aerobic Ferric Sulphide Complex \((a^3+a_3^3-H_2S)\)

This species was recognized by Keilin and Hartree (1938) and recently studied by Nicholls and co-workers (Nicholls, 1975; Nicholls et al., 1976; Nicholls and Hildebrandt, 1978). Its spectrum is given in Figures 19A and 19B, and shows a 420-nm peak in the Soret region and a 600-nm peak in the visible region. It gives e.p.r. signals at \(g = 3.0, 2.77, 2.0, 1.71\). The \(g = 3.0\) signal corresponds to the low-spin state of ferric haem \(a\), and \(g = 2.0\) signal is due to one of the two cupric atoms (detectable copper, \(Cu^{2+}\)). Extra signals which occur at \(g = 2.77, 2.23\) and \(1.71\) may be due to the presence of half-reduced enzyme (Shaw et al., 1978). The \(g = 2.77\) signal is due to a transient form of the enzyme. Under fully oxidizing conditions, in the presence of excess sulphide, the \(a_3\) centre is probably not e.p.r. detectable.

(ii) The Aerobic Mixed-Valence Sulphide Complex \((a^{2+}a_3^3+H_2S)\)

This species is produced from the preceding form (excess sulphide) by reduction (ascorbate, TMPD) under aerobic conditions (Nicholls, 1975; Nicholls et al., 1976; Nicholls and Hildebrandt, 1978). Figure 20A shows
### Table 3. General Classification of Species Formed in Cytochrome aa₃-Sulphide Interaction

<table>
<thead>
<tr>
<th>No.</th>
<th>Compound</th>
<th>Conditions for formation</th>
<th>Spectrum (nm)</th>
<th>Properties</th>
<th>e.p.r. spectrum (g values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$a_{3}^+a_{3}^+\cdot H_2S$</td>
<td>aerobic excess sulphide</td>
<td>420, 600&lt;sup&gt;(a)&lt;/sup&gt;</td>
<td>stable&lt;sup&gt;(a)&lt;/sup&gt;</td>
<td>2.77 (transient form), 2.23&lt;sup&gt;(c)&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$t_{1/2} \sim 1 \text{ min}$</td>
<td>2.0, 1.7 and 3.0</td>
</tr>
<tr>
<td>2</td>
<td>$a_{2}^+a_{3}^+\cdot H_2S$</td>
<td>aerobic excess sulphide ascorbate, TMPD</td>
<td>444, 603&lt;sup&gt;(a)&lt;/sup&gt;</td>
<td>stable&lt;sup&gt;(a)&lt;/sup&gt;</td>
<td>2.54, 2.23, 1.87&lt;sup&gt;(c)(d)&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$t_{1/2} \sim 60 \text{ min}$</td>
<td>2.57–2.54, 2.26–2.22, 1.9–1.8&lt;sup&gt;(e)&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>$a_{3}^+a_{3}^+(oxy)$</td>
<td>aerobic low conc. sulphide</td>
<td>433, 578&lt;sup&gt;(b)&lt;/sup&gt;</td>
<td>$t_{1/2} \sim 10 \text{ min}&lt;sup&gt;(b)&lt;/sup&gt;$</td>
<td>3.0, 2.54, 2.23, 2.0, 1.87&lt;sup&gt;(c)&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>decays to resting enzyme</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>$a_{3}^+a_{3}^+\cdot O_2$ (Compound C)</td>
<td>aerobic low conc. sulphide</td>
<td>436, 607&lt;sup&gt;(b)&lt;/sup&gt;</td>
<td>$t_{1/2} \sim 1 \text{ min}&lt;sup&gt;(b)&lt;/sup&gt;$</td>
<td>low-spin heme signal?&lt;sup&gt;(d)&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>decays to Compound C</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>$a_{3}^+a_{3}^+(H_2S)$</td>
<td>anaerobic low conc. sulphide</td>
<td>438, 587&lt;sup&gt;(b)&lt;/sup&gt;</td>
<td>stable?&lt;sup&gt;(b)&lt;/sup&gt;</td>
<td>not know</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$t_{1/2} \sim 30 \text{ min}$ reacts with O₂?</td>
<td></td>
</tr>
</tbody>
</table>

(a) Nicholls (1975)


(c) Shaw et al. (1978)

(d) Wever et al. (1975)

(e) Wilson et al. (1976)
its absorption peaks at 444 nm and 603 nm. These may be compared with those of fully reduced cytochrome c oxidase (Figure 19A). Thus, the spectrum of the cytochrome $a^2+3^+\cdot H_2S$ species, mixed-valence inhibited enzyme, shows a shift toward the blue region at both peaks. This species contains e.p.r.-detectable low-spin ferric haem presumably the $a_3$-sulphide centre, as shown by a $g = 2.54$ signal in the e.p.r. spectrum (Wever et al., 1975; Shaw et al., 1978).

(iii) Oxyferri (low spin) Ferric Enzyme ($a^{3+}\cdot a_3^+$ oxy) and Compound C Species

$a^{3+}\cdot a_3^+$ (oxyferri) (Nicholls and Chanady, 1981) which shows a peak at 578 nm in the visible region (Orii and King, 1972) is the form of oxidized enzyme produced by addition of stoichiometric levels of sulphide under aerobic conditions (Figures 23, 24).

$a^{3+}\cdot a_3^+\cdot O_2$ (Compound C) which has an $\alpha$-peak at 607 nm in the visible region (Chance et al., 1975a, b), is the probable precursor of the low-spin "oxyferri" form (Figs. 13, 22 and 23). When cytochrome c oxidase is reacted with low levels of sulphide, this 606-607 nm species is produced under aerobic conditions. This compound decays rapidly to give the oxyferri species and finally the low-spin sulphide complex (Fig. 23). The formation and decay of this compound seem to occur concurrently.
(iv) The Anaerobic Ferric Sulphide Complex (a^3+_{a_3}^3-(H_2S)_{anaer})

This species is formed upon addition of a stoichiometric amount of sulphide to cytochrome a_{a3} under anaerobic conditions. It may be postulated to exist in an equilibrium with a partially reduced form (a^3+_{a_3}^2+-SH^*). When oxygen is introduced into the system, the a^3+_{a_3}^+--SH^* species would be responsible for rapidly forming Compound C (cf. Fig. 22).

(B) Oxidation of Sulphide by Cytochrome c and Cytochrome c Oxidase

\[
\text{HS}^- \xrightarrow{e^-} \text{HS}^* \xrightarrow{2e^-} \text{S}^0 \rightarrow \text{S}_8
\]

Eq. (20)

Two pathways for oxidation of H_2S exist (Eq. (20)): one produces S^0 (elemental sulphur) in a two electron process, and the other one produces HS^* (hydrosulphide radical) as a product.

(i) One Electron Processes (HS^* generating):

One electron processes occur when Fe(CN)_6^{3+}, cytochrome c or cytochrome a react with sulphide (see Results B(ii)). The spectroscopic titration of ferricyanide with sulphide under aerobic conditions shows reduction of one equivalent of Fe(CN)_6^{3-} by one mole of sulphide (Fig. 27A). Under anaerobic conditions, the titration gives rise to a Fe(CN)_6^{2-}: sulphide ratio close to 2:1 (Fig. 31A). The initial one-electron processes (producing SH^*) is followed aerobically by oxygen uptake, according to Equation (21):
\[
1 \text{H}_2\text{S} + 1 \text{Fe(CN)}_6^{3-} + \frac{1}{2} \text{O}_2 \rightarrow [\text{S}] + \text{Fe(CN)}_6^{4-} + \frac{1}{2} \text{H}_2\text{O}_2 + \text{H}^+
\]

Eq. (21)

Titration of cytochrome c with sulphide shows a ratio between sulphide and cytochrome c of 1:2 (Fig. 28A), which can be expressed by the following equation:

\[
\text{H}_2\text{S} + 2 \text{C}^{3+} \rightarrow \text{S}^0 + 2 \text{C}^{2+} + 2\text{H}^+
\]

Eq. (22)

Evidently cytochrome c reacts more rapidly with HS• than does ferricyanide. The sigmoidal reduction of cytochrome c\(^{3+}\) (Fig. 28A) in these systems indicates the production of polysulphide (cf. Massey et al., 1971). This phenomenon will be discussed further in the next section.

Cytochrome c oxidase can also be involved in a one-electron process, when its cyanide complex is reduced to the mixed valence form (Fig. 21).

(ii) Chemistry of SH• and S0

Table 4 summarizes the reaction patterns shown by sulphide interacting with Fe(CN)\(_6^{3-}\), cytochrome c and cytochrome aa\(_3\).
Table 4. Products and Stoichiometry of the Reaction of Sulphide with Ferricyanide, Cytochrome $aa_3$ and Cytochrome $c$

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Sulphur-containing species produced</th>
<th>Stoichiometry of sulphide-oxidant:O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphide + Fe(CN)$_6^{3-}$</td>
<td>SH$^\cdot$, (S$^0$?)</td>
<td>1:1:0.5</td>
</tr>
<tr>
<td>Sulphide + Cytochrome $c$</td>
<td>SH$^\cdot$, S$^0$, polysulphide</td>
<td>1:1.9:0.05</td>
</tr>
<tr>
<td>Sulphide + Cytochrome $aa_3$</td>
<td>S$^0$, (SH$^\cdot$?)</td>
<td>1:1:2</td>
</tr>
</tbody>
</table>
Ferricyanide seems to react rather poorly with the SH• radical species (Figs. 27A and 29A). The first equivalent of sulphide is used for ferricyanide reduction aerobically, the second being transferred to oxygen. Thus, the following equations may be deduced:

\[ \text{Fe(CN)}_{\text{III}} + \text{SH}^- \rightarrow \text{Fe(CN)}_{\text{II}}^- + \text{SH}• \quad \text{Eq. (23A)} \]

\[ 2\text{SH}• + \text{O}_2 \rightarrow 2\text{S}^0 + (\text{H}_2\text{O}_2)? \quad \text{Eq. (23B)} \]

The reduction of cytochrome c gives rise to some SH• radicals or related species which can result in oxygen uptake (Figs. 28A and 30A). The stoichiometry observed in these experiments indicates that the majority of radicals formed in a reaction of sulphide with cytochrome c react with a second molecule of cytochrome c to give the overall process of Equation (22), only a small proportion (5%) escaping to induce oxygen uptake. Thus, these reactions may be expressed as follows:

\[ \text{H}_2\text{S} + \text{c}^{3+} \rightarrow \text{c}^{2+} + \text{SH}• + \text{H}^+ \quad \text{Eq. (24)} \]

\[ \text{SH}• + \text{O}_2 \rightarrow \text{S}^0 + (\text{H}_2\text{O}_2)? \quad \text{Eq. (25)} \]

\[ 8\text{S}^0 \rightarrow \text{S}_8 \quad \text{Eq. (26)} \]

The reaction between sulphide and cytochrome c oxidase is rather a complexity. Sulphide binds to the cytochrome a\text{3+} binding site (Nicholls, 1975). In order to obtain the half-reduced inhibited species, a^{2+}a^{3+}-\text{H}_2\text{S}, it requires about 3 moles of sulphide per mole of enzyme (Fig. 16A). Thus, the following equations may be written:
\[ \text{a}^{3+}\text{a}^{3+} + \text{H}_2\text{S} + \text{O}_2 \rightarrow \text{a}^{3+}\text{a}^{3+}(\text{oxy}) + \text{S}^0 + (\text{H}_2\text{O}_2)? \] Eq. (27)

\[ \text{a}^{3+}\text{a}^{3+}(\text{oxy}) + \text{H}_2\text{S} \rightarrow \text{a}^{2+}\text{a}^{3+} + \text{S}^0 \] Eq. (28)

\[ \text{a}^{2+}\text{a}^{3+} + \text{H}_2\text{S} \rightarrow \text{a}^{2+}\text{a}^{3+}-\text{H}_2\text{S} \] Eq. (29)

(iii) Two-Electron Processes (S⁰-producing)

When cytochrome c oxidase is titrated with progressive amounts of sulphide polarographically, oxygen uptake occurs. Initially, about 2 moles of oxygen are taken up per mole of sulphide added. Then a second slow phase appears in which 1 mole of oxygen is taken up per mole of sulphide added (Fig. 33A). The nature of this reaction suggests that one type of cytochrome c oxidase-sulphide interaction is a two-electron process.

Chance and co-workers previously proposed the following pathway (Chance et al., 1975; Chance and Leigh, 1977)

\[ \begin{align*}
\text{Cu}^{2+}\text{Cu}^+ & \quad \text{O}_2 \quad \text{Cu}^{2+}\text{Cu}^+ \\
\text{Fe}^{3+}\text{Fe}^{2+} & \quad \rightarrow \quad \text{Fe}^{3+}\text{Fe}^{2+}\cdot\text{O}_2 \quad \rightarrow \quad \text{Fe}^{3+}\text{Fe}^{2+}\cdot\text{O}_2^- \\
& \quad (\text{Compound A}) \quad (\text{Compound C})
\end{align*} \] Eq. (30)

This mechanism requires an initial two-electron reduction of the enzyme. Since both the mixed-valence CO compound and the 607 nm species (Compound C) can be produced by bubbling CO gas into the oxidized resting cytochrome c oxidase (Nicholls and Chanady, 1981), and the 607 nm species can also be produced when the enzyme interacts with low concentrations of sulphide, thus the following pathway can be proposed:
This proposed pathway involves a two-electron process. Thus, sulphide can produce reduced cytochrome $a_3$ without the reduction of the cytochrome $a$.

In order to measure the sulphide concentration, the sulphide solution was titrated with 0.1 N standard iodine ($I_2$) solution (see Methods (viii)a and Eq. 16). Equation 16 shows an example of a chemically simple two-electron process. $S^0$ (elemental sulphur) is generated as a product.

(C) Inhibition by Sulphide

Table 5 summarizes the kinetic values obtained in this study and compares them with previously reported values. In addition to the numbers collected here, the results have shown that a further important parameter not previously recognized is the number of sulphide molecules needed to inhibit one mole of cytochrome $aa_3$.
Table 5. Inhibition Constants for the Reaction Between Sulphide and Cytochrome c Oxidase

<table>
<thead>
<tr>
<th>No.</th>
<th>Enzyme</th>
<th>Conditions</th>
<th>$k_{on}$</th>
<th>$k_{off}$</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(M$^{-1}$s$^{-1}$)</td>
<td>(s$^{-1}$)</td>
<td>(µM)</td>
</tr>
<tr>
<td>1</td>
<td>cytochrome $aa_3$</td>
<td>cytochrome c steady state</td>
<td>$7.5 \times 10^3$ (a)</td>
<td>$5.5 \times 10^{-4}$ (b)</td>
<td>0.1-3.0 (e)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ascorbate</td>
<td>$1.5 \times 10^4$ (c)</td>
<td>$6.0 \times 10^{-4}$ (d)</td>
<td>0.24 (d)</td>
</tr>
<tr>
<td>2</td>
<td>cytochrome $aa_3$</td>
<td>O$_2$ kinetics</td>
<td>--</td>
<td>--</td>
<td>0.2 (f)</td>
</tr>
<tr>
<td>3</td>
<td>submitochondrial particles</td>
<td>ascorbate</td>
<td>$10^4$ (e)</td>
<td>--</td>
<td>0.1 (e)</td>
</tr>
</tbody>
</table>

(a) This thesis (see Methods (i))
(b) Dilution method (see Results B(i))
(c) Nicholls, 1975
(d) Nicholls, 1975: rate of reduction by Na$_2$S$_2$O$_4$
(e) This thesis (see Results A(i))
(f) Petersen, 1977
(i) Stoichiometry of the Cytochrome c Oxidase-Sulphide Reaction

Stoichiometric H₂S concentrations do not produce the sulphide complex formed by excess inhibitor (see Results B(i)). About 3 moles of sulphide are required to secure maximum spectroscopic change under catalytic conditions (Figs. 12A and 16A). This phenomenon can be explained by the following Equation (32):

\[
\begin{align*}
\text{a}^2+\text{a}^3+ & \xrightarrow{\text{H}_2\text{S}} \text{a}^2+\text{a}^3+ \xrightarrow{\text{O}_2} \text{a}^2+\text{a}^3+ - \text{H}_2\text{S} \\
(\text{ASC, TMPD}) & \xrightarrow{\text{H}_2\text{S}} \text{a}^3+\text{a}^3+(\text{oxy}) \xrightarrow{\text{H}_2\text{S}} \text{a}^3+\text{a}^3+ - \text{H}_2\text{S} \\
& \xrightarrow{\text{H}_2\text{S}} \text{a}^2+\text{a}^3+(\text{oxy}) \xrightarrow{\text{H}_2\text{S}} \text{a}^2+\text{a}^3+ - \text{H}_2\text{S} \\
& \text{Eq. (32)}
\end{align*}
\]

In order to obtain the $\text{a}^2+\text{a}^3+ - \text{H}_2\text{S}$ species, two moles of sulphide are needed per mole of cytochrome c oxidase (Eq. (32a)). There are two further pathways to produce enzyme-H₂S complexes (Eq. (32b,c)). One pathway requires one mole of H₂S plus reducing equivalent (TMPD)(32c) and the other pathway (32b) needs two moles of sulphide plus one mole of oxygen per mole of cytochrome $\text{aa}_3$ (Eq. (32b)). For the $\text{a}^2+\text{a}^3+ - \text{H}_2\text{S}$ species (Eq. (32c)), one mole of sulphide could be sufficient to transform one mole of cytochrome c oxidase through pathway Equation (32c), provided that ascorbate plus TMPD generates the sulphide-sensitive form.

Shaw et al. (1978) reported that the conversion of their transient high spin species by sulphide is near stoichiometric.
(ii) Rates of Inhibition and Release of Inhibitor at pH 7.4 and 30°C

Under catalytic conditions, the apparent association rate constant of sulphide and enzyme is close to $10^6$ M$^{-1}$ s$^{-1}$ (Table 5). As the direct spectroscopic titration of enzyme with sulphide gives essentially the same rate, the initial interaction between sulphide and cytochrome $c$ oxidase is probably the same in the two cases. Nevertheless, an equimolar mixture of cytochrome $c$ oxidase and sulphide shows enzymatic activity identical to that of the native enzyme (Fig. 18). It may be concluded that the initial product of sulphide-cytochrome $c$ oxidase interaction can give rise either to uninhibited, or to inhibited forms of the enzyme (Eq. 32). At a lower pH (6.65), the second order rate constant is about three times higher than that at pH 7.4 (see Results A(i)). According to Equation 2, these will be about 2.5 times the proportion of undissociated H$_2$S present in a solution at pH 6.65 compared to a solution at pH 7.4. It is concluded that undissociated H$_2$S is the probable inhibitory species. The other rate constant ($k_{off}$) of between $5.5 \times 10^{-4}$ s$^{-1}$ and $1.1 \times 10^{-3}$ s$^{-1}$ (Table 6) shows that the dissociation rate is rather slow.

(iii) $K_i$ Values for Sulphide Inhibition

The observed $K_i$ values are listed in Table 5. Interaction between enzyme and sulphide produces a relatively tight enzyme-inhibitor complex, $a^{2+}b^{3+}$-H$_2$S. The $K_i$ value is probably independent of cytochrome $c$ concentration, that is, the reaction is non-competitive towards cytochrome $c$. Towards oxygen, H$_2$S also behaves non-competitively (Petersen, 1977) like cyanide. This may be a reflection of mechanistic similarities between these two inhibitors.
(D) Proposed Reaction Scheme

Figure 35 summarized the proposed reactions. Sulphide produces two species, \( a^3+a_3^+{(H_2S)}_{anaer} \) and \( a^2+a_3^+ \), from resting cytochrome \( c \) oxidase. The \( a^2+a_3^+ \) species can produce the half-reduced, fully inhibited species, \( a^2+a_3^+{-H_2S} \), with excess sulphide. The "\( a^3+a_3^+(SH^\cdot)\)" species is a hypothetical intermediate formed from \( a^3+a_3^+{(H_2S)}_{anaer} \), postulated to account for the oxygen sensitivity of this species with which the proposed \( a^3+a_3^+(SH^\cdot) \) species may be in a pseudo-equilibrium. The 607 nm species, a probable half-reduced oxygenated compound (Compound C), is formed from \( a^3+a_3^+(SH^\cdot) \); subsequently a low-spin oxyferri form of the enzyme appears. With excess sulphide, \( H_2S \) may bind to \( a^3+a_3^+(oxyferri) \) to produce \( a^3+a_3^+{-H_2S}^{(oxy)} \) which is an inhibited but stable form of the enzyme and probably a low-spin species. Thus, two modes of \( H_2S \) reaction with cytochrome \( c \) oxidase are proposed: First an inhibitory mode requiring initial reduction of cytochrome \( a \), which gives the two fully inhibited species, \( a^2+a_3^+{-H_2S} \) and \( a^3+a_3^+{-H_2S} \). The second is the substrate mode, starting with resting ferric enzyme, in which partial reduction, reaction with oxygen, the dissociation of sulphur, and a return to an active enzyme species, \( a^3+a_3^+(oxyferri) \), occur in that order.
Figure 35. Proposed reaction scheme.

(See Discussion section for details.)
Fig. 35.
Chapter VI
Appendices
A. Smith-Conrad Assays of Cytochrome $\text{aa}_3$, $\text{aa}_3$-$\text{CN}^-$ and Sulphide-Treated $\text{aa}_3$ (Table 6)

In this series of experiments, the activity of the sulphide-treated enzyme was compared with that of the $\text{aa}_3$-$\text{CN}^-$ and the native enzyme. The initial activity was much higher than that of $\text{aa}_3$-$\text{CN}$ complex. The experimental procedure was as follows: 50 nmoles of cytochrome $\text{aa}_3$ was incubated with 150 nmoles of the inhibitors, cyanide and sulphide, in 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80, for 98 hours. After incubation, the excess inhibitor was removed by gel-filtration through Sephadex G-100 columns (20 x 1.6 cm) eluting with 67 mM sodium phosphate, pH 7.4, 0.25% Tween-80. The elution time was about a half hour. Then the enzymatic activities were measured according to the Smith-Conrad method (see Methods B. (v).a). All experiments were carried out at 4°C, except the activity test.
Table 6. The results of Smith-Conrad assays of cytochrome $\text{aa}_3$, $\text{aa}_3\text{-CN}^-$ and sulphide-treated cytochrome $\text{aa}_3$.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>$+ \text{CN}^-$</th>
<th>$+ \text{SH}^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of $\text{aa}_3$</td>
<td>50 nmoles</td>
<td>50 nmoles (+ 150 nmoles $\text{CN}^-$)</td>
<td>50 nmoles (+ 150 nmoles $\text{SH}^-$)</td>
</tr>
<tr>
<td>placed on column</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield (%)</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Spectrum of final species</td>
<td>$\text{aa}_3$</td>
<td>low-spin $\text{aa}_3$</td>
<td>low-spin $\text{aa}_3$</td>
</tr>
<tr>
<td>Initial activity (s$^{-1}$)</td>
<td>200</td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td>Final activity (s$^{-1}$)</td>
<td>100</td>
<td>15</td>
<td>60</td>
</tr>
</tbody>
</table>
(B) Anaerobic Reduction of Cytochrome c Oxidase by Reduced Cytochrome c

Under anaerobic conditions, the titration of oxidized cytochrome c oxidase with reduced cytochrome c causes the reduction of cytochrome a rapidly and the remaining centers more slowly (Fig. 37). Experimental conditions were as follows: Thunberg cuvettes containing resting ferric cytochrome c oxidase (5.35 μM) in the main vessel and various amounts (5.35, 10.7, 15.8, 20.9 μM) of the reduced cytochrome c solution (see Methods (ii)) in the side arm, were made anaerobic by repeated evacuation and flushing with nitrogen gas. The reduced cytochrome c solution was tipped into the main vessel and mixed thoroughly. Then the initial spectra were recorded. After the 1600-second-spectrum was obtained, air was introduced into the system. Then the aerobic spectrum of oxidized cytochrome c and aa3 was recorded. Finally, dithionite was added and the spectrum of the fully reduced species was obtained. Medium was 67 mM sodium phosphate, pH 7.4, at 30°C (Fig. 36). The results of several experiments are summarized in Figure 37.
<table>
<thead>
<tr>
<th>C:aa</th>
<th>Initial mix</th>
<th>Start</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.16 c2+</td>
<td>5.35 c2+ 5.35 a3</td>
<td>0.21 c2+ 1.1 a2+</td>
</tr>
<tr>
<td></td>
<td>1.2 c2+</td>
<td>5.25 c2+ 5.25 a3</td>
<td>1.57 c2+ 2.1 a2+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.7 c2+ 10.7 a3</td>
<td>2.3 c2+ 2.5 a2+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15.8 c2+ 15.8 a3</td>
<td>2.9 c2+ 2.9 a2+</td>
</tr>
<tr>
<td>1:1</td>
<td>2:1</td>
<td>3:1</td>
<td>4:1</td>
</tr>
<tr>
<td>5.58 c2+ 5.58 a2+</td>
<td>9.9 c2+ 9.9 a2+</td>
<td>2.76 c2+ 2.76 a2+</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 37.
(C) Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

The subunits of cytochrome c oxidase were separated by SDS-PAGE (see Methods (viii)). The acrylamide concentration was 10% and 0.1% SDS, and 8 M urea were added to the system. 30 μg of protein (cytochrome c oxidase) was applied to each gel column. Then, electrophoresis, staining and destaining processes were carried out in turn (see Methods (vii) for details). The gels were analyzed by a densitometer attached to the Gilford-2400 spectrophotometer, monitored at 550 nm. Beef heart cytochrome c oxidase has been resolved into seven subunits by acrylamide gel electrophoresis in highly cross-linked gels containing urea and sodium dodecyl sulphate (Downer et al., 1976; Chan and Tracy, 1978). It has been also reported to contain 8 subunits (Bucher and Penniall, 1975). In order to settle this discrepancy, Penttila et al. (1979) repeated the experiments with highly purified ox cytochrome c oxidase, and concluded that the enzyme contains six subunits. Recently, cytochrome c oxidase from rat liver was separated into up to 12 different protein subunits by the SDS-PAGE system (Merle and Kadenbach, 1980). As shown in Figure 38, the present preparation of beef heart cytochrome c oxidase contains up to nine subunits.
Chapter VII

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