

MECHANISMS of CARBON DIOXIDE FIXATION

and IRON OXIDATION

by Ferrobacillus ferrooxidans

by

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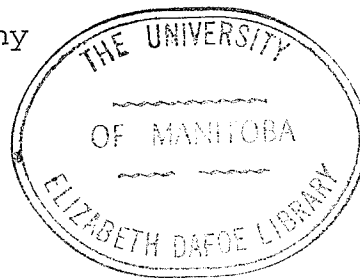
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ABSTRACT

Carbon dioxide fixation was studied in intact cells and cell-free extracts of Ferrobacillus ferrooxidans. The major pathways of fixation were found to be the carboxy-dismutase system and the phosphoenolpyruvate carboxylase system. Phosphoenolpyruvate carboxylase activity was shown to be under metabolic regulation, similar to the regulation established in heterotrophic microorganisms.

Acetyl coenzyme A stimulated phosphoenolpyruvate carboxylase activity, while the presence of aspartate was inhibitory. The Ferrobacillus ferrooxidans enzyme appeared to have a neutral or acidic pH optimum, in contrast to the same enzyme isolated from heterotrophs.

Although intact cells were able to oxidize ferrous iron with oxygen, crude sonicates and cell-free extracts lacked oxidase activity. The iron oxidation system involved the cytochrome system, namely cyt. c and an a-type cytochrome. The initial enzyme of the oxidation system was found to be ferrous iron-cytochrome c reductase, which linked the ferrous iron to the cell electron transport chain.

The oxidase system was extremely labile although the individual enzymes retained their activity after the system was destroyed. The inability to isolate cell-free extracts capable of oxidizing ferrous iron manometrically was found to be due to the disruption of the cytochrome chain alienating cytochrome c from the a-type cytochrome, thus terminating electron transport at cyt. c rather than at oxygen.

Ferrous iron-cytochrome c reductase was purified 60-70 fold from cell-free extracts. The enzyme was judged to be approximately 90% pure by disc electrophoresis, sedimentation, and DEAE-cellulose column chromatography. RNA, whose presence was obligatory for catalysis, was closely associated with the enzyme. The enzyme was dissociated into two subunits, one a protein devoid of catalytic activity and the other an RNA subunit. Molecular weight analyses of the holoenzyme and its protein and RNA subunits revealed weights of 100 - 110,000, 27-30,000, and 315 - 330,000 respectively.

Detailed kinetic analyses of the enzyme revealed the reaction mechanism to be Ping Pong Bi Bi. The K_m values for the substrates were found to be $K_{Fe^{++}} = 0.59$ mM and

$K_{\text{cyt. c}} = 0.085 \text{ mM}$. The presence of non-heme iron on the enzyme was implicated as the responsible factor for the oscillation of the enzyme between its two forms.

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ABBREVIATIONS

aceto-acetyl CoA	- aceto-acetyl coenzyme A
acetyl CoA	- acetyl coenzyme A
ADP	- adenosine 5'-diphosphate
AMP	- adenosine 5'-monophosphate
3'-AMP	- adenosine 3'-monophosphate
ATP	- adenosine 5'-triphosphate
C ₁	- one carbon
C ₃	- three carbon
C ₄	- four carbon
cyt. <u>a</u>	- cytochrome <u>a</u>
cyt. <u>c</u>	- cytochrome <u>c</u>
DEAE-cellulose	- diethylaminoethyl-cellulose
DNA	- deoxyribonucleic acid
DNase	- deoxyribonuclease
EDTA	- ethylenediamine tetracetic acid
E-4-P	- erythrose 4-phosphate
FAD	- flavin adenine dinucleotide
FDP	- fructose 1,6-diphosphate
FMN	- flavin mononucleotide
F-6-P	- fructose 6-phosphate

GA-3P	- glyceraldehyde 3-phosphate
GSH	- glutathione (reduced)
IDP	- inosine 5'-diphosphate
ITP	- inosine 5'-triphosphate
NAD	- nicotinamide adenine dinucleotide
NADP, NADP ⁺	- nicotinamide adenine dinucleotide phosphate
NADPH	- reduced nicotinamide adenine dinucleotide phosphate
PEP	- phosphoenolpyruvic acid
PGA, 3-PGA	- 3-phosphoglyceric acid
1,3-DPGA	- 1,3-diphosphoglyceric acid
RNA	- ribonucleic acid
RNAse	- ribonuclease
R-5-P	- ribose 5-phosphate
RuDP	- ribulose 1,5-diphosphate
Ru-5-P	- ribulose 5-phosphate
SDP	- sedoheptulose 1,7-diphosphate
S-7-P	- sedoheptulose 7-phosphate
TCA	- trichloroacetic acid
Tris	- tris(hydroxymethyl)aminoethane
X-5-P	- xylulose 5-phosphate

INTRODUCTION

Although the metabolic mechanisms and general physiology of autotrophic organisms are well established, virtually nothing is known of the autotrophic iron-oxidizers. This lack of knowledge is due partly to the belated isolation of these organisms (Temple and Colmer, 1951; Leathen, Kinsel, and Braley, 1956; Kinsel, 1960), and partly to the inability of researchers to obtain sufficient quantities of the cells for detailed enzyme studies. Research in this field was limited to physiological studies with intact cells until the recent development of Silverman and Lundgren (1959a;b), who were able to obtain sufficiently large yields of cells required for cell-free enzyme studies.

The major pathways of carbon dioxide fixation in autotrophic microorganisms have already been established, but this aspect has not been extensively studied in the iron-oxidizers. The recent work of Maciag and Lundgren (1964), using intact cells of Ferrobacillus ferrooxidans, and Gale and Beck (1966), using cell-free extracts of Thiobacillus ferrooxidans, indicated the presence of carboxydismutase and

the possible existence of an enzyme catalysing C_3 and C_1 condensation (Wood-Werkman). The first problem arising from these findings was to establish definitely the major pathways of CO_2 fixation in Ferrobacillus ferrooxidans.

Both intact cells and cell-free extracts were used to determine the major pathways of carbon dioxide fixation. Amarsingham (1959) demonstrated that the major pathway of C_4 synthesis feeding the tricarboxylic acid cycle in the Enterobacteriaceae was phosphoenolpyruvate carboxylase. More recent research (Cánovas and Kornberg, 1965; Sanwal and Maeba, 1965; 1966) indicated that this enzyme is under the control of intermediary metabolites.

Autotrophs utilize carbon dioxide exclusively as their sole carbon source. Since carboxydismutase is found solely in autotrophic organisms, it was decided that a study of the "heterotrophic" carbon dioxide fixing enzyme, phosphoenolpyruvate carboxylase, would be carried out so that comparisons could be made with the findings established in the Enterobacteriaceae.

The existing knowledge concerning the mechanism of iron oxidation in the chemoautotrophs is scant. Vernon et al. (1960) proposed a scheme of electron transport during

iron oxidation by Thiobacillus ferrooxidans which included the presence of both cytochrome c and cytochrome a. These workers proposed that the electrons originating at ferrous iron were transferred via cytochrome c and cytochrome a to oxygen. In 1964, Blaylock and Nason suggested the same scheme for Ferrobacillus ferrooxidans and in addition isolated a new enzyme, ferrous iron-cytochrome c reductase.

A study of the electron transport chain was undertaken to understand further the mechanism of iron oxidation and to attempt to elucidate the enzymes involved. To aid in the explanation of iron oxidation, ferrous iron-cytochrome c reductase was isolated and purified. The results of Blaylock and Nason (1964) and the initial results of this study indicated that a detailed kinetic analysis was required to explain the unusual properties of the enzyme and the reaction mechanism utilized in vivo.

Because the research encompassed a variety of subjects, this thesis has been divided into three sections: carbon dioxide fixation, iron oxidation, and a study of the initial enzyme of the iron oxidation scheme (ferrous iron-cytochrome c reductase).

HISTORICAL

HISTORICAL

In 1887 Winogradsky established the fundamental principles of autotrophy with his classic treatise on the sulphur bacteria. His original concept was that autotrophic bacteria adhered to two basic principles: (1) the only carbon source used for cell synthesis was CO_2 ; and (2) the sole source of energy was of an inorganic nature. In later work he discovered other bacteria capable of oxidizing ferrous iron (1888) and ammonia (1891). Although subsequent research revealed that his original work was carried out using the heterotrophic Beggiatoa, the original work can be justly called classic.

Winogradsky's original concept of autotrophy led to a belief that only the autotrophs were capable of fixing CO_2 . It was not until 1935 (Wood and Werkman) that this concept was challenged. Present knowledge reveals that all organisms fix CO_2 but that autotrophs are the only organisms capable of using CO_2 for all their carbon requirements. In 1954 autotrophic bacteria were re-defined into two classes: the chemolithotrophs and the photolithotrophs. The former

organisms gain their energy by the oxidation of inorganic compounds and the latter use light as an energy source; both classes of organisms use CO_2 as their sole source of carbon.

There is some controversy as to the rigidity of definition of inorganic energy source (Elsden, 1962; Bhat and Barker, 1948). Are carbon monoxide, methylamine, methanol, methane, and formate organic or can they be classed as inorganic? All of these compounds can supply certain bacteria with all of the energy required for growth, with CO_2 as the sole carbon source (Elsden, 1962). At the present time this controversy has not been settled and the definition of a chemolithotroph needs to be clarified.

The concept that growth of obligate autotrophs is inhibited by organic compounds arose with Winogradsky's original findings (1887). More recent studies show that although organic compounds alone cannot support the growth of chemoautotrophs, organic compounds can be metabolized into cell constituents. Waksman and Starky (1922; 1923) found that glucose increased the rate of sulfur oxidation in Thiobacillus thiooxidans and that the glucose slowly disappeared from the medium. More recently, Suzuki (1958)

found that Thiobacillus thiooxidans metabolized radioactive glucose into amino acids, sugars, and polysaccharides. These findings shed some light on the fundamental differences between heterotrophs and chemoautotrophs.

The metabolism of glucose by autotrophs shows that some organic compounds may enter the cell and be metabolized, but that the metabolism of organic compounds may not provide sufficient energy for the growth of the autotroph. The growth of autotrophs may in some cases be stimulated by the presence of a utilizable organic compound (Waksman and Starky, 1922; 1923; Remsen and Lundgren, 1963; Butler and Umbreit, 1965; London and Rittenberg, 1965; Krulwich and Funk, 1965). Although some carbohydrates can enter obligate autotrophs, the idea that autotrophs have cell walls or membranes impermeable to organic compounds cannot be completely discarded. The inability of obligate autotrophs to grow on organic compounds could be due either to the slow permeability of organic compounds or to the inability of the organic compounds to generate sufficient energy for growth.

This discussion will deal with the chemoautotrophs that oxidize ferrous iron compounds for their energy. The organisms are classified into two genera: Thiobacillus and

Ferrobacillus. For discussions on the thiobacilli and iron-oxidizers the reviews of Van Niel (1954), Lees (1955; 1960), Vishniac and Santer (1957), and Mulder (1964) should be consulted. Since the mechanisms of CO₂ fixation in bacteria have been ably reviewed by Elsdon (1962), and Wood and Stjernholm (1962), these details will be only briefly outlined.

Chemoautotrophic iron oxidizing bacteria

Although Winogradsky first reported growth of a chemoautotroph on ferrous iron in 1888 it was not until 1949 that a pure culture of such a bacterium was isolated (Colmer, Temple, and Hinkle, 1950; Temple and Colmer, 1951). The first iron-oxidizer isolated was named Thiobacillus ferrooxidans because of its close relationship to Thiobacillus thiooxidans in acid mine water (Temple and Colmer, 1951). Leathen, Braley, and McIntyre (1953) also isolated an iron-oxidizer from acid mine water and named the bacterium Ferrobacillus ferrooxidans (Leathen, Kinsel, and Braley, 1956). These authors found that this bacterium oxidized ferrous iron only and would not utilize either sulfur or thiosulfate as oxidizable substrates.

The isolation of other iron-oxidizers from mine water and sulfide ores continued. In 1958 Beck and Elsdon isolated another bacterium that oxidized ferrous iron and sulfur; this bacterium was thought to be similar to the bacterium isolated by Temple and Colmer (1951). Kinsel (1960) also isolated a similar bacterium which she named Thiobacillus sulfooxidans. In 1961 Unz and Lundgren questioned the taxonomic validity of the genus Ferrobacillus. They found that all of the above bacteria were capable of utilizing Fe^{++} and sulfur or thiosulfate as oxidizable substrates, although the generation time of these bacteria varied with the substrate. They suggested that the genus Ferrobacillus be abolished and that Ferrobacillus ferrooxidans be identified as a strain of Thiobacillus ferrooxidans. This controversy as to taxonomic position still has not been settled.

Thiobacillus ferrooxidans (Temple and Colmer, 1951; Beck, 1960), Ferrobacillus ferrooxidans (Leathen, Kinsel, and Braley, 1956), and Ferrobacillus sulfooxidans (Kinsel, 1960) are capable of autotrophic growth using ferrous iron as their sole energy source. Thiobacillus ferrooxidans (Colmer, Temple, and Hinkle, 1950; Temple and Colmer, 1951; Beck, 1960; Colmer, 1961) can, as well, utilize sulfur or thiosulfate as

an alternative energy source. Ferrobacillus sulfooxidans (Kinsel, 1960) exhibits the same ability to utilize an alternative substrate, in this case sulfur. The utilization of thiosulfate as an alternative substrate has been questioned because in acid conditions thiosulfate rapidly decomposes and the bacterium could then oxidize the released sulfur, further lowering the pH and increasing thiosulfate decomposition.

Previously, it was believed that the chemosynthetic iron oxidizers were obligate autotrophs, but in this connection Schnaitman and Lundgren (1965a) and Remsen and Lundgren (1963) showed that protoplasts and mildly sonicated cells of Ferrobacillus ferrooxidans were capable of heterotrophic growth on glucose. Remsen and Lundgren (1963) were able to train the bacteria to grow on glucose and contended that Ferrobacillus ferrooxidans is a facultative bacterium rather than an obligate autotroph. Schnaitman and Lundgren (1965b) found that pyruvic acid was excreted into the spent medium by autotrophically grown cells of Ferrobacillus ferrooxidans. This work has not been substantiated by other workers and the significance of pyruvate excretion is not understood.

All of the iron oxidizers use ammonium salts as their sole source of nitrogen. Temple and Colmer (1951) found that nitrates can replace ammonium in the growth of Thiobacillus ferrooxidans. Bryner and Jamieson (1958) reported that $(\text{NH}_4)_2\text{SO}_4$, urea, and cysteine can all supply the necessary nitrogen for growth of a bacterium similar to Thiobacillus ferrooxidans. Remsen and Lundgren (1963) found that alanine, glutamic acid, and lysine were capable of substituting for $(\text{NH}_4)_2\text{SO}_4$ during growth of Ferrobacillus ferrooxidans.

Lazaroff (1963) found that the presence of sulfate is obligatory for iron oxidation in Thiobacillus ferrooxidans. Dugan and Lundgren (1965) obtained a similar result in Ferrobacillus ferrooxidans. High concentrations of phosphate appear to inhibit oxidation and growth in Thiobacillus ferrooxidans (Razzell and Trussell, 1963) and Ferrobacillus ferrooxidans (Silverman and Lundgren, 1959a;b). Razzell and Trussell (1963) also reported that iron oxidation in Thiobacillus ferrooxidans is inhibited by chloride and thiosulfate and they suggested that high concentrations of anions are inhibitory.

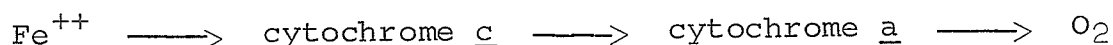
Mechanism of iron oxidation

Leathen, Braley, and McIntyre (1953), working with chemosynthetic autotrophs found in mine water, and Temple and Delchamps (1953), using Thiobacillus ferrooxidans also isolated from mine water, showed that iron is oxidized according to the following equation:



Silverman and Lundgren (1959b), using Ferrobacillus ferrooxidans, and Beck and Elsdon (1958), using a different isolate of Thiobacillus ferrooxidans, found the same stoichiometry.

Vernon, Mangum, Beck, and Shafia (1960) isolated cytochrome c and cytochrome a from Thiobacillus ferrooxidans and postulated that iron is oxidized through the cytochrome system with oxygen as the final electron acceptor.



In 1963, Blaylock and Nason proposed the same mechanism in Ferrobacillus ferrooxidans when they found that cell-free extracts contained cytochromes b, c, and a and an iron-

cytochrome c reductase. These authors also reported an iron oxidase system whose activity was measured by the decrease of ferrous iron concentration (colorimetrically not manometrically). Lundgren (1965) and Beck (1965) have not been able to find this iron oxidase activity, either colorimetrically or manometrically, in similar extracts of Ferrobacillus ferrooxidans or Thiobacillus ferrooxidans and the original report of Blaylock and Nason (1963) has not been further substantiated.

Blaylock and Nason (1963) advanced knowledge of the iron oxidation mechanism with their finding that the initial electron carrier of the system is cytochrome c. Their discovery of ferrous iron-cytochrome c reductase and cytochrome a (cytochrome c oxidase) has essentially substantiated the original hypothesis of Vernon, Mangum, Beck, and Shafia (1960) that the oxidation mechanism proceeds through the cytochrome chain with oxygen as the terminal electron acceptor.

Schnaitman and Lundgren (1965a) prepared protoplasts of Ferrobacillus ferrooxidans and showed that intact protoplasts were capable of oxidizing ferrous iron (manometrically) but lysed protoplasts lost almost all of their

oxidizing ability. This finding illustrates that the cell wall plays no part in the oxidation mechanism, but that the cytoplasmic membrane is the important structure in iron oxidation. Anderson and Lundgren (1965) manometrically detected iron oxidase activity in cell-free extracts of Ferrobacillus ferrooxidans but this finding has not been repeated (Lundgren, 1965).

In 1965, Dugan and Lundgren constructed a model to depict iron oxidation in intact cells of Ferrobacillus ferrooxidans. These authors envisaged ferrous iron complexing with oxygen (and phosphate ?) in the medium, thus oxidizing the ferrous iron to form a peroxide complex. They postulated that this complex then binds the cell membrane where iron oxygenase (or oxidase) would release an electron to a bound electron-deficient sulfate group (Lazaroff, 1963). Once the electron is released by the enzyme the complex splits and ferric iron is released from the membrane into the medium; the sulfate group then releases the electron into the electron transport chain (flavin or quinone). Alternatively, these authors suggest that the iron complex could also release its electron directly to the flavoprotein, thus bypassing sulfate. The oxygenase transferring the

electron from the complex to the cell membrane would split off one oxygen atom, which could act as the terminal electron acceptor, and the other atom would remain with the oxidized substrate (ferric iron). If the oxidase is the functional enzyme, then neither oxygen atom remains with the oxidized substrate and the ferric iron diffuses to react with water and sulfate in the external medium.

This model mechanism attempts to explain both the activation of the ferrous iron and the ability of the cell to transport rapidly the oxidized substrate so that the cell does not become clogged with excessive substrate. The electron transport chain involved does not differ substantially from the models of Vernon, Mangum, Beck, and Shafia (1960) or Blaylock and Nason (1963). The essential difference in this model is the requirement for oxygen in order for electron transfer to take place and the requirement for sulfate in the oxidation of the substrate.

Transfer of energy

Lees (1960, 1962) ably reviewed energy transfer and energetics in chemosynthetic systems. This author mentions the difficulty in explaining the ability of some

chemoautotrophs (most notably nitrite and ferrous iron oxidizers) to reduce pyridine nucleotides solely by the oxidation of their inorganic substrates. Iron oxidizers surmount a formidable energy gap ($E'_{\text{O}} \text{ Fe}^{++}/\text{Fe}^{+++} + 0.77 \text{ v}$, $E'_{\text{O}} \text{ red. cyt. c}/\text{ox. cyt. c} + 0.31 \text{ v}$, $E'_{\text{O}} \text{ NAD}/\text{NADH} - 0.32 \text{ v}$) that must be overcome in at least a two-step process (possibly five steps). The ferrous iron first reduces cyt. c (Vernon, Mangum, Beck, and Shafia, 1960) which lowers the redox potential but still leaves a potential gap of 0.6 v to overcome. The theoretical reversal of the electron transport chain from cyt. c to NAD requires a minimum of 27 kcal (Lees, 1962), which necessitates an energy input of 3-4 ATP molecules (ATP to ADP). The reversal of the electron transport chain in chemosynthetic autotrophs as yet has not been completely resolved.

Aleem, Lees, and Nicholas (1963) found that extracts of Nitrobacter, Nitrosomonas, and Ferrobacillus were able to reduce NAD in the presence of ATP and reduced cyt. c. The ATP:NAD ratio was 10-20:1 rather than 4:1. This system had an absolute requirement for ATP and reduced cyt. c and was inhibited by atabrin, thus implicating the involvement of flavin(s). Kiesow (1963, 1964) showed similar results with

Nitrobacter winogradskyi. These workers showed that both systems of energy transfer, production of ATP and reduction of NAD, were present in Nitrobacter. The system for NAD reduction in iron oxidizers, although present (Aleem, Lees, and Nicholas, 1963), has not been studied further.

Oxidative or substrate phosphorylation has never been demonstrated in the chemosynthetic iron oxidizers. Beck and Shafia (1964) reported an increased iron oxidation by aged cells of Thiobacillus ferrooxidans in the presence of phosphate. The same workers reported that 2,4-dinitrophenol inhibited CO₂ fixation but not iron oxidation. These results with intact cells imply the existence of an oxidative phosphorylation system, but the proof of such a system is delayed because of the inability of researchers consistently to find cell-free extracts capable of manometric iron oxidation (Lundgren, 1965; Beck, 1965).

Carbon dioxide fixation in chemoautotrophic bacteria

Although the ability of bacteria to fix carbon dioxide was discovered in the late 1800's (Winogradsky, 1887), it was not until 1935 (Wood and Werkman, 1935; 1938) that research on the processes involved received an impetus. With the

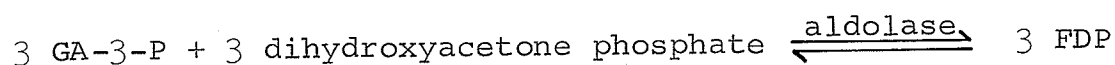
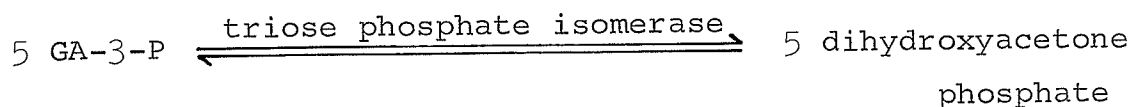
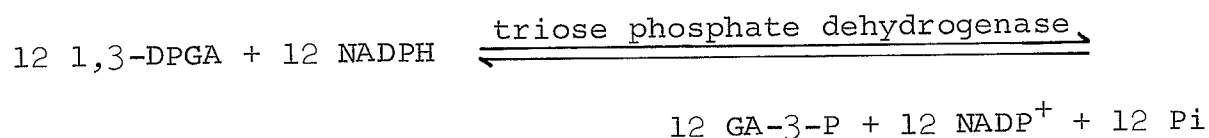
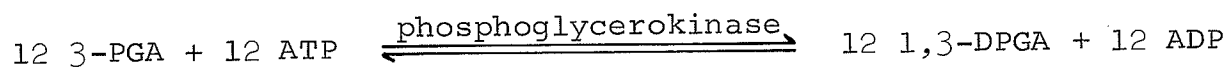
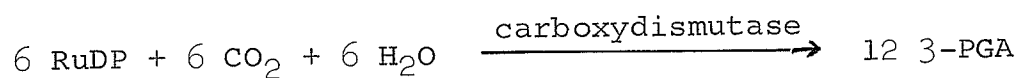
availability of C^{14} the field of autotrophic CO_2 fixation surged forward. The California group of Calvin, using new techniques of chromatography and radioautography, determined the sequence of formation of labelled products and intermediates after $C^{14}O_2$ incorporation by Chlorella.

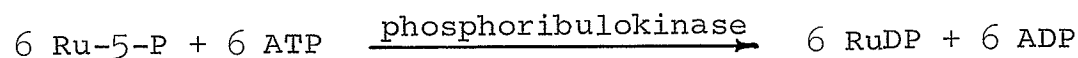
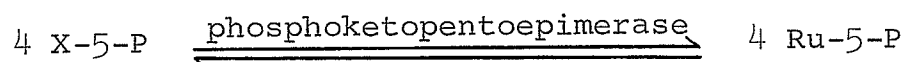
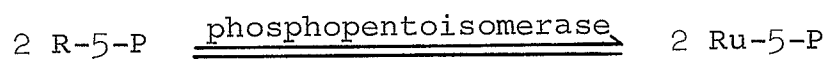
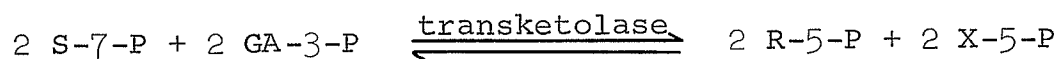
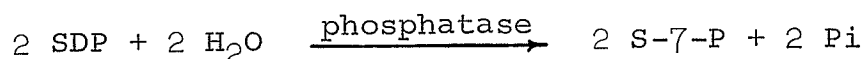
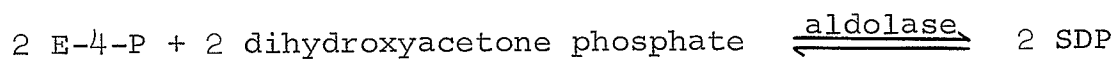
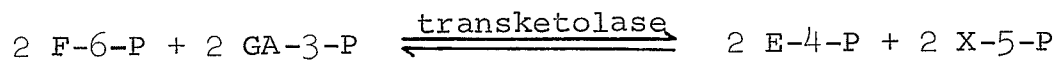
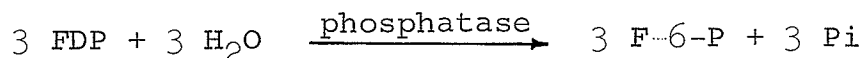
Calvin found that the first product of autotrophic CO_2 fixation was 3-PGA (Calvin and Benson, 1948). Calvin and Massini (1952) postulated that RuDP was the acceptor of the labelled CO_2 . With the sequence of labelled products known, enzymologists turned their efforts to finding the enzymes responsible for the cyclic mechanism. The primary carboxylation enzyme carboxylating RuDP was discovered in Chlorella (Quayle et al., 1954).

The formation of RuDP was discovered to be an enzymatic reaction catalyzed by phosphoribulokinase (Hurwitz et al., 1956). These two enzymes, the carboxylation enzyme (carboxydismutase) and phosphoribulokinase, are unique in autotrophic organisms. The discovery of the other enzymes involved in the cyclic mechanism either preceded the initial work of Calvin's group or was made later, but their occurrence is ubiquitous in nature.

Hexose phosphates are synthesized by a reversal of the

Embden-Meyerhof pathway using phosphoglycerokinase, NADP specific triose phosphate dehydrogenase, triose phosphate isomerase, and condensation by aldolase. Hexoses were formed by phosphatase action on the hexose phosphates. Pentose phosphates are regenerated by the action of transketolase, transaldolase, phosphopentoisomerase, and phosphoketopentoepimerase. The scheme originally postulated has recently been called the "Calvin-Bassham cycle" (Mahler and Cordes, 1966). The process is entirely cyclic and for each 6 turns around the cycle one extra 6 carbon unit is synthesized (see below).





SUM



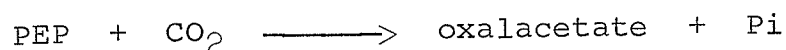
In 1955, Santer and Vishniac were the first to report a cell-free extract of a chemoautotroph (Thiobacillus thioparus) capable of fixing CO_2 . Using alumina extracts of Thiobacillus thioparus these authors reported the incorporation of

radioactive $C^{14}O_2$ into the carboxyl group of 3-PGA utilizing RuDP as the acceptor. In the same year Trudinger, working with extracts of Thiobacillus denitrificans, also demonstrated carboxydismutase and phosphoribulokinase activity in a chemoautotroph.

Trudinger (1956), following the pattern laid down in plant studies, was able further to demonstrate the following enzyme activities: phosphoglycerokinase, NAD triose phosphate dehydrogenase, triose phosphate isomerase, aldolase, hexose diphosphatase, transketolase, and transaldolase in extracts of Thiobacillus denitrificans. From these results Trudinger (1956) concluded that cell-free extracts were able to synthesize hexose phosphates from CO_2 by a mechanism similar to the mechanism established in Chlorella.

Working independently, the French group of Aubert (Aubert et al., 1956; 1957a,b) were able to show with time course studies using radioactive CO_2 and RuDP that the pattern of CO_2 fixation in whole cells of Thiobacillus denitrificans mimicked the results Calvin in his school obtained with Chlorella. Both Aubert and Calvin showed a high labelling content in aspartic acid. At that time the enzyme system forming C_4 compounds from C_3 compounds was still

unknown in chemoautotrophy. Bandurski and Greiner (1953), working with spinach leaves, first isolated an enzyme linking C_3 compounds to the Kreb's cycle. The enzyme catalyzed the following irreversible reaction:



In 1955 Bandurski purified the phosphoenolpyruvate carboxylase from spinach leaves. In later work the same enzyme was found in wheat germ extracts (Tchen and Vennesland, 1955) and succulent plant extracts (Walker, 1957). Utter and Kurahashi (1953; 1954a,b) demonstrated the activity of a reversible nucleotide-linked oxalacetic carboxylase in chicken liver. The enzyme required ITP or IDP for activity. These two enzymes, PEP carboxylase and oxalacetic carboxylase (PEP carboxykinase), differed in their requirement for nucleotides and their reversibility.

Suzuki and Werkman (1958a,b,c) demonstrated all the enzymes required for cyclic autotrophic CO_2 fixation in Thiobacillus thiooxidans. This intensive study clearly illustrated the ability of crude cell-free extracts to form hexose phosphates from R-5-P, ATP, and CO_2 as well as the enzymes required for the regeneration of the CO_2 acceptor.

These workers also were first to demonstrate two enzymes capable of C_3 to C_4 conversion in bacteria, PEP carboxylase and an ITP-linked oxalacetic carboxylase.

Carboxydismutase activity has also been reported in Hydrogenomonas facilis (Bergmann et al., 1958), Thiobacillus novellus (Aleem and Huang, 1965; Charles, 1966), and Nitrobacter agilis (Aleem, 1965). In the facultative thiobacilli, the synthesis of carboxydismutase is repressed in the presence of exogenous carbohydrates. Vishniac and Trudinger (1962), Aleem (1965), and Charles (1966) reported that the level of carboxydismutase activity during heterotrophic growth of Thiobacillus novellus was 2% or less of autotrophically grown cells.

In the iron oxidizers only two reports of CO_2 fixation have been published. Maciag and Lundgren (1964), using intact cells of Ferrobacillus ferrooxidans, were able to show that after 5 minutes in the presence of $C^{14}O_2$ and Fe^{++} the bacterium synthesized labelled phosphorylated carbohydrates, hexoses, amino acids and peptides, Kreb's cycle intermediates, and two and three carbon compounds. Gale and Beck (1966) showed that extracts of Thiobacillus ferrooxidans in the presence of R-5-P, ATP, and $C^{14}O_2$ were able to fix the

radioactive label. These authors found that AMP is competitive with ATP in the incorporation of radioactivity and postulated that AMP is a competitive inhibitor of phosphoribulokinase activity. This report is contradistinct from that of Aleem and Huang (1965) who reported that AMP inhibited carboxydismutase activity in Thiobacillus novellus.

MATERIALS AND METHODS

MATERIALS AND METHODS

Organism and medium

The organism used in this investigation, Ferrobacillus ferrooxidans, was originally isolated by W. W. Leathen (Leathen, Kinsel, and Braley, 1956) and was a gift of M.I.H. Aleem. The organism was grown on the 9K medium of Silverman and Lundgren (1959b). The medium contained: 4.5 g $(\text{NH}_4)_2\text{SO}_4$, 0.15 g KCl, 0.75 g K_2HPO_4 , 0.75 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ and distilled water to a final volume of 1,050 ml. The energy source, ferrous sulfate, was made by dissolving 66.33 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (14.74%) in 450 ml distilled water containing 1.5 ml 10 N H_2SO_4 . After 450 ml of the ferrous sulfate solution was added to the mineral-salts solution to give a total volume of 1,500 ml, 150 ml aliquots were sterile-filtered and placed in sterile 500 ml steel-capped Erlenmeyer flasks.

The organism was routinely maintained by twice a week transfer with a 1% inoculum and grown at 28° C in a gyrotary shaker. Large batches of cells were grown in 5 gallon polyethylene carboys containing 15 l of unsterilized 9K medium.

After inoculation with 150 ml freshly grown cells the carboys were aerated by passing compressed air through two sintered glass spargers (2 l/min) into the bottom of the medium. The carboys were incubated at 28° C and the ferrous iron content of the medium was assayed daily. When 75% of the original amount of ferrous iron present was utilized, 450 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in 700 ml distilled water and 10 ml 10 N H_2SO_4 was added (Blaylock and Nason, 1963). Again the ferrous iron content was assayed daily; when the ferrous iron content was again down to 25% of the original content another similar addition was made.

When the second ferrous iron addition had been utilized, the carboys were disconnected from the air lines and were incubated at 0-5° C for 12 hours. This storage in the cold allowed some precipitation of the ferric salts, thus facilitating subsequent recovery of the cells. The cells were harvested in a steam-driven Sharples centrifuge and suspended in cold $\text{H}_2\text{O}-\text{H}_2\text{SO}_4$ (pH 3.0).

After harvesting and prior to further use the cells were cleaned by centrifugation at 1,000 x g for 5 minutes to remove any ferric iron precipitate and centrifuged at 10,000 x g for 10 minutes to remove any soluble salts. The 10,000 x g

precipitate was resuspended again in cold $\text{H}_2\text{O}-\text{H}_2\text{SO}_4$ (pH 3.0) and this cleaning process was repeated 3-5 times. After cleaning, the cells were stored in $\text{H}_2\text{O}-\text{H}_2\text{SO}_4$ (pH 3.0) at $0-5^\circ \text{C}$.

If the "cleaned" cells were not used for any experiment before 4 days of storage had elapsed, then the cells were placed in 500 ml 9K medium and incubated at 28°C until 75% of the ferrous iron had disappeared. The revitalized cells were then harvested and cleaned in the normal manner.

CO_2 fixation by whole cells and crude extracts

Carbon dioxide fixation by whole cells and crude extracts was carried out in open test tubes at room temperature. The reaction mixtures contained: 0.62 ml 0.025 M citrate - 0.05 M phosphate buffer (pH 4.5), 20 mg (wet weight) intact cells or an equal amount of crude extract (prepared by sonicating a 10% (w/v) suspension of intact cells for 45 minutes in a 10 KC Raytheon), 2.5 μmoles sodium bicarbonate, 2.5×10^6 cpm $\text{NaHC}^{14}\text{O}_3$, and $\text{H}_2\text{O}-\text{H}_2\text{SO}_4$ (pH 4.5). The reaction was started by the addition of 10 μmoles

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ immediately followed by the radioactive bicarbonate. Other additions were 0.016 μmoles oxidized cyt. c where indicated. The final volume of the reaction was 1.0 ml.

At various time intervals the reaction was stopped by the addition of 0.5 ml 95% ethanol. Suitable samples were counted after acidification with 0.1 ml glacial acetic acid and drying the planchets at 60° C in a vacuum hood.

The radioactive samples were then extracted twice with 10 ml of 80% ethanol by heating to boiling. The residue was then discarded and the extract was dried and redissolved in water. The samples were treated batchwise with the H^+ form of Dowex 50W-X12 to remove cations. After drying, the samples were redissolved in water (1 ml) and used for chromatography and other experiments.

Paper Chromatography

Ascending one and two dimensional chromatograms were run in large round 5 gallon glass tanks at room temperature.

1. Paper

Whatman #1 paper was used in this investigation.

The paper was washed for 12 hours in 2 N acetic acid, then carefully rinsed with deionized water for 12 hours or until the rinses were a neutral pH. The paper was then air dried and stored before use.

2. Solvents

The following solvents were used: 80% phenol and ethyl acetate-acetic acid-H₂O (3:1:0.5). The phenol solvent was kept frozen and the ethyl acetate-acetic acid-H₂O was freshly prepared prior to use.

3. Sprays

A variety of sprays was used to locate various compounds on the chromatograms. Organic acids were detected by a mixed acid-base indicator as described by Aronoff (1956). Amino acids were found with ninhydrin reagent (Kornberg, 1958) and sugars were located by the aniline phthalate spray of Partridge (1949). Phosphorylated compounds were detected

by the procedure of Bandurski and Axelrod (1951) using an ammonium molybdate spray and U.V. light.

4. Location of radioactive compounds

The unknown radioactive compounds were located on the chromatograms with an Amperex GM tube fitted with a mica window and connected to a Phillips PW 4035 scaler. Strips were then cut out and fed into a Nuclear Chicago Model D47 gas flow, lead shielded GM counter fitted with a Nuclear Chicago Model C-100B Actigraph II ratemeter.

After elution of the spots, the eluted paper and the concentrated eluent were counted in a Nuclear Chicago D470 gas flow counter fitted with an end window. The papers were counted by cementing the paper to aluminum planchets and counting the samples directly with no correction for self absorption. Eluents of chromatograms (0.1 ml) were spread evenly over aluminum planchets with the aid of 0.5 ml acetone and dried at 80° C. The films were sufficiently thin to eliminate self absorption. All counts were corrected for background radiation.

5. Elution of isolated compounds

Compounds isolated by chromatography were eluted by cutting out radioactive spots and placing them in 100 ml of water overnight. The paper was then taken out, dried, and counted. The eluent was evaporated to a utilizable volume.

In some cases the radioactive spots were cut out as a narrow strip. The end of the strip closest to the isolated compound was cut into a wedge (V shaped) and the compound was eluted by a descending elution from a trough of water. When 10 ml was collected the paper was dried and counted, and the eluent was evaporated to a small volume.

6. Identification of radioactive compounds

The eluents were rechromatographed on a one dimension chromatogram and again located with an end-window counter. After a second elution the unknown radioactive compound was spotted alone and mixed with a known; the known alone was also spotted on the same chromatogram. The chromatogram was then developed, dried, and sprayed with the appropriate indicator. If the three spots coincided (R_f) and the first

two spots (unknown, and a mixture of the unknown and known) contained all the radioactivity, the compound was considered to be positively identified.

Carboxydismutase activity of cell-free extracts

Carboxydismutase activity of cell-free extracts was measured after the method of Suzuki and Werkman (1958a;c). The reaction mixture contained: 10 μ moles R-5-P, 15 μ moles ATP, 20 μ moles $MgCl_2$, 45 μ moles $NaHCO_3$, 120 μ moles Tris- H_2SO_4 buffer (pH 7.5), 10 μ moles GSH, 5 μ moles cysteine, 2 μ moles EDTA, 2.5×10^6 cpm $NaHC^{14}O_3$, cell-free extract (4.2 mg protein, prepared by sonicating a 10% suspension (w/v) of "washed" cells in 0.1 M Tris- H_2SO_4 buffer (pH 7.5) for 45 minutes in a 10 KC Raytheon and using the 10,000 x g supernatant after 15 minutes centrifugation), and water in a total volume of 2.0 ml. The reaction was carried out in double sidearm Warburg flasks stoppered with serum caps. The reaction vessels were flushed out for 5 minutes with N_2 gas and then the contents of both sidearms ($NaHC^{14}O_3$ and cell-free extract) were tipped into the main compartment.

After incubation for one hour at $28^\circ C$ the reaction

was stopped by the addition of 0.5 ml 50% TCA and the vessels were flushed with N_2 gas for an additional 5 minutes. The assay mixtures were centrifuged at $10,000 \times g$ for 15 minutes and suitable samples were placed in aluminum planchets with 0.5 ml acetone and dried at $60^\circ C$. The samples were then counted in a Nuclear Chicago Model D470 gas flow counter fitted with an end window. All counts were corrected for background radiation and the samples were sufficiently thin to exclude self absorption.

The assay mixtures were then treated batchwise with the H^+ form of Dowex 50W-X12 and chromatographed for identification of radioactive compounds.

Isotopic assay of cell-free phosphoenolpyruvate carboxylase activity

a. Phosphoenolpyruvate carboxylase activity of cell-free extracts was assayed by a modification of the method of Suzuki and Werkman (1958a;b). The complete reaction mixture contained: 10 μ moles PEP, 30 μ moles GSH, 3 μ moles $MgCl_2$, 45 μ moles $NaHCO_3$, 130 μ moles Tris- H_2SO_4 buffer (pH 7.5), 2×10^5 cpm $NaHC^{14}O_3$, cell-free extract (4.2 mg protein, see

carboxydismutase assay), and water in a total volume of 2.0 ml. The reaction was carried out in double sidearm Warburg vessels sealed with serum caps.

PEP and cell-free extract were each placed in separate sidearms and the flask was gassed with N_2 for 5 minutes. After tipping in the contents of the sidearms the reaction mixture was incubated for 1 hour at $28^\circ C$. The reaction was stopped by addition of 0.5 ml 50% TCA and the contents of the flask were gassed with "cold" CO_2 for 5 minutes.

The reaction mixtures were clarified and the radioactivity was determined as described in the previous section.

b. The same assay constituents as the spectrophotometric assay (see below) containing an addition of 2×10^5 cpm $NaHC^{14}O_3$ were also used to assay the enzyme activity isotopically. The reaction was run at $25^\circ C$ for 1 hour in N_2 filled Warburg vessels and was stopped with 0.5 ml 50% TCA. After clarification (see carboxydismutase assay) the samples were gassed with "cold" CO_2 for 5 minutes; then suitable aliquots were counted and treated with the H^+ form of Dowex 50W-X12 prior to chromatography for the identification of labelled compounds.

Spectrophotometric assay of cell-free phosphoenolpyruvate
carboxylase activity

Phosphoenolpyruvate carboxylase activity was measured by coupling the formation of oxalacetate to the oxidation of NADH and the production of malic acid in the presence of excess malic dehydrogenase. The reaction mixture contained: 300 μ moles Tris- H_2SO_4 buffer (pH 9.0), 0.3 μ moles NADH, 15 μ moles MgCl_2 , 30 μ moles NaHCO_3 , 7.5 μ moles PEP, enzyme, 5 μ g malic dehydrogenase, and water in a total volume of 3.0 ml (Sanwal and Maeba, 1966). The oxidation of NADH was followed at 340 m μ in a Gilford model 2000 spectrophotometer.

Preparation of cell-free Fe^{++} -cyt. c reductase

Fe^{++} -cyt. c reductase was prepared by sonicating a 10% (w/v) suspension of "washed" cells in 0.1 M Tris- H_2SO_4 buffer (pH 8.0) for one hour in a 10 KC Raytheon. After centrifugation at 25,000 x g for 15 minutes the precipitate was discarded and the supernatant was recentrifuged at 150,000 x g for one hour. The resultant supernatant was stored at 0-5° C and the precipitate was suspended in half the original volume of 0.1 M Tris- H_2SO_4 (pH 8.5). The particulate

suspension was resonicated for one hour; after recentrifugation at $150,000 \times g$ for one hour the supernatant solution was mixed with the first $150,000 \times g$ supernate and used as the crude enzyme source. The extract was stored at $-20^{\circ} C$ without any loss of activity.

Estimation of protein

Protein was determined by the colorimetric assay of Lowry et al. (1951) using crystalline bovine serum albumen as the standard.

Ferrous iron determination

Ferrous iron was determined by the acid-2,2'-dipyridyl method of Schulek and Flöderer (1939) as outlined by Snell and Snell (1949).

Manometry

All manometric experiments were carried out in a Barcroft-Warburg apparatus with double sidearm flasks. Standard techniques described by Umbreit et al. (1964) were used. The flasks were equilibrated at $30^{\circ} C$ for 10 minutes, 5 minutes open to the atmosphere and 5 minutes after the

flasks were closed, prior to the start of the reaction. Reactions were initiated by tipping in the contents of the sidearm(s) which contained either the substrate or the cells (extracts) studied.

Assay of ferrous iron-cytochrome c reductase activity

Fe^{++} -cyt. c reductase activity was measured by following the rate of reduction of horse heart cytochrome c spectrophotometrically at 550 m μ in a Unicam SP700 spectrophotometer (Blaylock and Nason, 1963). The reaction mixture contained: 24 μ moles Na barbital-acetic acid buffer (pH 7.0), 12 μ moles $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ in 0.15 ml H_2O - H_2SO_4 (pH 3.0), 0.45 μ moles cyt. c (Sigma, type III), enzyme, and water in a total volume of 3.0 ml.

The enzymatic and non-enzymatic reactions were followed against a control cuvette containing 0.45 μ moles cyt. c and water in a final volume of 3.0 ml. The reaction mixture minus the ferrous iron was incubated for 6 1/2 minutes at 25 $^\circ$ C prior to the start of the reaction. The reaction was initiated by the introduction of ferrous iron and the change in optical density at 550 m μ was followed for two minutes. The reaction was linear only for a short

time, so only the linear initial velocities or rates were recorded (Blaylock and Nason, 1963).

Iron oxidase activity

Iron oxidase activity was assayed colorimetrically following the disappearance of ferrous iron by a modification of the method described by Blaylock and Nason (1963). The reaction mixtures contained 6 times the volume of the Blaylock and Nason method and the ferrous iron remaining after the reaction was stopped was measured by the method of Schulek and Flöderer (1939) as described elsewhere in Materials and Methods. The reaction mixture contained: 0.6 ml enzyme, 1.8 ml 0.0025 M citrate-0.005 M phosphate buffer (pH 5.0), 0.6 ml 10^{-2} M $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 2.5 with H_2SO_4). Manometrically iron oxidase activity was assayed using the same reactants. Spectrophotometrically iron oxidase activity was measured by following the reduction and subsequent oxidation of cellular cytochrome c in an Aminco Dual Wavelength spectrophotometer. (see Figs. 3 and 4).

Cytochrome oxidase activity

Cytochrome oxidase activity was assayed spectrophotometrically by a modification of the method of Blaylock and Nason (1963). The reaction mixture contained 3 ml of enzyme, 0.025 M citrate-0.05 M phosphate buffer (pH 4.5), and 2 μ moles reduced cytochrome c. The activity was assayed in 10 mm cuvettes at 550 m μ in a Unicam SP700 spectrophotometer. Activity was also assayed at pH 5.7 in 0.01 M Na acetate-acetic acid buffer in an Aminco Dual Wavelength spectrophotometer (see Fig. 5). Reduced cytochrome c was prepared by reduction of oxidized cytochrome c (Sigma, type III) dissolved in water with excess sodium ascorbate. After the reduction was completed (measured spectrophotometrically) the solution was dialysed in two changes of 4 l of distilled water prior to dilution to achieve the required concentration.

Dual wavelength spectrophotometry

Dual wavelength spectrophotometry was carried out in an Aminco-Chance Dual Wavelength spectrophotometer. All reactions (aerobic or anaerobic) were performed in 10 mm

cuvettes fused at the base of a Thunberg tube so that gas evacuation of the tube was possible. In anaerobic experiments the gas phase was evacuated by means of a water aspirator and the tube was filled with N_2 gas. This process was repeated three times to ensure anaerobiosis.

The substrate used was in the sidearm of the tube and after the machine was adjusted the substrate was tipped in and the reaction was followed on a transmission strip chart recorder and visually on the oscilloscope screen. The response of the machine was adjusted to the appropriate setting (0-5%, 0-10% etc. full scale deflection) and the reaction was followed with extreme sensitivity.

Spectroscopy

The absorption spectrum of whole cells was observed in a Hartridge Reversion Spectroscope manufactured by Beck (London). The spectroscopy was carried out in evacuated Thunberg tubes containing 50 mg (wet weight) intact cells in 3.9 ml $H_2O-H_2SO_4$ (pH 3.0). Ten μ moles Fe^{++} or sodium dithionite (0.1 ml $H_2O-H_2SO_4$ (pH 3.0)) was placed in the sidearm prior to evacuation of the tube. The tubes were

then evacuated and the absorption spectrum recorded before and after the addition of Fe^{++} or dithionite.

Spectrophotofluorometry

Spectrophotofluorometric quenching studies were carried out after the method of Velick (1958). The fluorescence of the enzyme was measured in an Aminco-Bowman spectrophotofluorometer. The excitation wavelength was varied from 200-800 $\text{m}\mu$ and fluorescence spectra were recorded from 200-800 $\text{m}\mu$. The enzyme fluorescence had a peak at 350 $\text{m}\mu$ when the enzyme was excited at 270-290 $\text{m}\mu$ (280 $\text{m}\mu$ peak). The fluorescence quenching was corrected for dilution by using ordinary buffer instead of the substrates or end-products of the enzyme. Quenching by non-specific reactions was checked for by using a concentration of tryptophan exhibiting the same fluorescence as the enzyme and adding the same quantities of substrates and end-products used to quench the enzyme.

Determination of sulfide

Sulfide content of Fe^{++} -cyt. c reductase was assayed by the method of Fogo and Popowsky (1949); the colorimetric

assay was performed using 10 mm cuvettes in a Unicam SP700 spectrophotometer.

RNA determination

RNA content was estimated using the orcinol method as described by Schneider (1957). Yeast nucleic acid, 3' AMP, and ribose were used as standards.

RNA was assayed spectrophotometrically after the method described by Magasanik and Chargaff (1951). These workers calculated that an extinction coefficient ($\epsilon(P)$) of 10,000 at 260 m μ for intact RNA corresponded to a quantitative phosphate analysis of the RNA assayed. Based on this extinction coefficient for each nucleotide residue and assuming each enzyme molecule contains one protein molecule and one RNA molecule, the calculation of the number of nucleotide residues per protein molecule is simply a division of the number of nucleotide residues by the moles of protein. Assuming an average nucleotide (P) weight of 340, the molecular weight of the RNA molecule was calculated by multiplying the number of moles of nucleotide residue per mole of protein by 340. The weight of RNA (g) was calculated by multiplying the moles of RNA (moles of protein) by the

molecular weight of the RNA (calculated).

moles of nucleotide (P) per protein =

$$\frac{\text{moles of nucleotide found}}{\text{moles of protein}}$$

M.W. RNA = moles of nucleotide per protein $\times$ 340

RNA (g) = moles of RNA (moles of protein) $\times$ M.W. of RNA

DNA determination

The DNA content of Fe⁺⁺-cyt. c reductase was assayed by the diphenylamine method as described by Schneider (1957).

Iron content of Fe⁺⁺-cyt. c reductase

The iron content of the enzyme was assayed by a modification of the methods of Rajagopalan and Handler (1964) and Massey (1957) as described by Suzuki and Silver (1966).

When large concentrations of the enzyme were used, 1 ml of enzyme was mixed with 1 ml of saturated 2,2'-dipyridyl and allowed to incubate for one hour in the dark. After 0.2 ml saturated ammonium acetate and 0.3 ml H₂O were added the mixture was incubated in the dark for an additional hour. The optical density of the solution was read at 520 mμ in a Unicam SP700 spectrophotometer against an H₂O blank in 10 mm

cuvettes. This value was the ferrous iron content.

The total iron content was determined by adding 0.1 ml 2% $\text{Na}_2\text{S}_2\text{O}_4$ and incubating the mixture in the dark for one hour. After dilution with H_2O to a 10 ml final volume the optical density was again read against a water blank at 520 m μ in 40 mm cuvettes.

Molecular weight determinations

Molecular weight determinations of the enzyme and enzyme subunits were carried out by the sucrose density gradient method described by Martin and Ames (1961). A 4-20% sucrose gradient was prepared in 5 ml lusteroid Spinco centrifuge tubes using cold (0-5° C) sucrose solutions dissolved in 0.01 M sodium barbital-acetate buffer (pH 7.0). The sample solutions (0.2 ml) were then carefully layered on the top of the gradients and the tubes were centrifuged for 12 hours at 39,000 rpm in a SW39L rotor (Model L Spinco).

The deceleration of the rotor was not braked. When the tubes had come to rest the bottom of the tubes was pierced and two drop fractions were collected. The fractions were either assayed for enzyme activity or diluted to 2 ml with H_2O and the optical density of the diluted fractions was

recorded in a Gilford model 2000 spectrophotometer. The enzyme (Fe^{++} -cyt. c reductase) and its subunits were found by recording the optical density of the diluted fractions at 260 and 280 m μ . Both marker proteins, cyt. c and hemoglobin, were found by recording the optical density of the diluted fractions at 407 m μ (γ absorption band).

All tests were run separately. There was no mixing of marker and unknown to preclude the possibility of an interaction between them. In one case both enzyme subunits were run together when reconstitution of the holoenzyme was attempted.

Disc polyacrylamide gel electrophoresis

Disc electrophoresis was used to study the purity of the Fe^{++} -cyt. c reductase preparations. The polyacrylamide gel electrophoresis was performed after the method described by Orenstein (1964) and Davis (1964). The electrophoresis was carried out in a trial kit purchased from Canalco (Bethesda) and was run at pH 8.3 in Tris-glycine buffer.

Samples of enzyme (50-150 μg) were used and the electrophoresis was run for 30-60 minutes. The gels were then stained with amido black to dye the protein and

destained using 20% acetic acid in the same current as the electrophoresis was run. After destaining, the gels were stored in water and were viewed in a background of light to provide sharp contrast. Since no other electron acceptor could replace cyt. c, no specific enzyme stain was used to find the location of the active band or fraction.

Preparation of cytochrome c monomer and dimer

Monomer and polymers of cytochrome c were prepared by a modification of the method of Margoliash and Lustgarten (1962). Monomer was prepared by dissolving cytochrome c (type III, Sigma) in water and lowering the pH to 2.0 with the addition of glacial acetic acid. After 10 minutes incubation the solution was dialysed for 18 hours in 0.01 M Na acetate-acetic acid buffer (pH 5.7). The monomer was then diluted to the proper concentration with the same buffer.

Cyt. c dimer was prepared by making a cyt. c solution (type III, Sigma) 60% with respect to ethanol concentration, and allowing the solution to incubate for one hour at room temperature. The solution was cooled to 0-5° C and enough concentrated NaCl solution was added to make the cytochrome solution 0.015 M NaCl. After centrifugation at 25,000 x g

for 15 minutes the precipitate was washed twice in a 70% ethanol solution by repeated resuspension and centrifugation. After the second washing the precipitate was dissolved in a minimal volume of 0.5 N NH_4OH . The solution was then dialysed for two hours in deionized water and lyophilized. The cyt. $\underline{c}$ was weighed, dissolved in water, and dialysed in 0.01 M Na acetate-acetic acid buffer (pH 5.7) for 18 hours prior to dilution with the same buffer to make up the required concentration of cytochrome.

Chemicals

Standard reagent grade chemicals were used. Type III horse heart cytochrome $\underline{c}$, hemoglobin, protease type VII (subtilisin), triose phosphate dehydrogenase, NADH, N-ethylmaleimide, $\underline{p}$ -hydroxymercuribenzoate, 3'AMP, aceto-acetyl CoA, acetyl CoA, phosphoenolpyruvate, ribose-5-phosphate, ATP, 3-PGA, yeast RNA, DEAE-cellulose, and $\text{Ca}_3(\text{PO}_4)_2$ gel were purchased from Sigma Chemical Company. Ribonuclease, carboxypeptidase, and malic dehydrogenase were obtained from Worthington Biochemicals; and trypsin, α -chymotrypsin, and malic dehydrogenase from Boehringer and Soehne (Germany). $\text{Na}_2\text{C}^{14}\text{O}_3$ was obtained from the Radiochemical

Centre, Amersham, England; quinacrine hydrochloride (atabrin), Mann Research Laboratories; powdered bovine albumen, Armour Laboratories, and benzyl viologen, phenazine methosulfate, and 2,2'dipyridyl, British Drug Houses.

RESULTS

RESULTS

I. Mechanisms of Carbon Dioxide Fixation

Carbon dioxide fixation by Ferrobacillus ferrooxidans was studied in intact cells, crude extracts, and cell-free extracts.

Carbon dioxide fixation by whole cells and crude extracts

Intact cells and crude extracts of Ferrobacillus ferrooxidans both fixed radioactive $C^{14}O_2$ in the absence of an added energy source. The amount of $C^{14}O_2$ fixed was very small and could be accounted for by the presence of endogenous metabolites (Table I). In the presence of an energy source, ferrous iron, only intact cells showed an appreciable increase in the amount of $C^{14}O_2$ incorporated. The inability of crude extracts to incorporate additional $C^{14}O_2$ in the presence of ferrous iron is another evidence of the failure of crude extracts to metabolize ferrous iron which leads to the exhaustion of intermediates for CO_2 fixation and ATP (see Tables I, II, and III).

TABLE I

CO₂ fixation by whole cells and crude extracts
(counts per minute)

Time	Whole cells + Fe ⁺⁺	Crude extract	Crude extract + Fe ⁺⁺	Crude extract + cyt. c	Crude extract + cyt. c + Fe ⁺⁺
0	930	1,010	890	990	1,030
30 sec	1,020	970	750	980	970
3 min	770	790	960	730	990
10 min	1,230	1,000	900	960	970
30 min	1,230	940	950	950	800

The reaction was carried out under the conditions described in Materials and Methods.

The incorporation of $C^{14}O_2$ shows that metabolizing intact cells have the ability to regenerate the necessary intermediates of CO_2 fixation and also illustrates the ability of intact cells to generate ATP while metabolizing ferrous iron. Typical results are found in Table I.

Separation and identification of radioactive compounds

Ethanol extracts of the 30 second fixation by intact cells metabolizing ferrous iron (Table I) were separated by two dimension ascending chromatography. The following labelled compounds were found: 3-PGA, aspartic acid, glutamic acid, and phosphorylated sugars. The majority of the radioactivity was found to be in the 3-PGA, which contained approximately 79% of the $C^{14}O_2$ fixed. Aspartic acid made up 7% of the activity fixed and glutamic acid contained 2%.

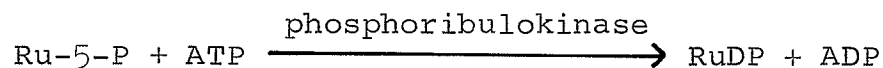
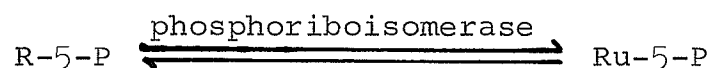
The appearance of 3-PGA indicates an autotrophic CO_2 fixation mechanism similar to that first found in plants (Calvin and Benson, 1948; Bassham et al., 1950; Benson et al., 1950) and in other chemolithotrophic bacteria (see Elsdén, 1962). The high activity in aspartic and glutamic acids indicates the operation of the heterotrophic Wood-Werkman

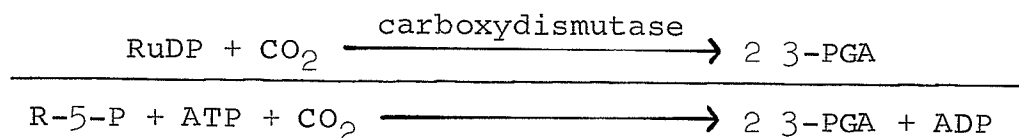
reaction (PEP carboxylase) first shown in bacteria by Suzuki and Werkman (1958a,b).

The formation of 3-PGA by cell-free extracts

Autotrophic CO₂ fixation mechanisms, cyclic mechanisms where the CO₂ acceptor is regenerated, contain two enzymes, phosphoribulokinase and carboxydismutase, not found in any other metabolic system (Fager, 1954; Quayle et al., 1954; Weissbach et al., 1954; Hurwitz et al., 1956; Weissbach et al., 1956). The autotrophic system is able to regenerate RuDP from 3-PGA. The other enzymes found in the regeneration mechanism are not unique to autotrophic systems and appear ubiquitous.

The formation of PGA by cell-free extracts was studied by the method of Suzuki and Werkman (1958a,c). The reaction mixture contained R-5-P and ATP, thus necessitating the presence of another enzyme, phosphoriboisomerase. The following equations describe the system studied (Hurwitz et al., 1956; Weissbach et al., 1956):





The results are shown in Table II. The necessary requirements of the system were: cell-free extract, Mg^{++} , ATP, and R-5-P. EDTA, glutathione, and cysteine appear to inhibit rather than stimulate the reaction. This result is contradistinct from the purified system from spinach leaves (Hurwitz et al., 1956; Weissbach et al., 1956), and similar to the same system found in Thiobacillus thiooxidans (Suzuki and Werkman, 1958a,c).

The contents of tube 7 (Table II) were desalted then chromatographed to identify the end-product of the reaction. After chromatography in ethyl acetate:acetic acid: H_2O the radioactive spot was cut out and eluted. The eluent was rechromatographed by spotting the eluent, eluent and 3-PGA, and 3-PGA alone. After development in the same solvent the chromatogram was dried and sprayed with the mixed acid-base indicator of Aronoff (1956). All three spots had the same R_f and radioactivity was found only in the location of the spots; thus the sole labelled end-product was positively identified as 3-PGA.

TABLE II

Carboxydismutase activity of cell-free extracts

Test	Omission	Activity fixed (cpm)
1	None	10,020
2	Cell-free extract	0
3	Mg ⁺⁺	0
4	ATP	0
5	R-5-P	0
6	EDTA	14,200
7	GSH, cysteine	29,320

The reaction was carried out under the conditions described in Materials and Methods with 0.5 ml cell-free extract (4.2 mg protein).

The carboxydismutase activity of cell-free extracts was also assayed spectrophotometrically by using "cold" bicarbonate in place of HC^{14}O_3 and adding 10 μmoles ATP, 0.5 μmoles NADH, and triose phosphate dehydrogenase (Sigma) to the isotopic reaction mixture contents. The reaction was carried out in 10 mm cuvettes at 340 m μ in a Gilford model 2000 spectrophotometer. This assay provided evidence for the presence of phosphoriboisomerase, phosphoribulokinase, carboxydismutase, and phosphoglycerokinase in cell-free extracts. Extracts also exhibited very weak glyceraldehyde-3-phosphate dehydrogenase activity using 3-PGA, ATP, and NADH as substrates.

Extracts of Ferrobacillus ferrooxidans contained all the enzymes necessary for the autotrophic fixation of CO_2 in addition to the enzymes required to generate glyceraldehyde-3-phosphate necessary for the isomerization and condensation into a hexose in order to make the fixation process cyclic.

The formation of oxalacetate

The results of C^{14}O_2 fixation by whole cells indicated a $\text{C}_3 + \text{C}_1$ reaction feeding the Krebs cycle, leading to the formation of labelled aspartic and glutamic acids. This

"heterotrophic" CO_2 fixation is common to all organisms and is required for replenishment of necessary intermediates of the Krebs cycle. In bacteria four major systems of CO_2 fixation (or exchange) feeding in necessary intermediates have been found: the malic enzyme, PEP carboxylase, PEP carboxykinase, and pyruvate carboxylase.

Kornberg (1965) discussed the "anaplerotic" function of PEP carboxylase and has reviewed evidence that the only enzyme responsible for feeding C_4 compounds into the Krebs cycle in many bacteria is PEP carboxylase. Single mutants of E. coli (Amarsingham, 1959; Ashworth and Kornberg, 1963) and S. typhimurium (Theodore and Englesberg, 1964) lacking PEP carboxylase were unable to grow on pyruvate or its precursors unless Krebs cycle intermediates were added to the growth medium.

Suzuki and Werkman (1958a,b) first demonstrated the existence of PEP carboxylase in cell-free extracts of bacteria (Thiobacillus thiooxidans) along with an ITP-linked oxalacetic decarboxylase. Since the Enterobacteriaceae contain the malic enzyme, PEP carboxylase and an ATP-linked oxalacetic decarboxylase and only one enzyme, PEP carboxylase, serves an "anaplerotic" function, this same premise may hold true for

chemolithotrophs (chemoautotrophs).

The PEP carboxylase activity of cell-free extracts was assayed by $C^{14}O_2$ incorporation in the presence of PEP and Mg^{++} (Mn^{++}). The results are shown in Table III. The tests indicated a requirement for PEP, Mg^{++} or Mn^{++} , and cell-free extract. Test 7 illustrated a lack of pyruvic carboxylase in the cell-free extracts.

Other experiments to investigate the possible metabolic controls exhibited on PEP carboxylase were carried out linking the enzyme to malic dehydrogenase and NADH oxidation. The reaction was measured by following the rate of NADH oxidation or measuring the amount of radioactivity incorporated. The PEP carboxylase of E. coli (Cánovas and Kornberg, 1965; Nishikido et al., 1965) and S. typhimurium (Sanwal and Maeba, 1965; 1966) was activated by acetyl CoA, fructose-1,6-diphosphate, and inhibited by aspartate.

Cell-free extracts of Ferrobacillus ferrooxidans contained PEP carboxylase activity and the sole end-product of the linked reaction was malic acid (test 1, Table IV). The results shown in Tables IV, V, and VI illustrate the effects of various intermediates of the Krebs cycle, amino acids of the aspartic family, glutamic acid, aceto-acetyl CoA,

TABLE III

Phosphoenolpyruvate carboxylase activity of cell-free extracts

Test	Omission	Addition	Activity fixed (cpm)
1	None	None	8,520
2	Cell-free extract	None	0
3	Mg ⁺⁺	None	0
4	Mg ⁺⁺	Mn ⁺⁺ (3 μ moles)	8,140
5	Phosphoenolpyruvate	None	0
6	Phosphoenolpyruvate	Pyruvate (100 μ moles)	0
7	Phosphoenolpyruvate	Pyruvate (100 μ moles), ATP (3 μ moles)	0
8	None	ADP (3 μ moles)	5,770

The reaction was carried out as described in Materials and Methods (see isotopic assay a) with 0.5 ml cell-free extract (4.2 mg protein)

TABLE IV

Effect of various compounds on cell-free
phosphoenolpyruvate carboxylase activity.

Test	Addition	Activity fixed (cpm)	Relative activity (%)
1	None	1,930	100
2	Citrate (15 μ moles)	2,100	109
3	Glutamate (15 μ moles)	2,310	119
4	Acetyl phosphate (1.5 μ moles)	2,070	107
5	CoA (1.5 μ moles)	2,080	107
6	CoA (1.5 μ moles), Acetyl phosphate (1.5 μ moles)	1,960	101
7	α -ketoglutarate (15 μ moles)	1,950	101
8	Aceto-acetyl CoA (1.5 μ moles)	4,900	254
9	Acetyl CoA (1.5 μ moles)	4,780	248
10	Aspartate (15 μ moles)	2,070	107

The reaction was carried out as described in Materials and Methods (see isotopic assay b) with 0.5 ml cell-free extract (4.2 mg protein).

TABLE V

Effect of potential activators on
phosphoenolpyruvate carboxylase activity*

Test	Addition	Concentration	Relative activity (%)
1	None	-	100
2	Aceto-acetyl CoA	6.6×10^{-5} M	750
3	Acetyl CoA	6.6×10^{-5} M	800
4	CoA	1.0×10^{-4} M	100
5	ATP Acetyl CoA	6.0×10^{-3} M 6.6×10^{-5} M	760
6	Acetyl phosphate	1.0×10^{-4} M	100
7	Acetyl phosphate ATP CoA	1.0×10^{-4} M 1.0×10^{-4} M 1.0×10^{-4} M	100
8**	Pyruvate	6.0×10^{-3} M	0
9**	Pyruvate ATP	6.0×10^{-3} M 6.0×10^{-3} M	0

*Assayed spectrophotometrically as outlined in Materials and Methods with 0.4 ml cell-free extract (3.9 mg protein).

** These tests lacked phosphoenolpyruvate.

TABLE VI

Effect of activators and potential inhibitors on
phosphoenolpyruvate carboxylase activity*

Test	Addition	Concentration	Relative activity (%)
1	None	-	100
2	Acetyl CoA	5×10^{-4} M	740
3**	Acetyl CoA	5×10^{-4} M	0
	Pyruvate	6×10^{-3} M	
	ATP	6×10^{-3} M	
4	Aspartate	5×10^{-3} M	30
5	Acetyl CoA	5×10^{-4} M	230
	Aspartate	5×10^{-3} M	
6	Acetyl CoA	5×10^{-4} M	750
	Threonine	5×10^{-3} M	
7	Acetyl CoA	5×10^{-4} M	760
	Citrate	5×10^{-3} M	
8	Acetyl CoA	5×10^{-4} M	730
	α -ketoglutarate	5×10^{-3} M	
9	Acetyl CoA	5×10^{-4} M	750
	Succinate	5×10^{-3} M	

*Assayed spectrophotometrically as outlined in Materials and Methods with 0.4 ml cell-free extract (3.2 mg protein).

**This test lacked phosphoenolpyruvate.

and acetyl CoA.

The reaction was similarly activated by aceto-acetyl CoA and acetyl CoA (Table IV), unlike the S. typhimurium enzyme where aceto-acetyl CoA could not completely substitute for acetyl CoA (Maeba, 1966). The PEP carboxylase activity was inhibited by aspartate in the presence or absence of acetyl CoA (Table VI). This result is in agreement with the results of Sanwal and Maeba (1965). Thus it appears that the "heterotrophic" CO₂ fixation mechanism of a chemoautotroph is subjected to the same control mechanisms as heterotrophic bacteria. Once this similarity was found, the question of control was not further pursued.

The PEP carboxylase of Ferrobacillus ferrooxidans has a pH optimum close to pH 7.0 or on the acid side of neutrality. This is illustrated in Table VII. It was not determined whether the actual carbon moiety fixed was CO₂ or HCO₃⁻ but the results indicate that the substrate fixed may be CO₂ rather than HCO₃⁻. Keeping in mind that the bacterium grows optimally at an acid pH this result would not be unexpected. On the other hand it has never been demonstrated that PEP carboxylase fixed free CO₂.

The cell-free extracts containing PEP carboxylase

TABLE VII

Effect of pH on phosphoenolpyruvate carboxylase activity*

pH	Relative activity (%)
9.0	100
8.5	111
8.0	167
7.5	256
7.0	422

*The reaction was assayed spectrophotometrically in 10 mm cuvettes at 340 m μ in a Gilford model 2000 spectrophotometer. The reaction mixture contained: 600 μ moles Tris-HCl buffer, 1.5 μ moles acetyl CoA, 15 μ moles MgCl₂, 10 μ moles PEP, 45 μ moles NaHCO₃, 1.5 μ moles NADH, 5 μ g pig heart malic dehydrogenase (Worthington), cell-free extract (3.2 mg protein) and water in a final volume of 3.0 ml.

activity were tested for malic enzyme activity spectrophotometrically, using both NAD and NADP coenzymes but no activity was found. Some heterotrophic bacteria have been found to contain the malic enzyme, but rather than act as a pathway into C_4 metabolism the enzyme replenished C_3 compounds when the bacteria were grown on Krebs cycle intermediates (Wood and Stjernholm, 1962; Kornberg, 1965).

II. Iron Oxidation

Iron oxidation was studied in both intact cells and cell-free extracts in an attempt to elucidate the oxidation mechanism.

Electron transport components

Vernon, Mangum, Beck, and Shafia (1960) first demonstrated the existence of cytochromes in an iron oxidizer. These workers, who found that cell-free extracts of Thiobacillus ferrooxidans contained cytochromes c and a, purified the cytochrome c. Blaylock and Nason (1963) found cytochromes b, c, and a in extracts of Ferrobacillus ferrooxidans and postulated that iron oxidation involved only cytochromes c and a. The cytochrome content of Ferrobacillus

ferrooxidans was investigated spectroscopically in intact cells and spectrophotometrically in cell-free extracts (sonic extracts).

When intact cells were treated with either ferrous iron or sodium dithionite spectroscopy revealed absorption bands at 409-410 m μ , 510-525 m μ , and 552-555 m μ , which coincide with the γ , β , and α bands of reduced cytochrome c; and at 420-425 m μ and 602-605 m μ , which coincide with the γ and α bands of a reduced a-type cytochrome (Smith, 1961). Contrary to the results of Blaylock and Nason (1963) there was no evidence of the presence of cytochrome b. Spectrophotometrically, sonic cell-free extracts exhibited the same type of absorption spectrum. The results of the spectroscopic studies are shown in Table VIII.

Intact cells and sonic extracts were extracted with 40% butanol. The butanol layer was dried and redissolved in butanol prior to spectrophotometric analysis. The yellow colour of the dissolved substance and the spectrophotometric absorption spectrum (263, 271, and 280 m μ absorption peaks) indicated the presence of a quinone-type compound (Bishop, Pandya, and King, 1962). Attempts to reduce the compound with potassium borohydride failed and the spectrum remained

TABLE VIII

Absorption spectrum of whole cells after anaerobic incubation with ferrous iron and sodium dithionite.

Addition	Absorption lines (mμ)	
None	406,421	
Fe ⁺⁺	409,425	522,555,604
Na dithionite	410,425	523,554,605

The absorption spectrum was measured as described in Materials and Methods using a Hartridge Reversion Spectroscope.

unchanged unlike the coenzyme Q of Thiobacillus thiooxidans (Cook and Umbreit, 1963). The compound resembled the K vitamins of E. coli (Bishop, Pandya, and King, 1962). The role of this quinone-type compound in the electron transport chain was not pursued further.

Oxidation of ferrous iron by intact cells

Reported optimum conditions for ferrous iron oxidation by Thiobacillus ferrooxidans and Ferrobacillus ferrooxidans vary considerably (Silverman and Lundgren, 1959a,b; Beck, 1960; Razzell and Trussell, 1963; Landesman, Duncan, and Walden, 1966). Experiments designed to find the optimum pH and iron concentration for intact cell iron oxidation were performed. Using 'washed' intact cells, the optimum pH (initial) in a water-sulfuric acid mixture was pH 3.0 (Fig. 1). This pH optimum coincides with the results of Silverman and Lundgren (1959b) who used the same organism. At an initial pH of 3.0 the optimum ferrous iron content was found to be 250 $\mu\text{moles FeSO}_4 \cdot 7\text{H}_2\text{O}$ in a volume of 2.8 ml (Fig. 2). This result is at variance with the results of Landesman, Duncan, and Walden (1966) who reported an optimum iron concentration of 400-800 μmoles for Thiobacillus ferrooxidans.

Fig. 1. Effect of pH on the oxidation of ferrous iron by intact cells.

The reaction mixtures contained: 0.2 ml 20% KOH in the centre well, 3.5 mg (wet weight) whole cells and 250 μ moles Fe^{++} in 2.8 ml $\text{H}_2\text{O}-\text{H}_2\text{SO}_4$ at the proper pH. The reaction was started by the addition of ferrous iron and oxygen uptake was measured manometrically at 15 minute intervals for 1 hour at 30° C. The oxygen uptake after 1 hour was used to compare activity. (relative activity = 1 was 170 μ l O_2 /hour).

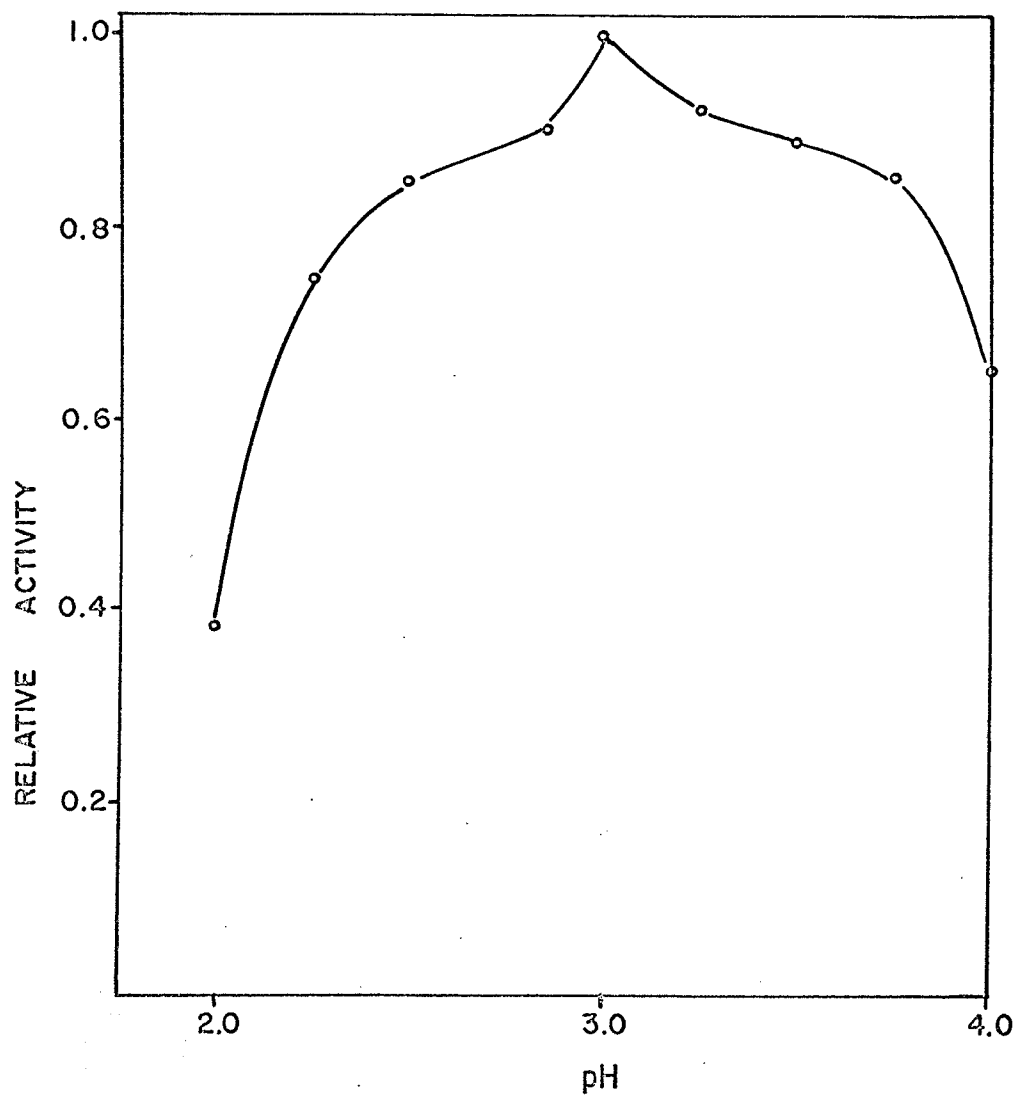


Fig. 2. Effect of ferrous iron concentration on iron oxidation by whole cells.

The reaction mixture contained: 0.2 ml 20% KOH in the centre well, 5 mg (wet weight) whole cells in 0.5 ml $\text{H}_2\text{O}-\text{H}_2\text{SO}_4$ (pH 3.0) in the sidearm, and Fe^{++} in 2.3 ml $\text{H}_2\text{O}-\text{H}_2\text{SO}_4$ (pH 3.0) in the main compartment. The reaction was initiated by the addition of the cells from the sidearm. Oxygen uptake was manometrically measured at 30° C.

A = 10 $\mu\text{moles Fe}^{++}$

B = 100 $\mu\text{moles Fe}^{++}$

C = 250 $\mu\text{moles Fe}^{++}$

D = 500 $\mu\text{moles Fe}^{++}$

E = 750 $\mu\text{moles Fe}^{++}$

F = 1000 $\mu\text{moles Fe}^{++}$

G = 0 $\mu\text{moles Fe}^{++}$

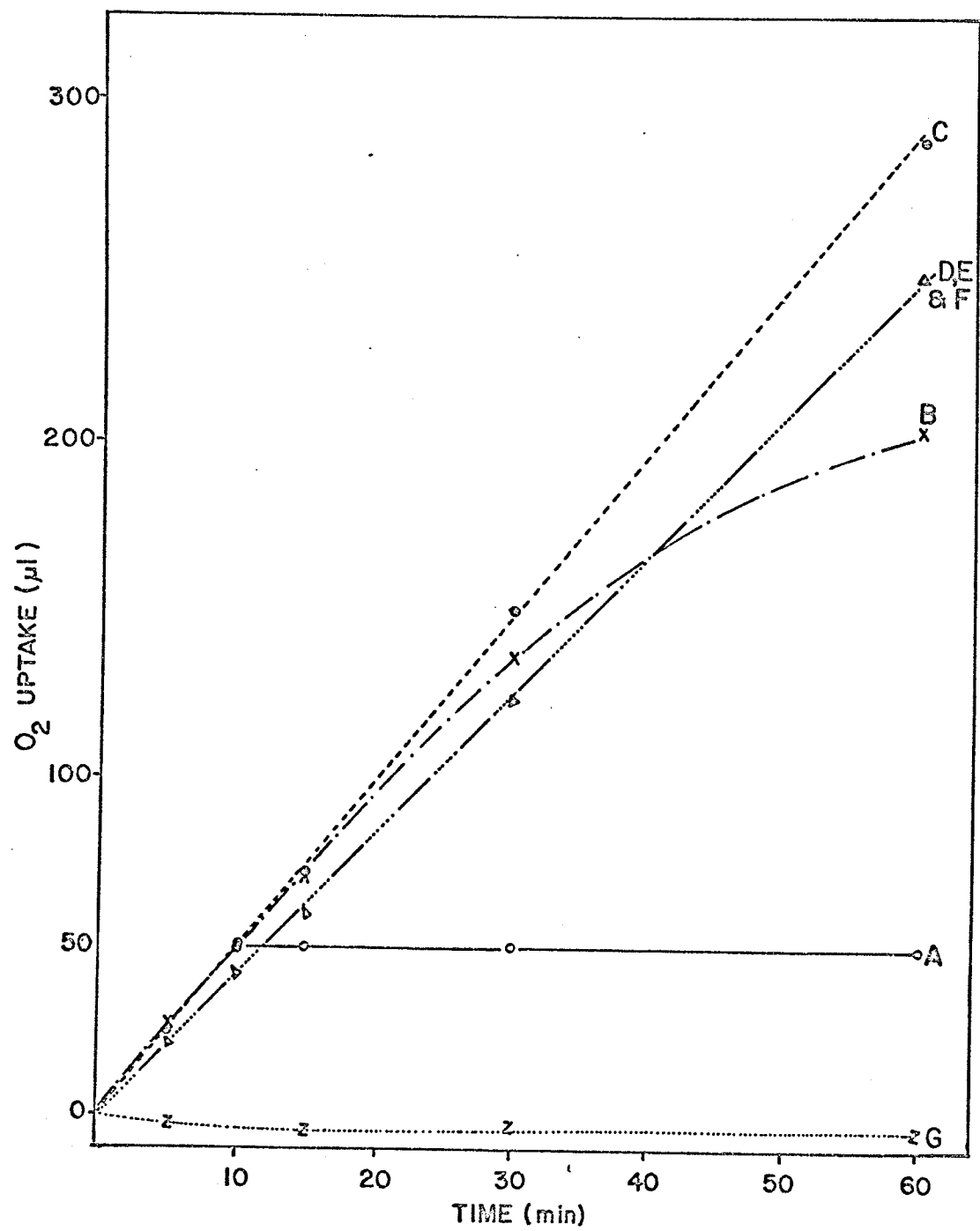


Figure 2 also shows that there is no lag in oxygen uptake as was experienced by Landesman, Duncan, and Walden (1966).

Intact cells of Ferrobacillus ferrooxidans did not oxidize Mn^{++} , Co^{++} , or Cu^+ (30 μmoles); and Mg^{++} , Mn^{++} , Co^{++} , Cu^+ , and Cu^{++} (30 μmoles) had no effect on iron oxidation. The stoichiometry of the reaction was found to be the same as that reported by Beck and Elsdon (1958) and Silverman and Lundgren (1959a). The oxygen uptake was 90% or more of the theoretical oxygen uptake (Fig. 2). The following equation describes the reaction:

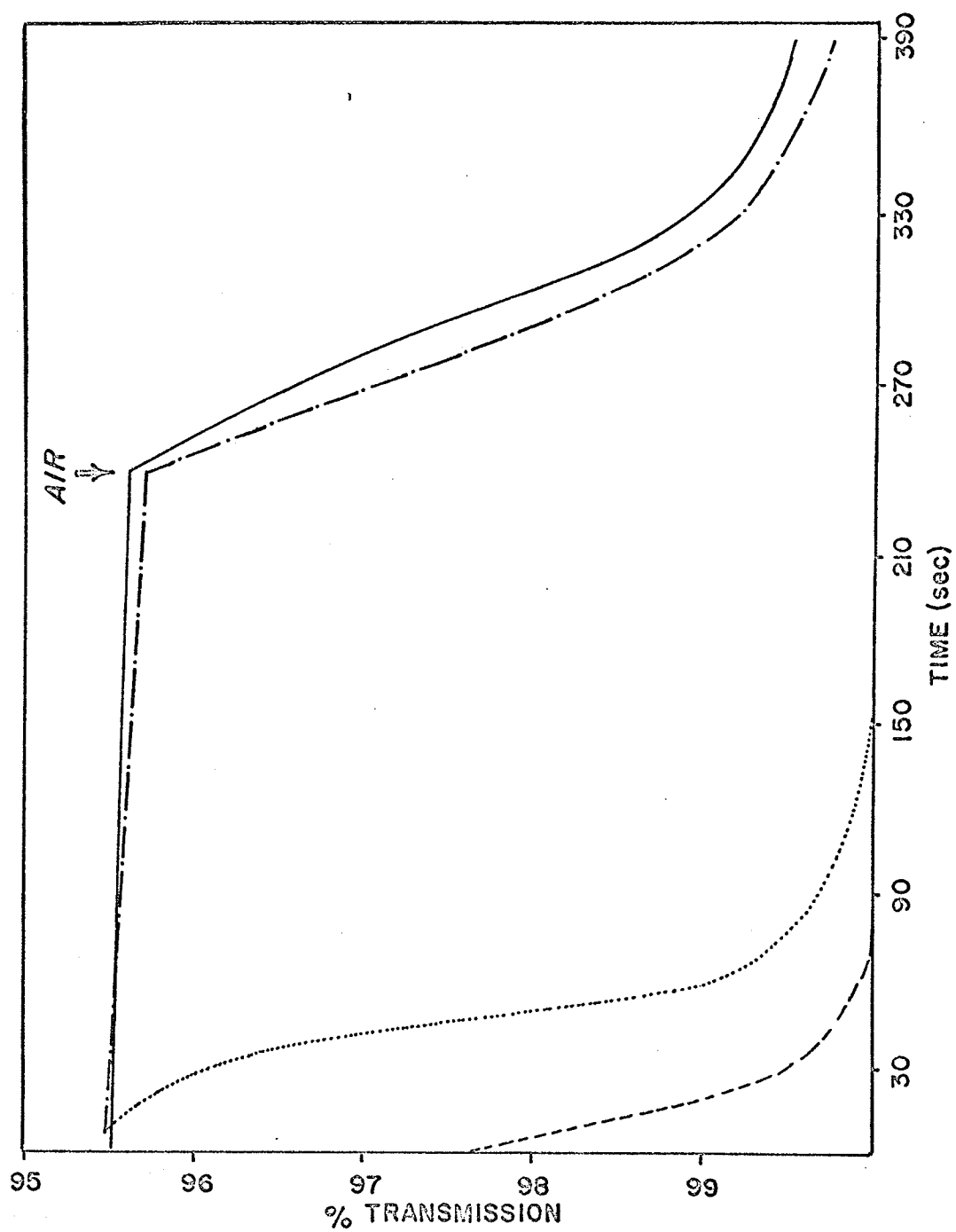


The colorimetric measurement of iron oxidation was inhibited 95% by 10^{-4} M azide, thus implicating the requirement of O_2 and the participation of cytochrome oxidase. Manometric studies exhibited similar inhibition. The involvement of oxygen was also demonstrated by Dual Wavelength spectrophotometry. 'Washed' intact cells showed cellular cytochrome c reduction in the presence of ferrous iron or ascorbate (Fig. 3). In the absence of oxygen the cellular cytochrome c was rapidly reduced but was not reoxidized; after the admittance of air the reduced cellular

Fig. 3. The oxidation of ferrous iron and ascorbate by whole cells.

The reaction mixture contained: 60 mg (wet weight) whole cells, 39 μ moles Na acetate-acetic acid buffer (pH 5.7), 1 μ moles Fe^{++} or ascorbate, and H_2O in a final volume of 4.0 ml. The reaction was followed in an Aminco Dual Wavelength spectrophotometer with wavelength settings at 540 and 550 m μ .

	Aerobic	Anaerobic
Fe^{++}	-----	_____
Ascorbate		-.-.-.-.



cytochrome c was rapidly oxidized (Fig. 3). These results indicate a two-step process in the oxidation scheme: (1) the reduction of cytochrome c by ferrous iron; and (2) the subsequent reoxidation of the reduced cytochrome c in the presence of oxygen via cytochrome oxidase, an a-type cytochrome.

Intact cells were stored for months at 0-5° C and still retained some oxidizing ability. The rate of iron oxidation by intact cells decreases with increased duration of storage. Landesman, Duncan, and Walden (1966) found that Thiobacillus ferrooxidans cells stored for longer than four days lost a large percentage of their oxidizing ability, so old cells were revitalized prior to use in all experiments (see 'Materials and Methods'). Intact cells lose 90-100% of their oxidizing ability upon freezing at -20° C, even for brief periods (5-10 minutes) (Figs. 4 and 5). The loss in activity could not be recovered by incubating the cells with ferrous iron. This freezing phenomenon is also exhibited by Nitrosomonas (Lees, 1964) and is not unique in the iron oxidizers.

Although frozen cells lose their ability to oxidize ferrous iron, crude sonicates of these same inactive cells

Fig. 4. The oxidation of ferrous iron by whole cells and crude extracts.

The reaction mixture contained: 60 mg (wet weight) whole cells or crude sonicate, 39 μ moles Na acetate-acetic acid buffer (pH 5.7), 1 μ mole Fe^{++} , and H_2O in a final volume of 4.0 ml.

The reaction was followed in an Aminco Dual Wavelength spectrophotometer with wavelength settings at 540 and 550 m μ .

_____	whole cells
-.-.-.-..	frozen cells
- - - -	crude sonicate

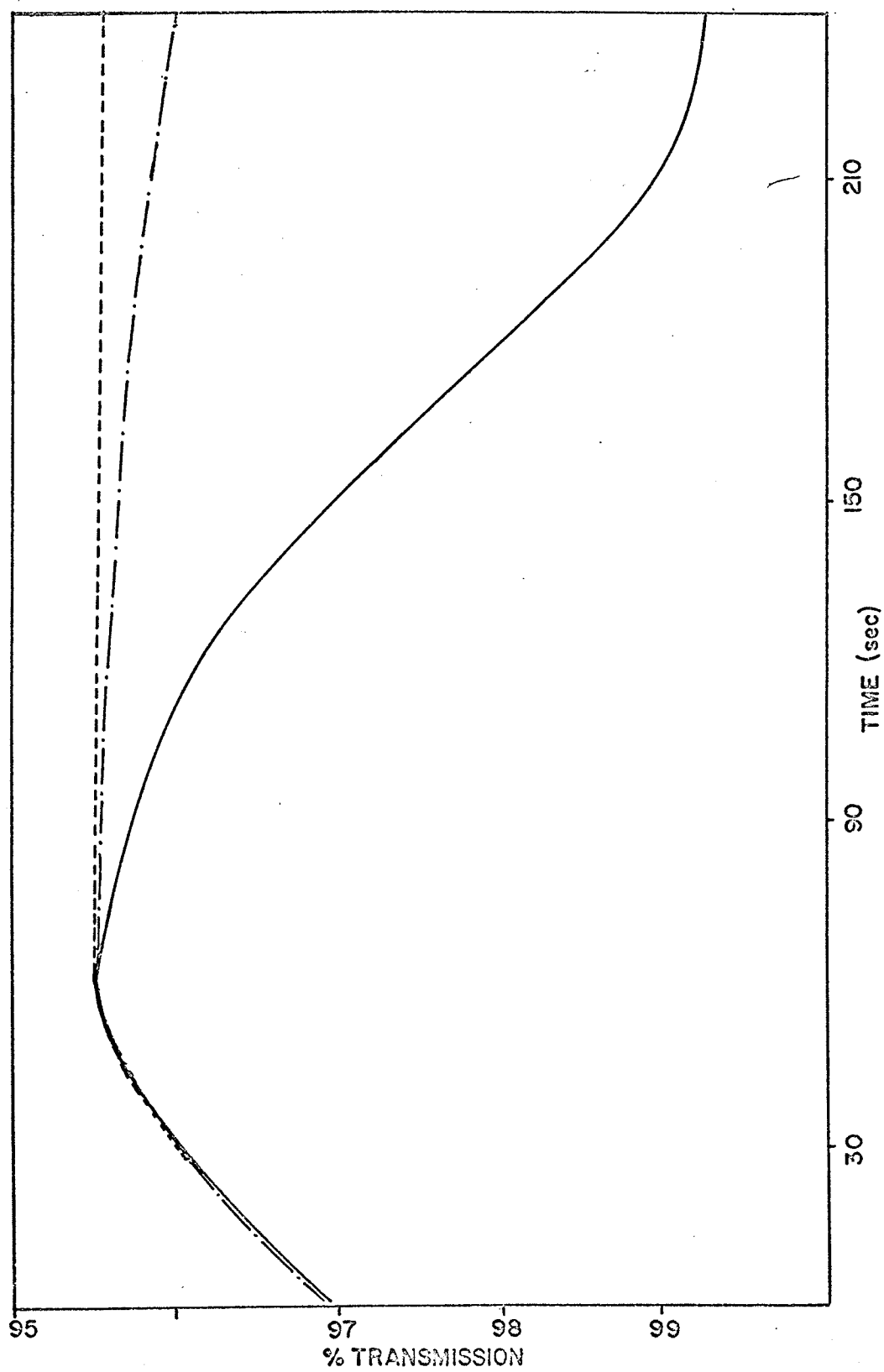
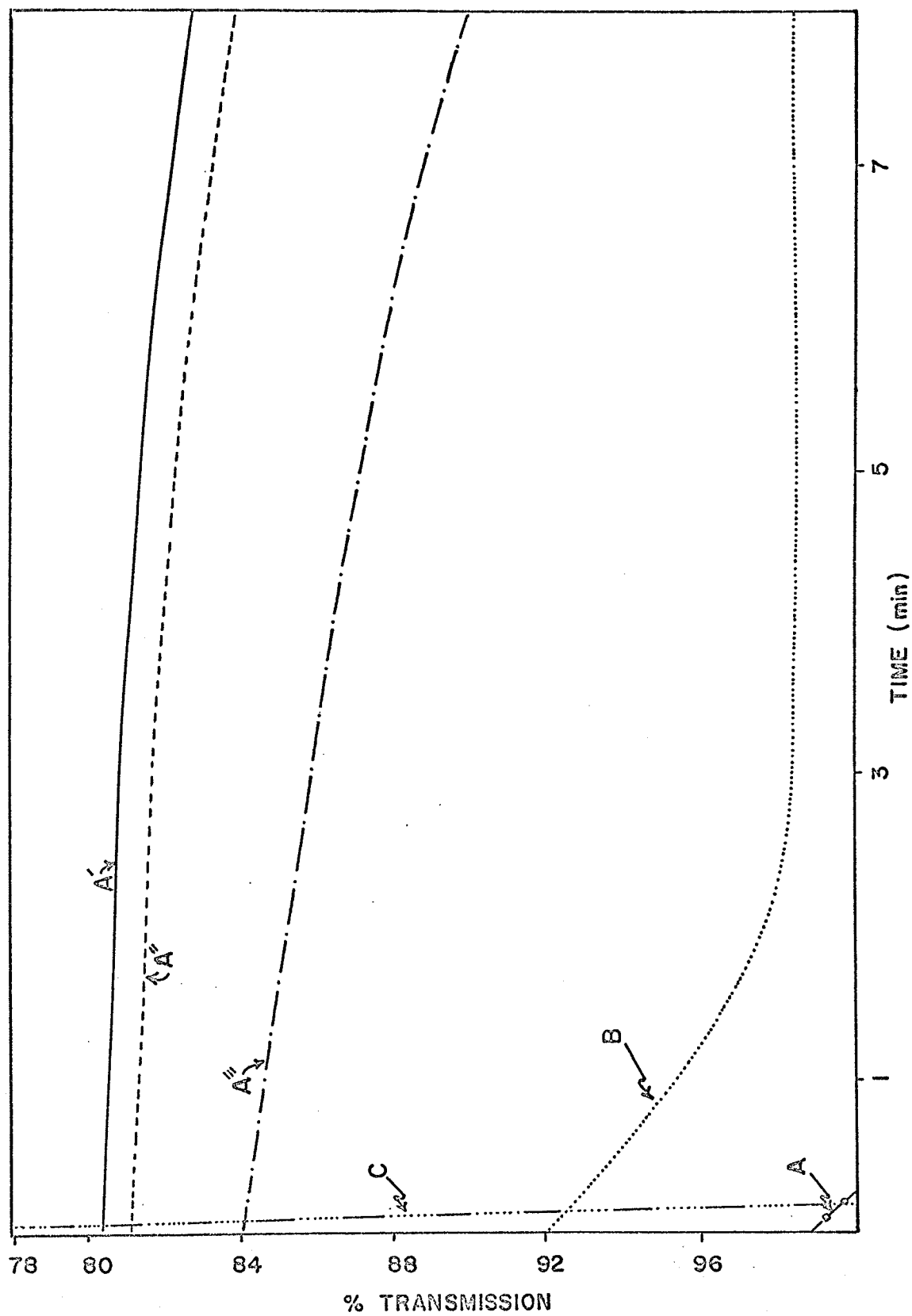


Fig. 5. The oxidation of reduced cyt. c by whole cells and crude extracts.

The reaction mixture contained: 60 mg (wet weight) whole cells or crude sonicate, 39 μ moles Na acetate-acetic acid buffer (pH 5.7), reduced cyt. c, and H₂O in a final volume of 4.0 ml. The reaction was followed in an Aminco Dual Wavelength spectrophotometer with wavelength settings at 540 and 550 m μ .

- A = sonicated cells (6.75 μ mole reduced cyt. c)
- A' = whole cells (6.75 μ moles reduced cyt. c)
- A'' = sonicated cells, anaerobic (6.75 μ moles reduced cyt. c)
- A''' = frozen cells (6.75 μ moles reduced cyt. c)
- B = sonicated cells diluted 1/10 (10.125 μ moles reduced cyt. c)
- C = sonicated cells (33.85 μ moles reduced cyt. c)



exhibited both partial reactions of the oxidation mechanism (Figs. 4 and 5). These extracts contain both ferrous iron-cytochrome c reductase and cytochrome oxidase activities. The loss of iron oxidizing ability by frozen intact cells must mean that the electron transport chain is interrupted or broken between cytochrome c and cytochrome a.

Oxidation of ferrous iron by cell-free extracts

Blaylock and Nason (1963) reported that they were able to isolate cell-free extracts of Ferrobacillus ferrooxidans capable of oxidizing ferrous iron (colorimetric assay). These authors showed that oxidation by cell-free extracts required oxygen and was inhibited by cytochrome oxidase inhibitors. Attempts by others to duplicate these results failed (Aleem, 1964; Beck, 1965; Lundgren, 1965; Silverman, 1965). In this investigation, use of the same methods as those described by Blaylock and Nason (1963) failed to uncover the activity of an iron oxidase that requires oxygen as its terminal electron acceptor.

'Washed' intact cells were sonicated for various time intervals and iron oxidase activity was measured at pH 3.0, manometrically, and at pH 5.0, colorimetrically and

manometrically. Oxygen uptake at pH 3.0 decreased with increase of sonication time; after 10 minutes sonication the extracts exhibited no oxygen uptake. There was no retention or increase of oxygen uptake at pH 5.0; the manometric oxidation at pH 5.0 paralleled the results obtained at pH 3.0. The results are illustrated in Table IX. Similar results were obtained when cell-free extracts rather than crude extracts were utilized (there was no oxygen uptake at any time).

Colorimetrically, crude and cell-free extracts oxidized iron at pH 5.0, but the oxidation of ferrous iron was not oxygen dependent (see Fig. 4). Ferrous iron oxidation by cell extracts was dependent on cellular cytochrome c reduction. The oxidation of iron and the reduction of cyt. c took place equally as well anaerobically as aerobically. Once the cellular cyt. c was reduced the reaction stopped and there was no reoxidation of the reduced cyt. c, although cytochrome oxidase was present in the extracts (Figs. 4 and 5).

Attempts to prepare cellular extracts capable of manometric iron oxidation failed although all of the extracts contained both ferrous iron-cyt. c reductase and cytochrome

TABLE IX

Effect of sonic oscillation on iron oxidase activity

Duration of oscillation (min)	μ l O ₂ consumed per hour per 20 mg (wet weight) extract ^a	
	pH 3.0 ^d	pH 5.0 ^c
0	280 ^b	160
10	60 ^b	30
20	0	0
30	0	0
40	0	0
50	0	0
60	0	0

^a The extract was prepared by sonicating a 1:10 suspension of intact cells (wet weight) in 0.1 M Na acetate-acetic acid buffer (pH 5.5) in a 10 KC Raytheon for various time intervals.

^b These readings were obtained with a 1:4 dilution of the sonicated cells in 0.1 M Na acetate-acetic acid buffer (pH 5.5).

^c Assayed manometrically as described in Materials and Methods.

^d For assay see Figs. 1 and 2.

oxidase activities. The inability of cellular extracts to oxidize ferrous iron manometrically seems to be due to a blockage of the electron transport chain between cyt. c and cyt. a. FAD, FMN or the quinone isolated from intact cells did not overcome this blockage in the electron transport chain.

III. Ferrous Iron-Cytochrome c Reductase

Since studies with whole cells and crude extracts indicated that the primary enzyme in ferrous iron oxidation was Fe^{++} -cyt. c reductase, this enzyme was purified and the mechanism of the reaction was studied. Ferrous iron-cyt. c reductase was found to be tightly bound to the cell and the enzyme was not easily solubilized, thus necessitating the use of extreme conditions for extraction (a high pH extraction buffer and an extended duration of sonication).

Enzyme purification

All manipulations during the enzyme purification were carried out at $0-5^{\circ}\text{C}$ unless otherwise stated. Crude cell-free extracts of Ferrobacillus ferrooxidans (150,000 x g supernatant, see Materials and Methods) were adjusted to

pH 7.0 with 10 N sulfuric acid. The extract was then heated rapidly to 90-95° C in a boiling water bath and maintained at this temperature for five minutes. The extract was cooled quickly to 5° C in an ice-water bath and was centrifuged at 25,000 x g for 15 minutes.

The resultant 25,000 x g supernatant was adjusted to pH 5.7 with 1 N acetic acid. Calcium phosphate gel suspended in 0.01 M acetate-acetic acid buffer (pH 5.7) was added slowly, with stirring, until the concentration of gel:protein was 5:1 (dry weight). The mixture was stirred slowly for 30 minutes, then centrifuged at 25,000 x g for 15 minutes. The precipitate was discarded and cold ethanol (-20° C) was added with constant stirring to the enzyme incubated in an ice-salt bath. The enzyme temperature was maintained below 0° C during the ethanol addition.

There was no formation of precipitate until the ethanol concentration reached 60%. Since the precipitate was slow in forming, the ethanol-enzyme solution was incubated at -20° C for 12 hours to allow maximum precipitation to occur. The ethanol precipitate was centrifuged down in a pre-cooled centrifuge (-20° C) at 25,000 x g for 15 minutes. The precipitated enzyme was

solubilized with 5 ml of 0.01 M acetate-acetic acid buffer (pH 5.7). The enzyme was then dialysed for 12 hours against 2 changes of 4 l 0.01 M acetate-acetic acid buffer (pH 5.7). Further purification was attempted using $(\text{NH}_4)_2\text{SO}_4$ precipitation but the enzyme was labile at high salt concentrations and no further purification was achieved.

DEAE-cellulose adsorbed the enzyme very strongly. A 1 x 10 cm DEAE-cellulose column prepared by the method of Sober et al. (1956) was equilibrated with 0.01 M acetate-acetic acid buffer (pH 5.7). After the enzyme was adsorbed (1-2 mg protein) a linear gradient of 0-2 M NaCl in 0.01 M acetate-acetic acid buffer (pH 5.7) was applied to the column in order to elute the enzyme. The DEAE-cellulose chromatography resulted in two patterns of elution. When the column was tightly packed and slow-flowing two peaks were eluted, while only one peak was eluted from a loosely packed, fast-flowing column (Figs. 6 and 7). The peaks were found by measuring the optical density at 260 and 280 m μ of 5 ml eluent fractions.

The fractions constituting the peaks were pooled and concentrated by dialysis in a saturated solution of sucrose (0.01 M acetate-acetic acid buffer (pH 5.7)). The sucrose

Fig. 6. Gradient elution of Fe^{++} -cyt. c reductase from a fast-flowing (2.0 ml/min.) DEAE-cellulose column.

260 m μ —●—●—

280 m μ ..●.....●..

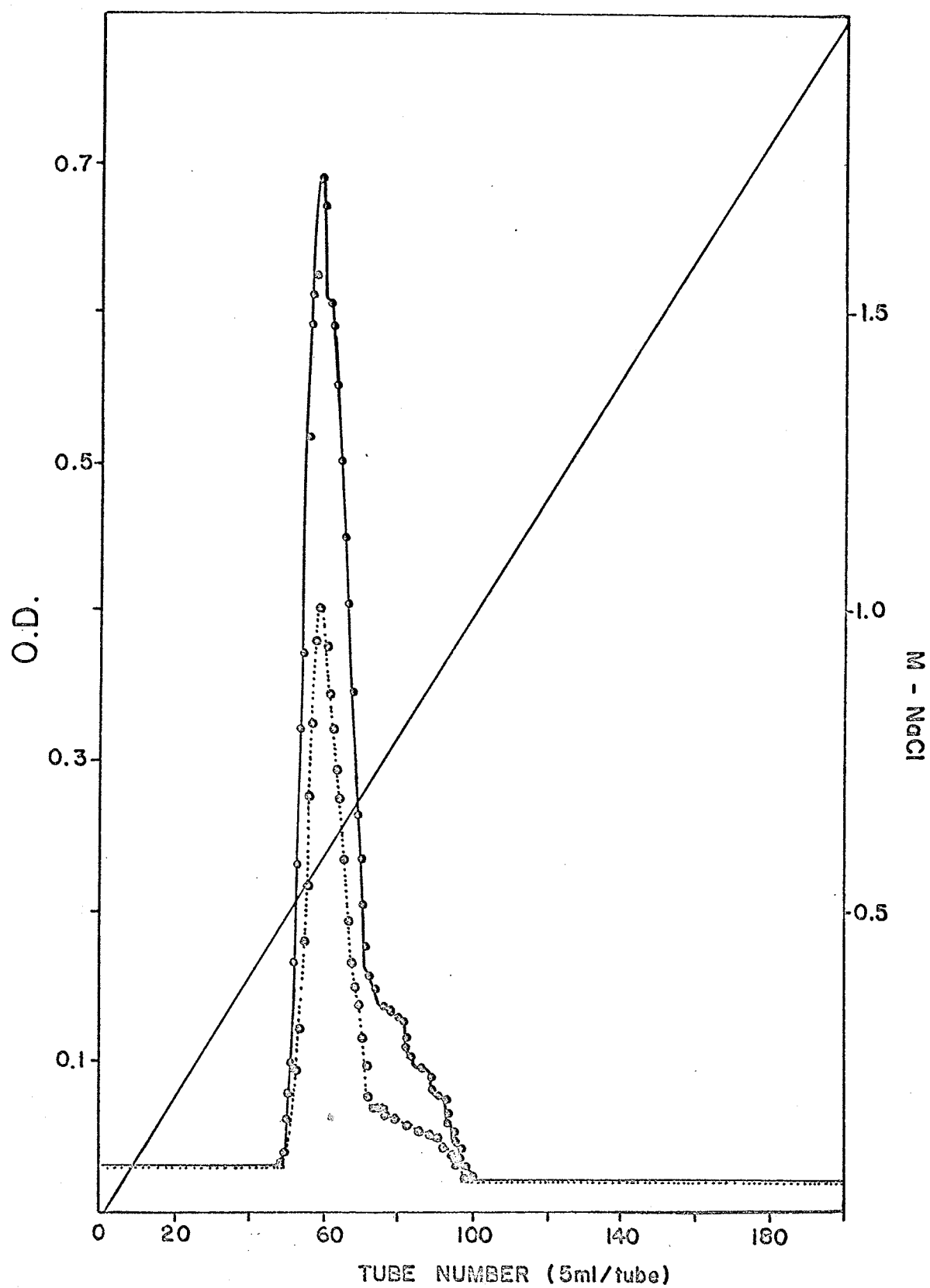
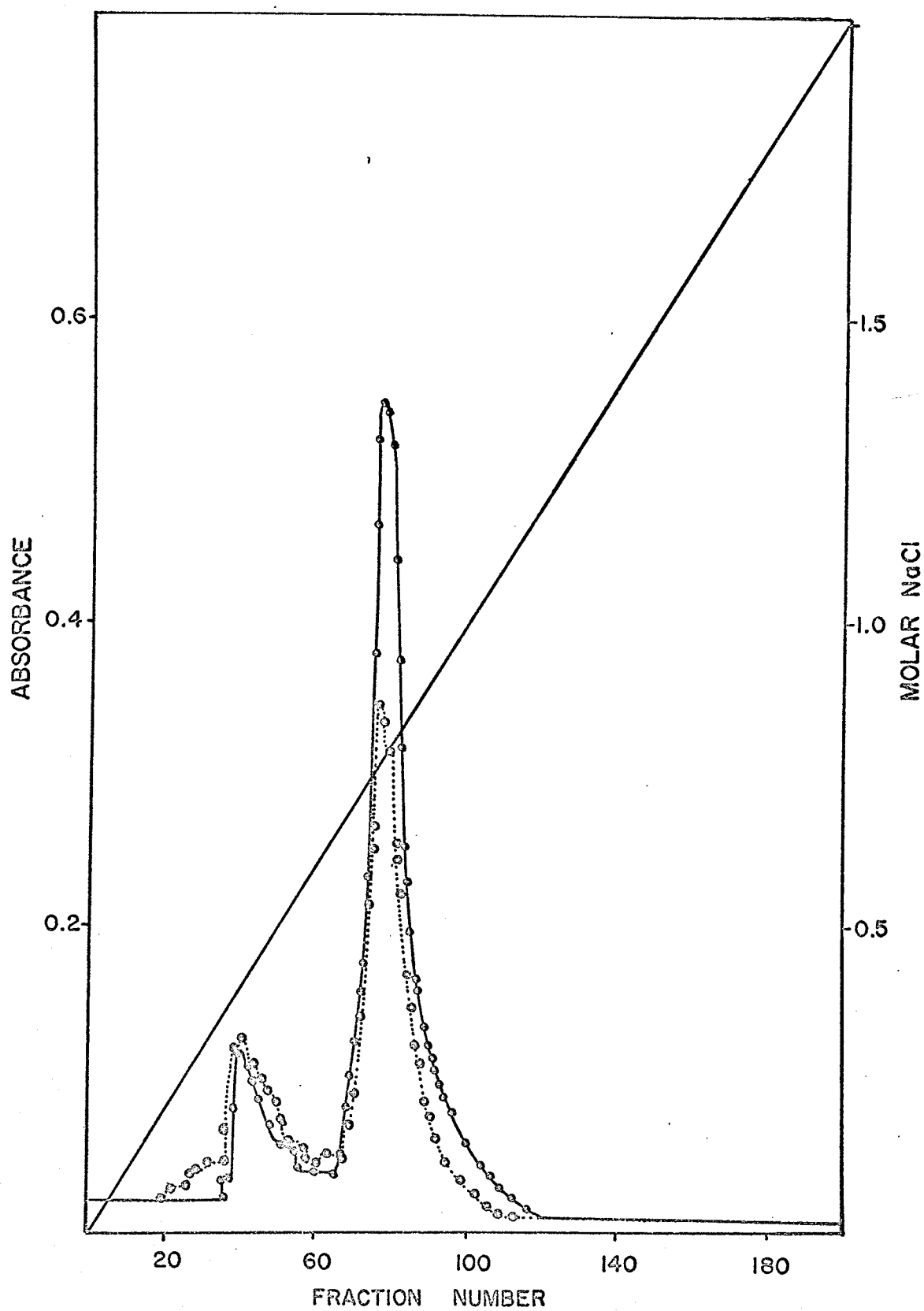


Fig. 7. Gradient elution of Fe^{++} -cyt. c reductase from a slow-flowing (0.5 ml/min.) DEAE-cellulose column.

260 m μ —●—●—

280 m μ ..●.....●..



was then dialysed out against 2 changes of 4 l 0.01 M acetate-acetic acid buffer (pH 5.7). Only the single peak eluted from the fast-flowing column exhibited enzyme activity. Neither peak eluted from the slow-flowing column exhibited any enzyme activity and attempts to mix the two peaks to reactivate the enzyme failed.

Because the single peak eluted from the fast-flowing column showed no increase in specific activity, DEAE-cellulose chromatography was not included in the purification scheme. The ethanol-precipitated enzyme was purified 60-70 times with a 20% recovery and the specific activity was greater than that achieved by Blaylock and Nason (1963). The purification scheme is outlined in Table X.

Enzyme storage

Fe^{++} -cyt. c reductase could be stored at $0-5^{\circ}\text{C}$ for one week without any loss in activity. At $0-5^{\circ}\text{C}$ the enzyme was dialysed against 0.01 M acetate buffer (pH 5.7) for periods up to 24 hours without subsequent loss in activity. At room temperature the enzyme retained complete activity after six hours incubation. Routinely, the enzyme was stored either undiluted or diluted at -20°C without any

TABLE X

Purification of Fe^{++} -cyt. c reductase

Fraction	Total protein (mg)	Total activity (units) ^a	Specific activity (units/mg protein)	% Recovery	Purification
150,000 x g supernatant	370.6	345,000	930	100	1.0
Heat stable supernatant	35.0	243,000	6,940	70.4	7.4
$\text{Ca}_3(\text{PO}_4)_2$ supernatant	4.55	135,000	29,700	39.1	31.9
Ethanol precipitate 0-60%	1.09	63,000	58,100	18.3	63.4

^aOne unit of enzyme is defined as that amount of enzyme which causes an increase in optical density of 0.001 per minute at 550 mμ.
The enzyme was assayed as described in Materials and Methods.

appreciable loss in activity for periods up to 6 months.

Purity of Fe^{++} -cyt. $\underline{c}$ reductase

All preparations of purified Fe^{++} -cyt. $\underline{c}$ reductase contained nucleic acid similar to the enzyme purified by Blaylock and Nason (1963). The presence of extraneous proteins in the purified enzyme was determined by disc electrophoresis. Samples (50-150 μg protein) were run in Tris-glycine buffer (pH 8.3), then stained with Amido black. The resultant gels revealed one dark, broad band and two faint, narrow bands. These results indicated that the enzyme preparation was approximately 90-100% pure. The results of the gel electrophoresis were supported both by DEAE-cellulose chromatography (fast-flowing column), where only one band was eluted (Fig. 6), and by molecular weight studies, where all the protein was found in only one band (Figs. 17 and 18). No attempt was made to stain specifically the active enzyme band in the gel since no artificial electron acceptor could be found for the enzyme.

Effect of pH on enzyme activity

The effect of pH on enzyme activity was tested using

various buffers; only one buffer proved to be satisfactory. Blaylock and Nason (1963) routinely assayed Fe^{++} -cyt. c reductase in 8 mM acetate buffer (pH 5.7) and showed that the enzyme activity has no pH optimum in 8 or 80 mM acetate buffers. These authors reported that a plateau was evident at pH 5.7-6.0 at a concentration of 8 mM. In this investigation similar results were obtained using acetate buffer at the same concentrations (8 and 80 mM). The results obtained with acetate buffer are illustrated in Figs. 8 and 9. The higher concentration acetate buffer (80 mM) increases the non-enzymatic reaction and decreases the enzymatic reaction (ion effect).

When cytochrome c, an extremely basic protein, was added to the 8 mM acetate buffered reaction mixture, the pH immediately rose to pH 7.5-7.8, depending on the initial pH of the acetate buffer (pH 5.25 - 6.5). In the presence of such a high concentration of cyt. c the 8 mM acetate buffer proved to be too weak to maintain a constant pH. The pH values reported in Fig. 8 represent the initial pH of the acetate buffer rather than the actual pH of the reaction mixture. Although the 80 mM acetate buffer was shown to have better buffering action, its use was limited because of

Fig. 8. Relation of Fe^{++} -cyt. c reductase activity to pH using 24 μmoles Na acetate-acetic acid buffer.

Non-enzymatic reaction —●—●—

Total reaction —x—x—

Enzymatic reaction —o—o—

The reaction was carried out as described in

Materials and Methods with 6 μg protein.

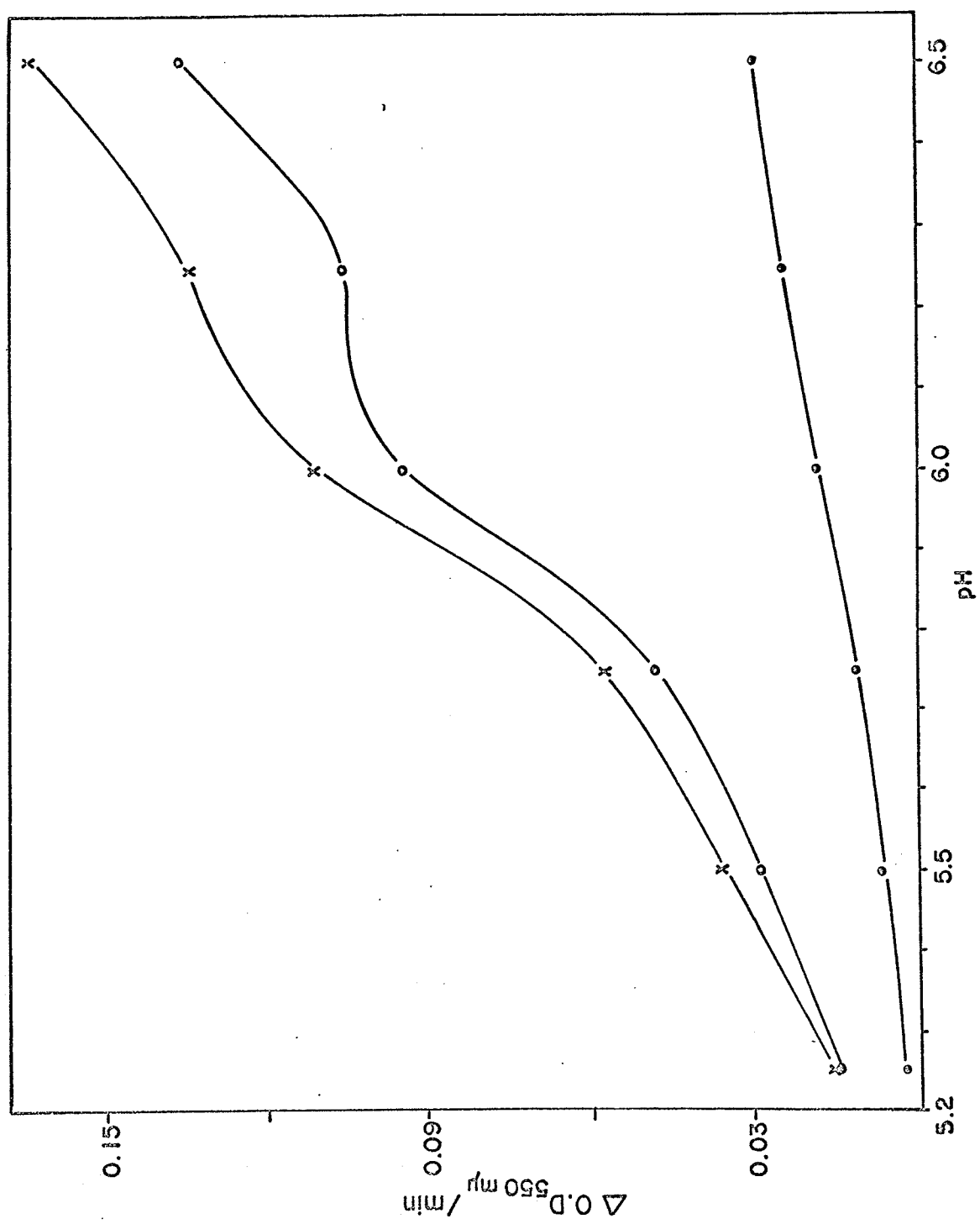


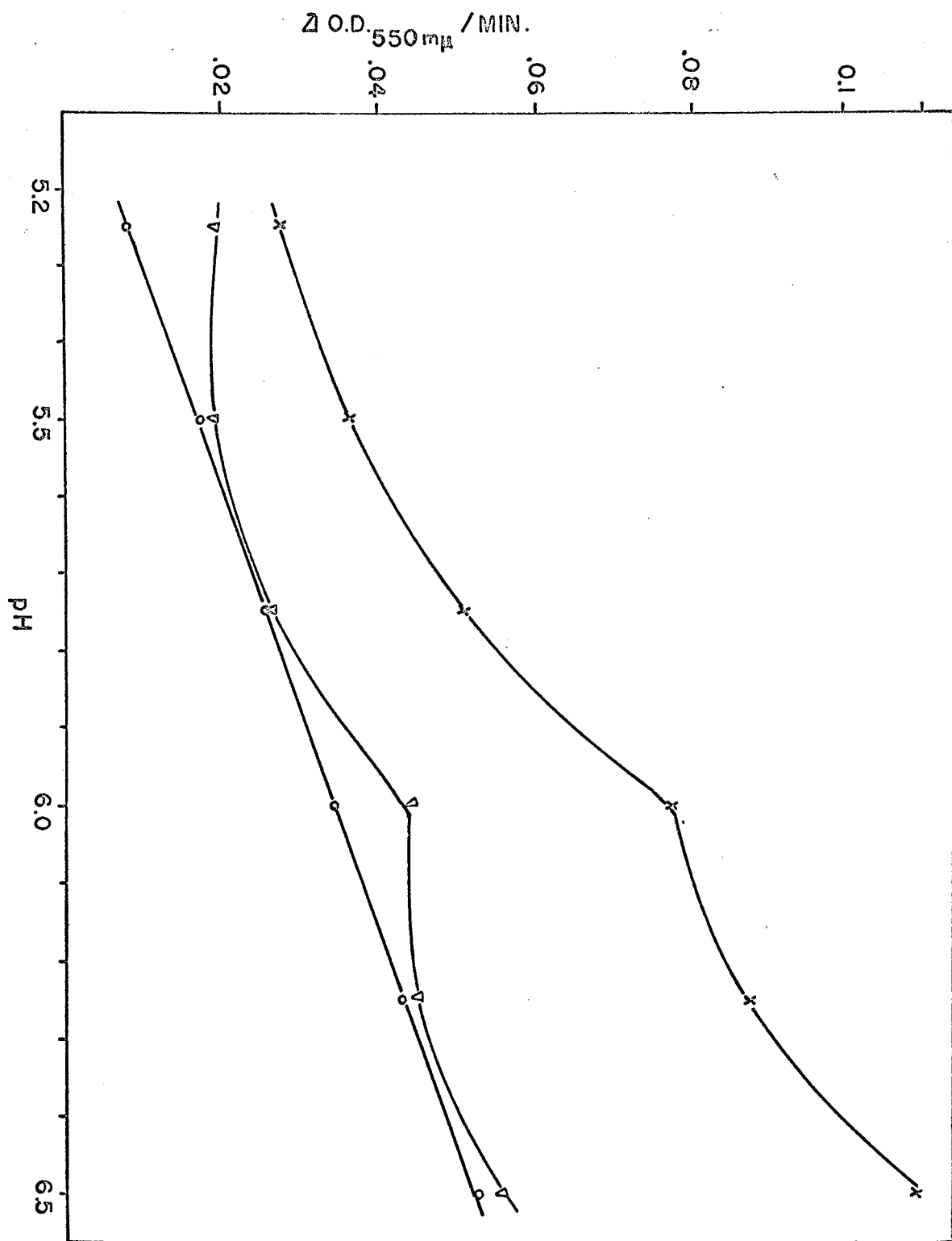
Fig. 9. Relation of Fe^{++} -cyt. c reductase activity to pH
using 240 μmoles Na acetate-acetic acid buffer.

Non-enzymatic reaction —●—●—

Total reaction —x—x—

Enzymatic reaction —v—v—

The reaction was carried out as described in
Materials and Methods with 6 μg protein.



its inhibitory effect on enzyme activity (Fig. 9).

Other buffers were tried since it was necessary to find a good buffer system for kinetic studies where varied concentrations of cyt. c were often used. Sodium barbital buffer was found to be most satisfactory in maintaining a constant pH level in the reaction mixture. Cyt. c addition to the reaction mixture had no effect on the initial pH of the assay mixture and the enzyme, when aerobically assayed, exhibited an optimum activity at pH 7.0 (Fig. 10). Anaerobically, barbital buffer (8 mM) exhibited a reduced non-enzymatic reaction which did not rise above pH 7.0 as rapidly as under aerobic conditions. There was no optimum pH evident anaerobically in the pH range studied. The enzyme was routinely assayed aerobically in 8 mM sodium barbital buffer (pH 7.0). The results obtained using barbital buffer are shown in Figs. 10 and 11.

Effect of substrate concentration on enzyme activity

Ferrous iron-cytochrome c reductase catalyses the following reaction:

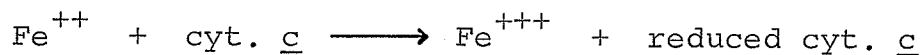


Fig. 10. Relation of Fe^{++} -cyt. c reductase activity to pH
using $24 \mu\text{moles}$ Na barbital-acetic acid buffer.

Non-enzymatic reaction -o—o-

Total reaction -x—x-

Enzymatic reaction -v—v-

The reaction was carried out as described in
Materials and Methods with $4.5 \mu\text{g}$ protein.

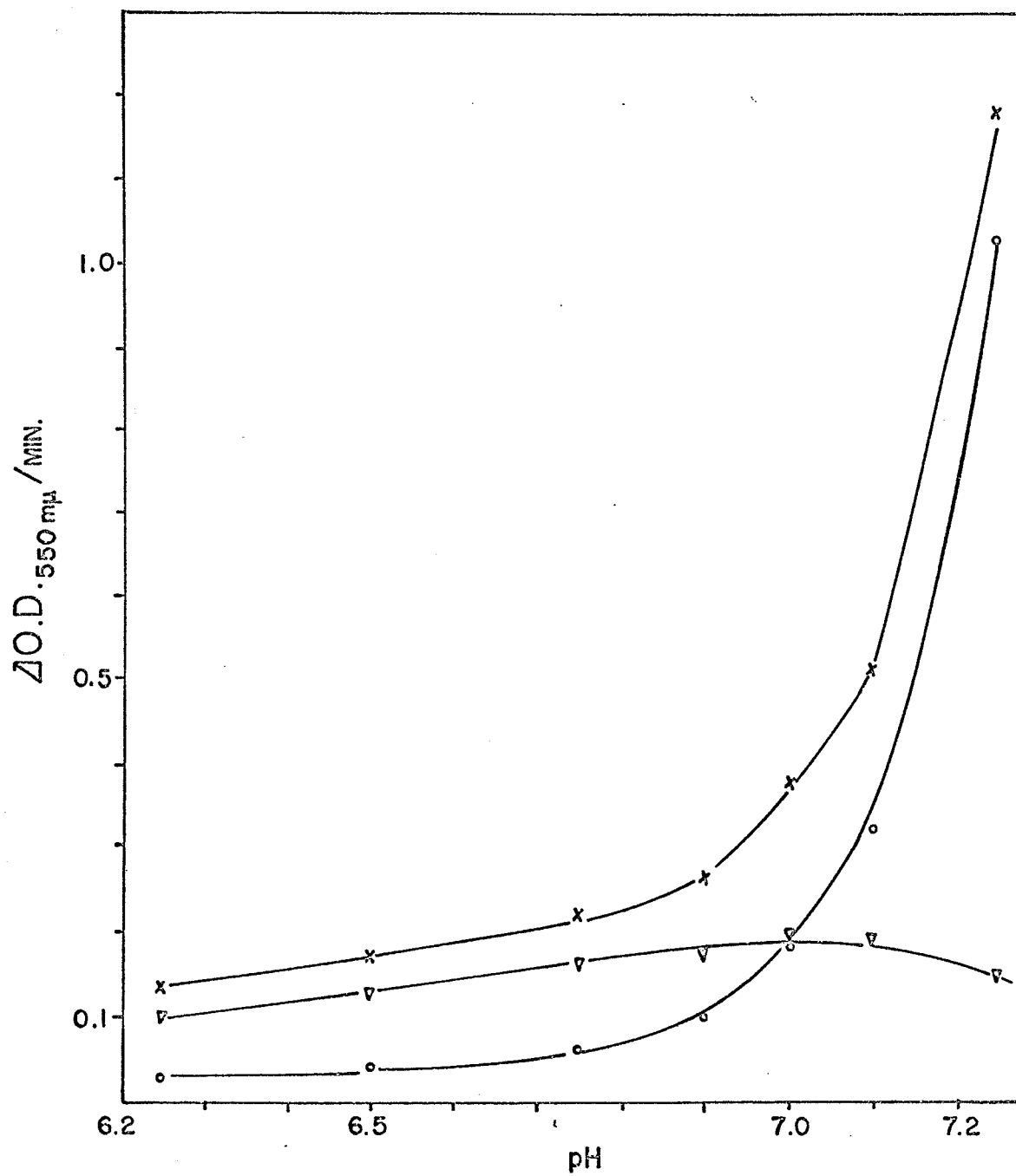


Fig. 11. Relation of Fe^{++} -cyt. c reductase activity to pH using 24 μmoles Na barbital-acetic acid buffer under anaerobic conditions.

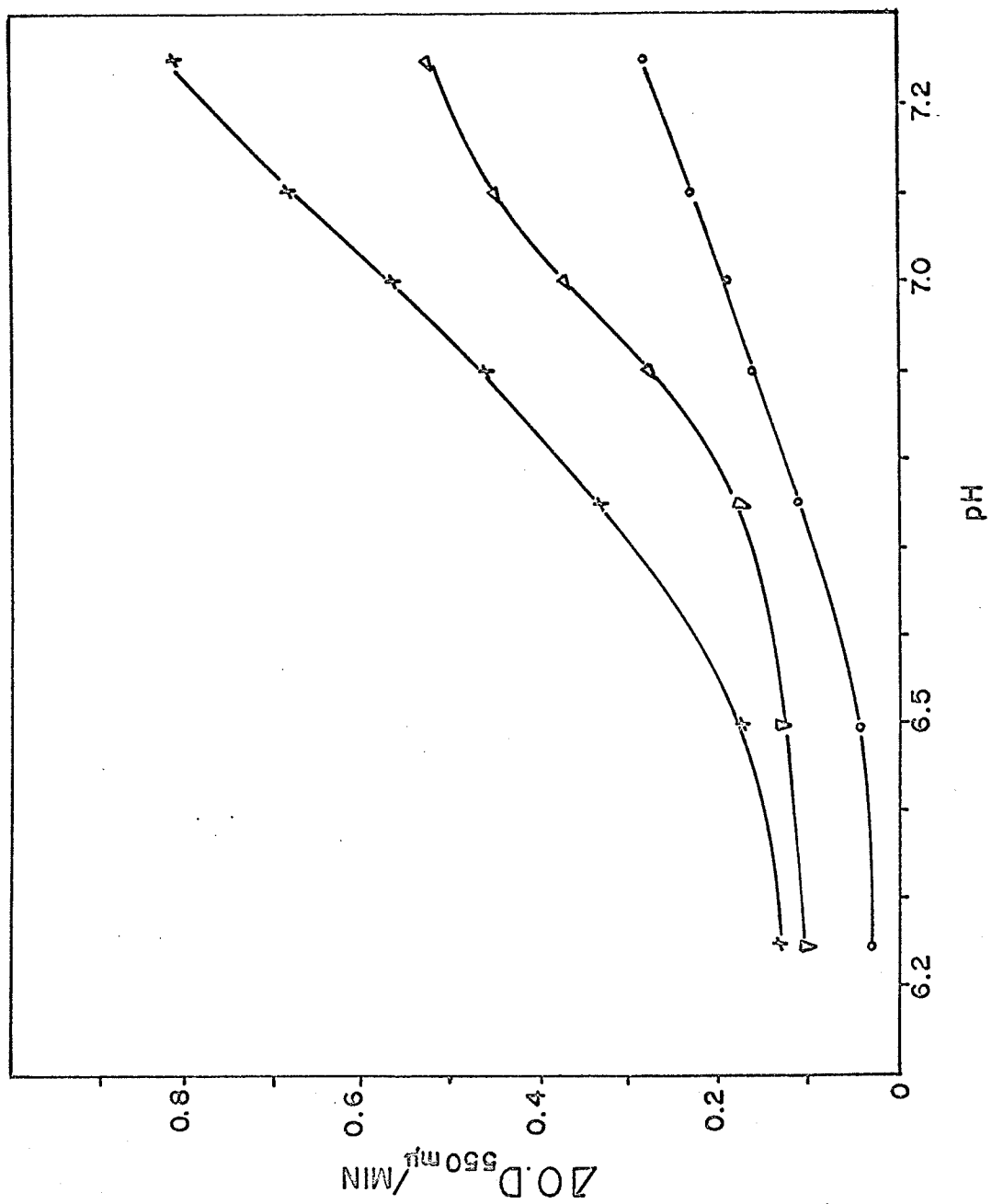
Non-enzymatic reaction -o-o-

Total reaction -x-x-

Enzymatic reaction -v-v-

The reaction was carried out as described in

Materials and Methods with 4.5 μg protein.



The effect of both substrates, ferrous iron and cytochrome c, on enzyme activity was measured by assaying the initial reaction velocity while varying the concentration of one substrate. At the same time, the concentration of the second substrate was maintained at a constant level. Under these conditions the concentration of the first substrate (S) is related to the initial velocity (v) according to the equation:

$$v = \frac{V'_f}{1 + \frac{K'_s}{(S)}}$$

This is easily transposed to the double reciprocal form of Lineweaver and Burk (1934)

$$\frac{1}{v} = \frac{K'_s}{V'_f} \frac{1}{(S)} + \frac{1}{V'_f}$$

where K'_s is the apparent Michaelis constant of the varied substrate (S) (either ferrous iron or cytochrome c) and V'_f is the apparent maximum velocity. When data is plotted in the double reciprocal form, K'_s values may be calculated from intercept on the horizontal axis, where $\frac{1}{v} = 0$ and $\frac{1}{(S)} = -\frac{1}{K'_s}$

(see Figs. 13 and 15).

The results of the effect of ferrous iron on enzyme activity are shown in Figs. 12 and 13. At low ferrous iron concentrations enzyme activity increased in a normal manner, but at higher concentrations the curve deviated from the normal hyperbolic function and exhibited substrate inhibition. The enzyme was saturated by 4 mM ferrous iron. Similar results were obtained with cyt. c, which indicated that the enzyme was saturated at a 0.15 mM concentration (see Figs. 14 and 15).

The double reciprocal replots of Figs. 12 and 14 revealed apparent Michaelis constants of 1.0 mM and 0.095 mM for ferrous iron and cyt. c respectively. Blaylock and Nason (1963) reported apparent K_m 's of 0.15 mM and 0.08 mM for ferrous iron and cyt. c. Their results of substrate saturation are at variance with the results of this investigation. The concentrations of substrates that saturated the enzyme (Figs. 12 and 14) were used in all routine assays (4 mM ferrous iron and 0.15 mM cytochrome c, see Materials and Methods).

Other substances were tried in attempts to substitute for ferrous iron but no other electron donor could replace

Fig. 12. Effect of ferrous iron concentration on Fe^{++} -
cyt. c reductase activity.

The reaction mixture contained: 0.6 μmoles oxidized cyt. c, 24 μmoles Na barbital-acetic acid buffer (pH 7.0), 12 μg protein, Fe^{++} as indicated, and H_2O in a final volume of 3.0 ml.

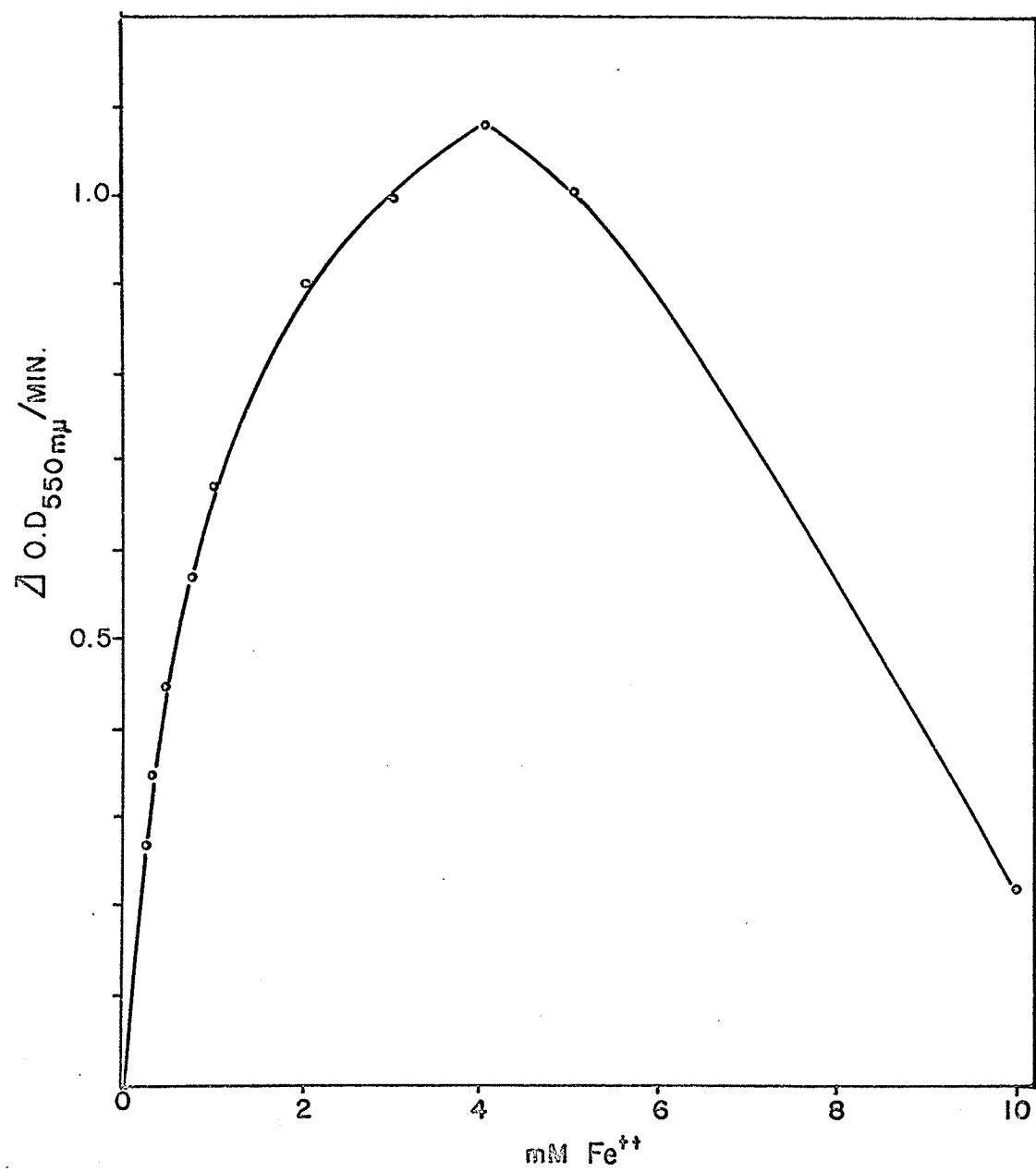


Fig. 13. Double reciprocal plot of Fig. 12.

v = difference in $OD_{550 \text{ m}\mu}/\text{min.}$

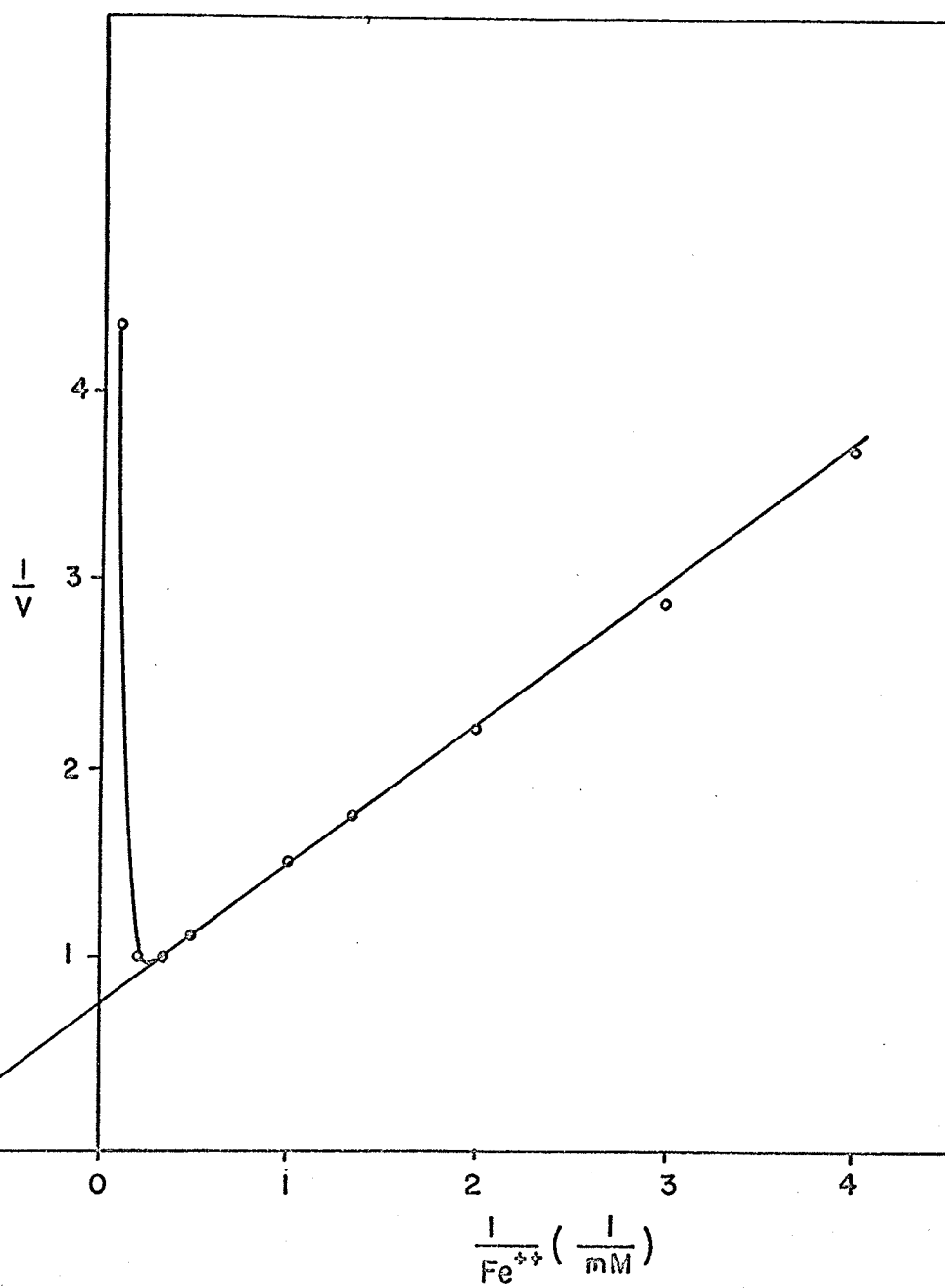


Fig. 14. Effect of oxidized cyt. c concentration on Fe^{++} -
cyt. c reductase activity.

The reaction mixture contained: 7.5 μmoles Fe^{++} ,
24 μmoles Na barbital-acetic acid buffer (pH 7.0), 12 μg
protein, oxidized cyt. c as indicated, and H_2O in a final
volume of 3.0 ml.

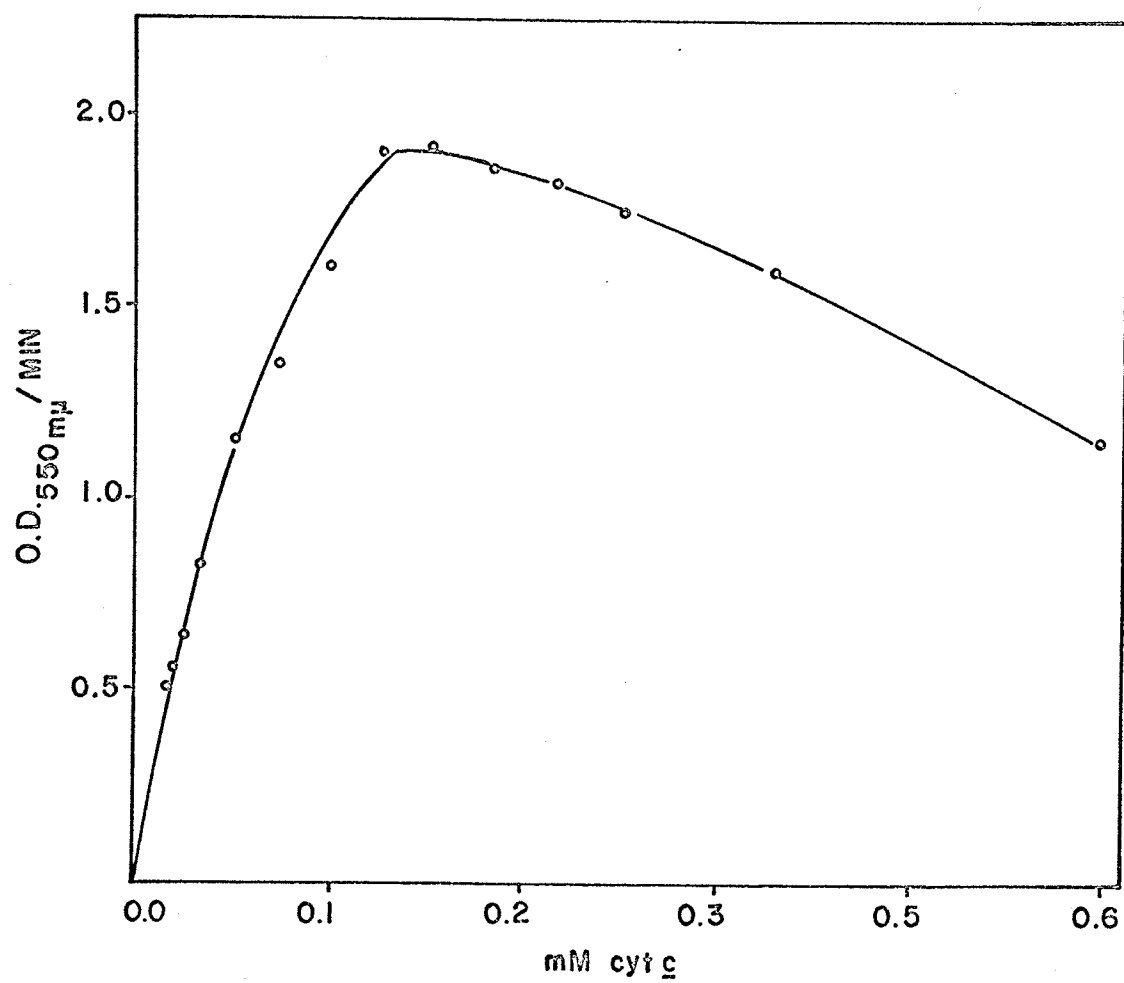
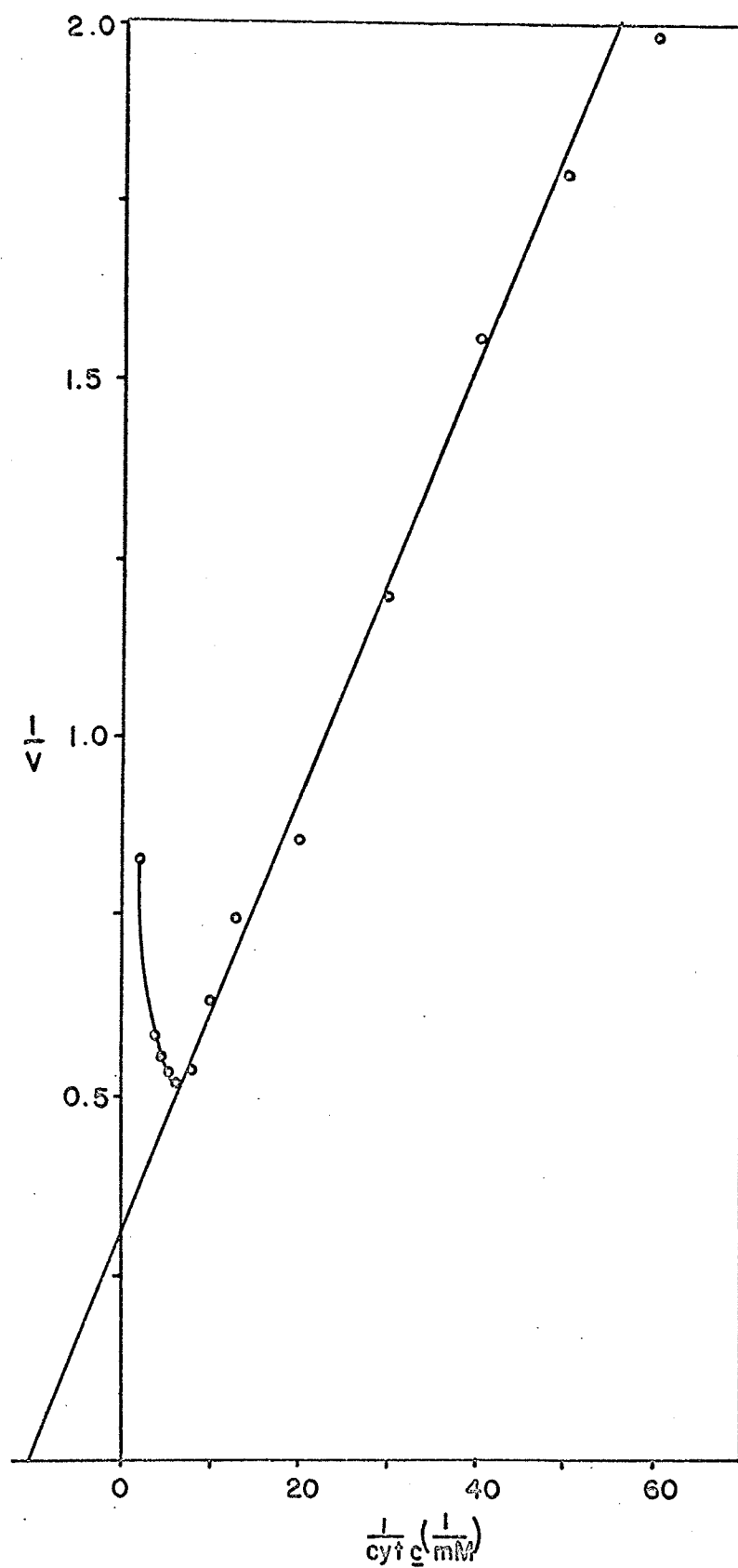


Fig. 15. Double reciprocal plot of Fig. 14.

v = difference in $OD_{550 \text{ m}\mu}/\text{min.}$



ferrous iron. The following substances were tried: NADH, NADPH, Co^{++} , Mn^{++} , Cu^+ , and Hg^+ . Artificial acceptors - dichlorophenolindophenol, phenazine methosulfate, methylene blue, tetrazolium chloride, and benzyl viologen - would not substitute for cytochrome c; consequently it was impossible to use an easily permeable electron acceptor for identification of the active enzyme in gel electrophoresis.

Effect of inhibitors

Ferrous iron - cyt. c reductase was weakly inhibited by sulfhydryl inhibitors. This inhibition appeared to be ionic rather than specific binding to sulfhydryl groups, as demonstrated by results with mercuric chloride (Table XI). When mercuric chloride was added to the assay mixture the reaction was substantially inhibited, but upon dialysis in 0.01 M acetate-acetic acid buffer (pH 5.7) the enzyme activity returned to normal levels. If the mercury was bound to the sulfhydryl group(s) of the enzyme, the inhibition should have remained after dialysis. The ability of the enzyme to overcome the inhibition of mercury salts after dialysis indicates that sulfhydryl groups are not required for catalysis.

Blaylock and Nason (1963) reported that Fe^{++} -cyt. c reductase was inhibited by atabrin, thus indicating the possible involvement of flavin(s) in catalysis. However, these authors were unable to demonstrate the presence of flavin(s) in the enzyme. Although atabrin does inhibit the enzyme, this inhibition also appears to be ionic rather than specific binding of the enzyme at the flavin site(s). The inability of ultra violet light, a potent inhibitor of flavin enzymes, to inhibit Fe^{++} -cyt. c reductase indicates that flavins are not involved in the reaction. When Fe^{++} -cyt. c reductase was excited at 350-375 m μ the enzyme did not fluoresce. This fluorescence, at 350-375 m μ excitation, is characteristic of some flavin enzymes (Massey, 1963). These results lead to the conclusion that Fe^{++} -cyt. c is not a flavin enzyme. As a further proof, the absorption spectrum (Fig. 16) of Fe^{++} -cyt. c reductase failed to exhibit any of the spectral properties of flavin enzymes. The results of inhibition by flavin and sulfhydryl inhibitors are shown in Table XI.

The effect of proteolytic enzymes and ribonuclease (RNase) on Fe^{++} -cyt. c reductase was tested by incubating 100 μg of the various enzymes with Fe^{++} -cyt. c reductase for

TABLE XI

Effect of sulphhydryl and flavin inhibitors
on Fe^{++} -cyt. c reductase activity

Inhibitor	Concentration	Activity (% of control)
N-Ethylmaleimide	10^{-3} M	81
	10^{-4} M	92
<u>p</u> -Hydroxymercuribenzoate	10^{-3} M	61
	10^{-4} M	96
HgCl_2	10^{-3} M	16
	10^{-4} M	28
	10^{-4} M (dialysed)	104
Atabrin	10^{-3} M	37
	10^{-4} M	74
U.V. light ^a	1 hour	100

The reaction was carried out under standard conditions as described in Materials and Methods with 20 μg protein.

^a20 μg protein (0.15 ml) and 0.4 ml 0.01 M Na barbital-acetic acid buffer (pH 7.0) were placed 4 inches below a Westinghouse germicidal lamp and allowed to incubate for one hour prior to assay of the enzyme.

one hour prior to enzyme assay. None of the proteases - trypsin, α -chymotrypsin, carboxypeptidase, and subtilisin - affected enzyme activity. Ribonuclease, on the other hand, exhibited a 25-30% inhibition of enzyme activity. This inhibition was immediate, requiring no preincubation, thus leading to the conclusion that it was not due to the hydrolysis of the RNA-portion of the enzyme. Blaylock and Nason (1963) also reported that Fe^{++} -cyt. c reductase showed remarkable stability in the presence of various proteases, DNase, and RNase. Table XII illustrates the effect of proteolytic enzymes and RNase on Fe^{++} -cyt. c reductase activity. Fe^{++} -cyt. c reductase activity was also inhibited by ionic inhibitors (Na acetate, NaCl, etc.). This ionic inhibition was evident when enzyme activity was compared in 8 and 80 mM acetate buffer (see Figs. 8 and 9). The inhibition by sodium chloride was studied in more detail and the results are shown in Figs. 31 and 32 (see Kinetic Analysis).

Effect of some potential activators on enzyme activity

Mora, Creskoff, and Person (1965) reported that certain carbohydrates were able to reduce cytochrome c.

TABLE XII

Effect of proteolytic enzymes and ribonuclease
on the activity of Fe^{++} -cyt. c reductase

Enzyme	Incubation time (minutes)	Activity (% of control)
Trypsin	60	100
Carboxypeptidase	60	100
α -Chymotrypsin	60	100
Subtilisin	60	100
Ribonuclease	60	70
	0	77

The reaction was carried out under standard conditions as described in Materials and Methods with 34.5 μg protein. The enzymes (100 μg) were incubated with Fe^{++} -cyt. c reductase (0.15 ml) in a total volume of 0.55 ml Na barbital-acetic acid buffer (pH 7.0) at 25° C prior to the start of the standard assay.

This reduction of cytochrome c was a time-dependent reaction in contrast to the instantaneous oxidation or reduction caused by oxidizing or reducing agents (ascorbate and ferricyanide). More recently, Person, Fine, Mora, and Zipper (1965) reported on the reversible polyion reduction and oxidation of cytochrome c. These workers found that polycations reduced cytochrome c, while polyanions oxidized reduced cytochrome c. Since these results were unexpected and not completely explained, the effect of some of the substances causing reduction of cytochrome c was tested with Fe^{++} -cyt. c reductase in a hope that these compounds may convert cyt. c to a more readily reducible conformation.

None of the sugars used - ribose, glucose, or arabinose - had any effect on Fe^{++} -cyt. c reductase activity. Only RNase had any effect, and this effect was inhibitory rather than stimulatory. Possibly, the reason for the absence of stimulation of enzyme activity was the short duration of assay (1 minute), whereas reduction of cyt. c with these compounds was observed by the above workers only after a long duration (2-24 hours).

The effect of cytochrome c monomer and polymers (Margoliash and Lustgarten, 1962) on Fe^{++} -cyt. c reductase

was also assayed to determine which form the enzyme preferred as its substrate. Although the dimer form (polymer) was not purified, the enzyme showed no preference for either form (monomer or polymer). The results of the effects of these substances on Fe^{++} -cyt. c reductase activity are shown in Table XIII.

The formation of enzyme subunits

Fe^{++} -cyt. c reductase is associated with nucleic acid when isolated (Blaylock and Nason, 1963). The enzyme was dissociated into subunits by adsorption on a DEAE-cellulose column and by slow elution with 0-2 M NaCl in 0.01 M acetate-acetic acid buffer (pH 5.7) (see Fig. 7). A similar result was obtained when the enzyme was treated with DEAE-cellulose in a batch process. Fe^{++} -cyt. c reductase (1-2 mg) was incubated with DEAE-cellulose (5 ml bed volume in a total volume of 10 ml 0.5 M NaCl, 0.01 M acetate-acetic acid buffer (pH 5.7)) in the cold ($0-5^{\circ}\text{C}$) and stirred slowly for 12 hours.

The enzyme-DEAE-cellulose mixture was then poured into a 1 x 10 cm column and was slowly eluted with 10 ml 0.5 M NaCl, 0.01 M acetate-acetic acid buffer (pH 5.7).

TABLE XIII

Effect of some potential activators
on Fe^{++} -cyt. c reductase activity

Activator	Concentration	Relative activity (%)
None	-	100
Cyt. <u>c</u> monomer ^a	1.5×10^{-4} M ^b	100
Cyt. <u>c</u> dimer ^a	1.5×10^{-4} M ^b	100
D-Ribose	1.2×10^{-3} M	100
D-Glucose	1.2×10^{-3} M	100
D-Arabinose	1.2×10^{-3} M	100
Ribonuclease	100 μg /assay	77

^aUsed in place of untreated cyt. c utilized in standard assays.

^bBased on a molecular weight of 12,270 (Type III cyt. c, Sigma).

The reaction was carried out as described in Materials and Methods with 19.2 μg protein.

When the liquid level reached the top of the DEAE-cellulose the elution was stopped. This first elution at 0.5 M NaCl contained the protein portion of the enzyme and corresponded to the first peak eluted from the slow-flowing DEAE-cellulose column during chromatography (Fig. 7).

The column was next eluted with 15 ml 1.0 M NaCl, 0.01 M acetate-acetic acid buffer (pH 5.7). This second fraction, consisting of the RNA portion of the enzyme, corresponded to the second peak eluted from a slow-flowing DEAE column (see Fig. 7). These results were quite similar to those of Symons (1965), who used DEAE-cellulose chromatography to separate the DNA component from yeast cytochrome b_2 .

The two fractions were then concentrated by dialysis in a saturated sucrose solution (0.01 M acetate-acetic acid buffer (pH 5.7)); the sugar and NaCl were dialysed out against 2 changes of 4 l 0.01 M acetate-acetic acid buffer (pH 5.7).

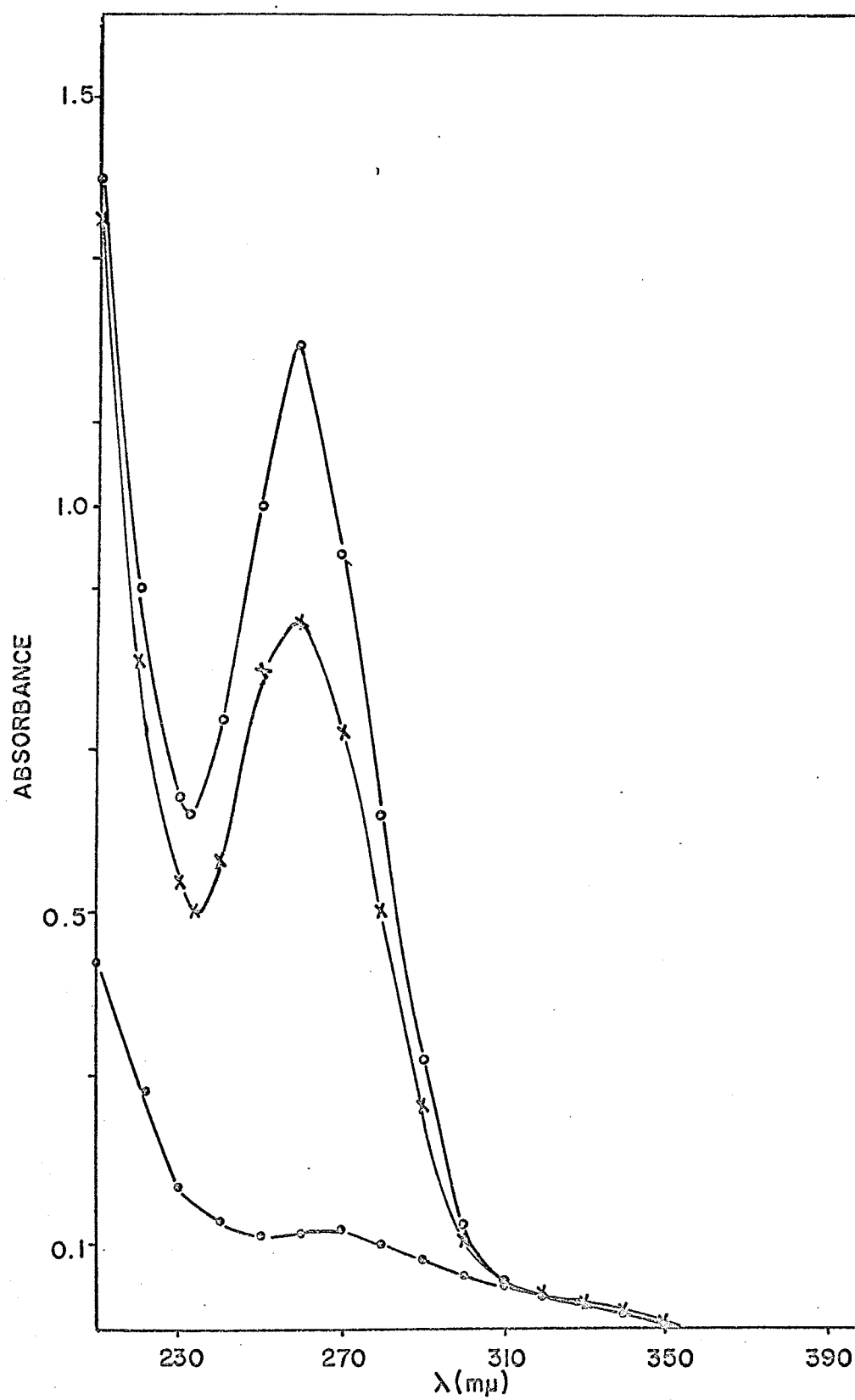
Absorption spectra of the enzyme and its subunits were recorded in a Unicam SP700 spectrophotometer which revealed differences in the compositions of the subunits (Fig. 16). Both methods of forming enzyme subunits were

Fig. 16. Absorption spectra of Fe^{++} -cyt. c reductase holoenzyme and its protein and nucleic acid subunits.

Holoenzyme -x—x-

RNA subunit -o—o-

Protein subunit -●—●-



equally effective; however, the batch method was much simpler and the results were always consistent.

The first fraction eluted from the DEAE-cellulose contained all of the protein adsorbed (see Table XIV), but no Fe^{++} -cytochrome c reductase activity was found in this subunit. Since the second subunit contained RNA (see Table XV), attempts were made to anneal the protein and RNA subunits by the method of Hall and Spiegelman (1961). Mixtures of the two subunits were boiled and slowly cooled, or heated to 70°C and slowly cooled, in attempts to reconstitute the original holoenzyme.

Restoration of the original catalytic activity of the holoenzyme, by mixing equal quantities of the subunits or by annealing, was not successful. Incubation of the two subunits in dioxane 5-25%, ethanol 10-60%, and β -mercapto-ethanol 10^{-4} M also failed to reactivate the original catalytic activity. The subunits were mixed in 0.5 M NaCl, and 1.0 M NaCl, 0.01 M acetate-acetic acid buffer (pH 5.7) and allowed to incubate for 6 hours prior to dialysis against 2 changes of 0.01 M acetate-acetic acid buffer (pH 5.7). These attempts also failed to reactivate the enzyme. Molecular weight determinations indicated that

there was no change in the original subunits and that no new species was formed.

Yeast RNA and ribose did not restore the original catalytic activity to the protein subunit. Later studies showed that the RNA subunit formed a higher molecular weight species than the original holoenzyme. All of the methods attempted failed to split the RNA subunit into the smaller molecular weight species necessary, perhaps, for restoration of the original enzyme structure and catalysis.

Composition of Fe^{++} -cyt. c reductase and its subunits

Fe^{++} -cyt. c reductase must be preincubated with cyt. c prior to assay or the reaction rate is not linear (Blaylock and Nason, 1963). During the preincubation period the optical density at 550 m μ was recorded and it was noticed that there was an ever decreasing oscillation in the optical density, which was finally damped after 4-5 minutes. The optical density showed an increase above the original optical density of the cytochrome c solution. An absorption spectrum was recorded before and after the addition of enzyme; it was observed that after preincubation there was some reduction of the cytochrome c.

The amount of cyt. c reduction varied with the enzyme preparation. This reduction of cyt. c by the enzyme led to the belief that some substrate might be bound to the enzyme or that the enzyme existed in two stable forms, an oxidized and a reduced form. Assays were performed to determine the amount of iron bound to the enzyme. Since the presence of iron on an enzyme is usually accompanied by sulfide (Miller and Massey, 1965; Brumby, Miller, and Massey, 1965), the sulfide content of the enzyme was also assayed.

Iron and labile sulfide content

The total iron content of the holoenzyme was constant although the ferrous:ferric ratio varied considerably. Despite the fact that the iron bound to the holoenzyme was non-dialysable, the protein subunit retained only 10% of the bound iron. This indicated that only 1 mole of the iron was extremely tightly bound to the enzyme. Assuming a molecular weight of 30,000 for the protein portion of the enzyme, the iron:protein ratios of the holoenzyme and the protein subunit were 10:1 and 1:1 respectively.

The presence of tightly-bound iron is characteristic of non-heme iron proteins (Mason, 1965), the majority of

which contain sulfide closely linked to the iron on the protein structure. Sulfide analysis failed to detect any sulfide in either the holoenzyme or protein subunit. If the ratio of protein:iron:sulfide was 1:1:1, then the amounts of sulfide that should have been present were well within the limits of the assay and should have been detected (King, 1964). The results of the iron and sulfide assays are shown in Table XIV.

An attempt was made to determine if the iron bound to the enzyme was replaced slowly or if the binding was instantaneous. To remove the bound iron, the enzyme was incubated with saturated 2,2' dipyridyl and the mixture was dialysed against 2 changes of 4 l 0.01 M acetate-acetic acid buffer (pH 5.7). When the iron-free enzyme was assayed there was no detectable lag in cyt. c reduction compared with the normal holoenzyme. This result indicated that the tightly bound iron was rapidly replaced and the iron-protein interaction was not time-dependent, which is true for some enzyme-cofactor systems.

Protein and nucleic acid content

Since the presence of nucleotide was obligatory for

TABLE XIV

Iron and labile sulfide content of Fe⁺⁺-cyt. c reductase

Fraction	μg	Protein moles ^a	Ferrous iron (moles)	Total iron (moles)	Fe ⁺⁺ :Fe ⁺⁺⁺	Iron:protein	Labile sulfide (moles)
Holoenzyme 1	20	6.6×10^{-10}	1.4×10^{-9}	5.8×10^{-9}	1:3.1	8.8:1	0
Holoenzyme 2	48	1.6×10^{-9}	4.3×10^{-9}	1.8×10^{-8}	1:3.2	10.6:1	0
Holoenzyme 3	300	1.0×10^{-8}	5.0×10^{-8}	1.0×10^{-9}	1:1	10:1	0
Holoenzyme 4 ^b	315	1.05×10^{-8}	3.5×10^{-8}	1.1×10^{-7}	2.1:1	10:1	0
Protein ^b subunit	300	1.0×10^{-8}	6.0×10^{-9}	1.0×10^{-8}	1.5:1	1:1	0

^aBased on a molecular weight of 30,000 for the protein moiety (see Figs. 19 and 20).^bThe protein subunit was derived from holoenzyme 4 with a 95.2% protein yield.

enzyme activity, the protein and nucleic acid contents of the enzyme were assayed. The constant 260:280 $m\mu$ ratio of purified Fe^{++} -cyt. c reductase meant that different batches of enzyme had the same protein-nucleic acid composition. When the nature of nucleic acid was studied, only RNA was found; there was no DNA present (Table XV). Since the enzyme was found to be rather resistant to RNase hydrolysis (Table XII), this resistance was tested in the holoenzyme and RNA subunit. RNA hydrolysis was measured by dialysing the hydrolysed nucleotides from the reaction mixture and assaying these nucleotides by the orcinol method (Table XV).

Alkali completely hydrolysed the enzyme-RNA as well as the RNA subunit. RNase, on the other hand, hydrolysed the complete RNA subunit but only 50% of the RNA of the holoenzyme. This resistance to RNase hydrolysis by the holoenzyme must mean that the RNA is an integral part of the enzyme and the complete structure is not accessible to RNase action. Assuming a molecular weight of 30,000 (see Figs. 19 and 20) for the protein portion of the holoenzyme, the molecular weight of the RNA-portion of the enzyme was spectrophotometrically calculated to be approximately 65,000. The combined molecular weights of the protein subunit and

RNA-portion of the enzyme closely approximated the molecular weight of the holoenzyme (see Figs. 17 and 18). The results of the protein and nucleotide assays are shown in Table XV.

Molecular weight studies

Schachman (1959) calculated the molecular weights of macromolecules by means of sedimentation equilibrium.

Schachman, in his ultracentrifugation study, showed that the sedimentation constants of two kinds of macromolecules were related to their respective molecular weights according to the following equation:

$$(1) \quad \frac{S_1}{S_2} = \left(\frac{MW_1}{MW_2} \right)^{2/3}$$

where S = sedimentation constant

MW = molecular weight

Martin and Ames (1961) demonstrated that molecular weights of protein mixtures could be determined by a measurement of sedimentation velocity after centrifugation in a sucrose gradient. The distance (D) a protein travelled from the meniscus was a direct measure of its sedimentation velocity and S_1/S_2 equalled D_1/D_2 . Substituting in (1),

TABLE XV

Protein and nucleotide content of Fe⁺⁺-cyt. c reductase

Fraction	Protein μg	Protein moles ^a	OD ₂₆₀	RNA μg	Nucleotide found moles	Reaction	RNA hydrolysed μg %	Diphenyl- amine test
Holoenzyme	20.5	6.5 x 10 ⁻¹⁰	0.415	42.25 ^b 40 ^c	12.45 x 10 ⁻⁸	Alkaline hydrolysis (12 hours)	40 100	Negative
Holoenzyme	20.5	6.5 x 10 ⁻¹⁰	0.415	40 ^c	12.45 x 10 ⁻⁸	RNase hydrolysis (1 hour)	0 0	Negative
Holoenzyme	20.5	6.5 x 10 ⁻¹⁰	0.415	40 ^c	12.45 x 10 ⁻⁸	RNase hydrolysis (12 hours)	18.8 47	Negative
RNA subunit	-	-	0.34	31.1 ^c	10.20 x 10 ⁻⁸	Alkaline hydrolysis (12 hours)	29.8 96	Negative
RNA subunit	-	-	0.34	31.1 ^c	10.20 x 10 ⁻⁸	RNase hydrolysis (12 hours)	31.7 102	Negative

continued

TABLE XV (continued)

- ^aBased on a molecular weight of 30,000 for the protein subunit (see Figs. 19 and 20).
^bBased on a molecular weight of 65,000 as calculated from spectrophotometric data showing 192 mgles P per mole of protein and using an average nucleotide molecular weight of 340 (see ^c)
^cOrcinol assay
^dBased on an $\epsilon(P)$ of 10,000

$$(2) \quad \frac{D_1}{D_2} = \left(\frac{MW_1}{MW_2} \right)^{2/3}$$

where D = distance of travel
from the meniscus

Thus it is possible to determine the weight of an unknown molecular weight protein from a mixture of proteins of known and unknown molecular weights.

Utilizing the density gradient method of Martin and Ames (1961), molecular weight determinations of Fe^{++} -cyt. c reductase and its subunits were carried out with cyt. c (MW 12,270) and hemoglobin (MW 67,000) as reference proteins. Fe^{++} -cyt. c reductase, the protein subunit, and the RNA subunit were estimated to have the following molecular weights: 103 - 110,000, 27 - 30,000, and 314 - 329,000 respectively. The results of the sucrose density gradients are illustrated in Figs. 17-20.

Enzyme fluorescence

Velick (1958) utilized spectrofluorometric quenching to study coenzyme binding in dehydrogenases. He found that substances which bind the enzyme quench its fluorescence. When Fe^{++} -cyt. c reductase was excited at 280 mμ the enzyme

Fig. 17. Molecular weight determination of Fe^{++} -cyt. c reductase using oxidized cyt. c as marker.

260 mμ ---

280 mμ —○—○—

407 mμ ———

//// active fractions

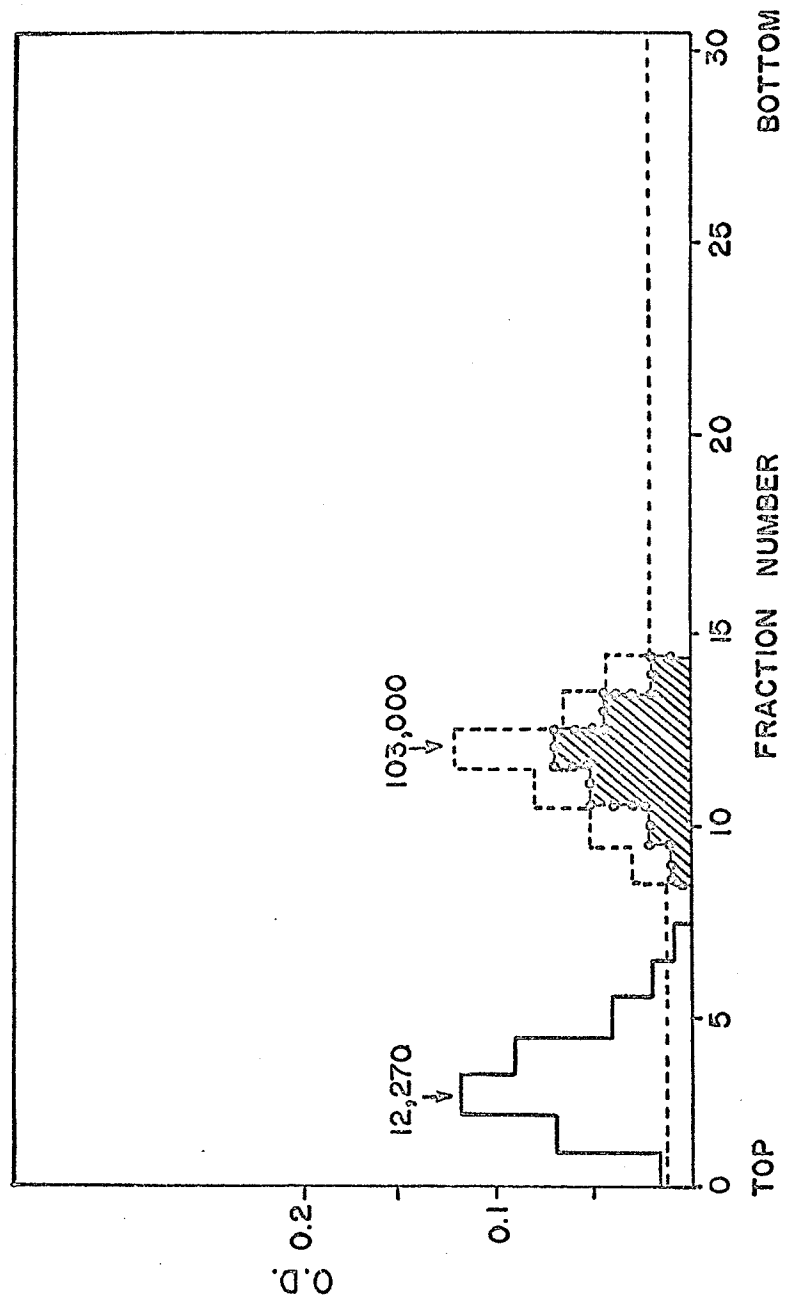


Fig. 18. Molecular weight determination of Fe^{++} -cyt. c
using hemoglobin as marker.

260 $\text{m}\mu$ - - -

280 $\text{m}\mu$ -○-○-

407 $\text{m}\mu$ ———

//// active fractions

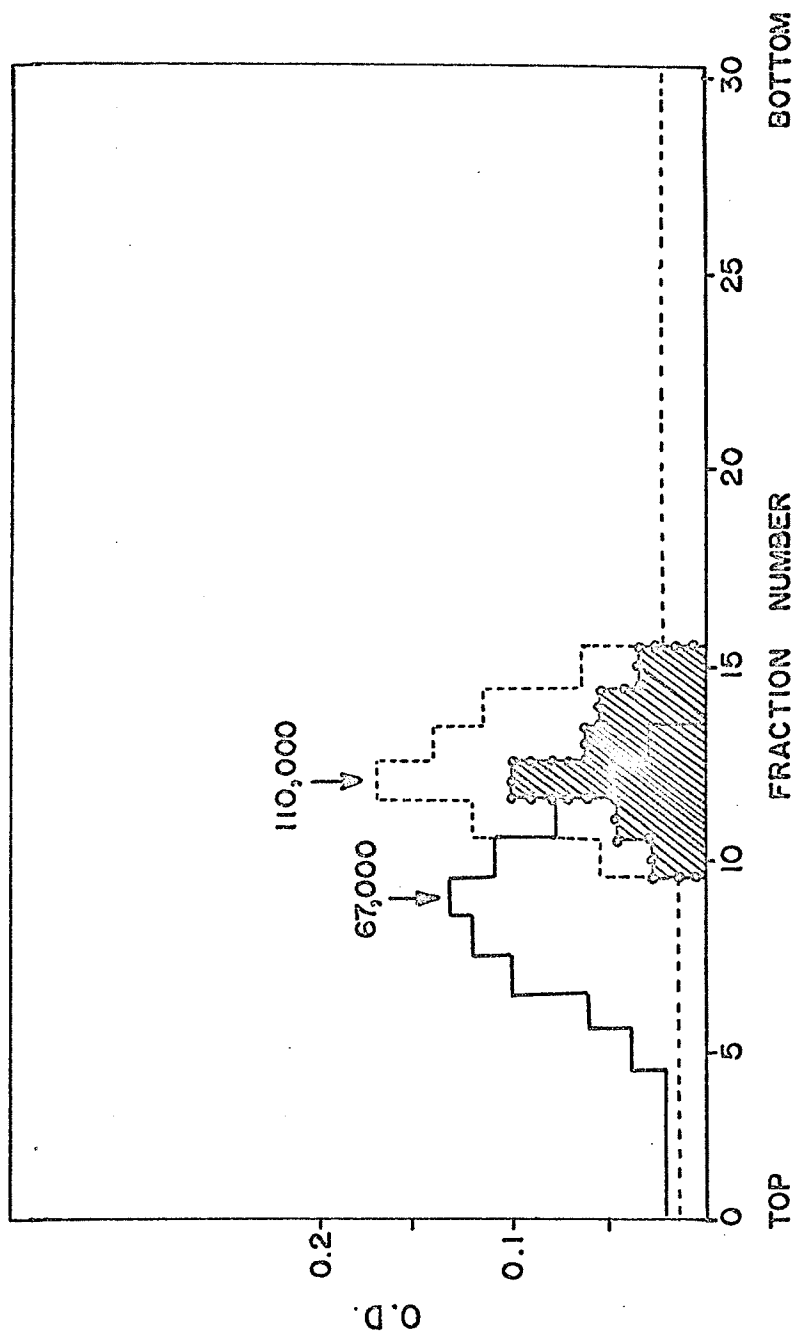


Fig. 19. Molecular weight determination of Fe^{++} -cyt. c reductase subunits using oxidized cyt. c as marker.

260 $m\mu$ — — —

280 $m\mu$ —○—○—

407 $m\mu$ —————

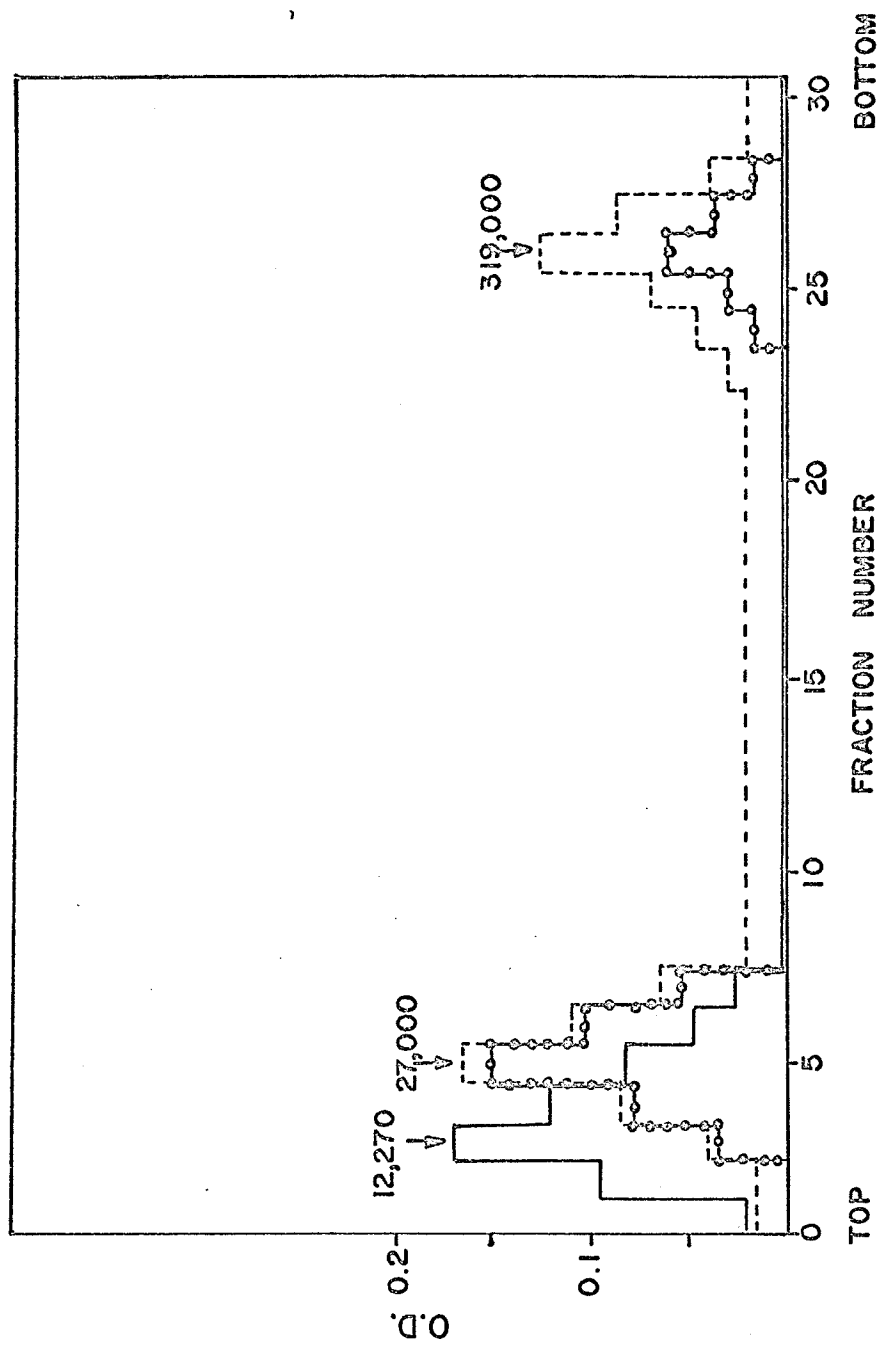
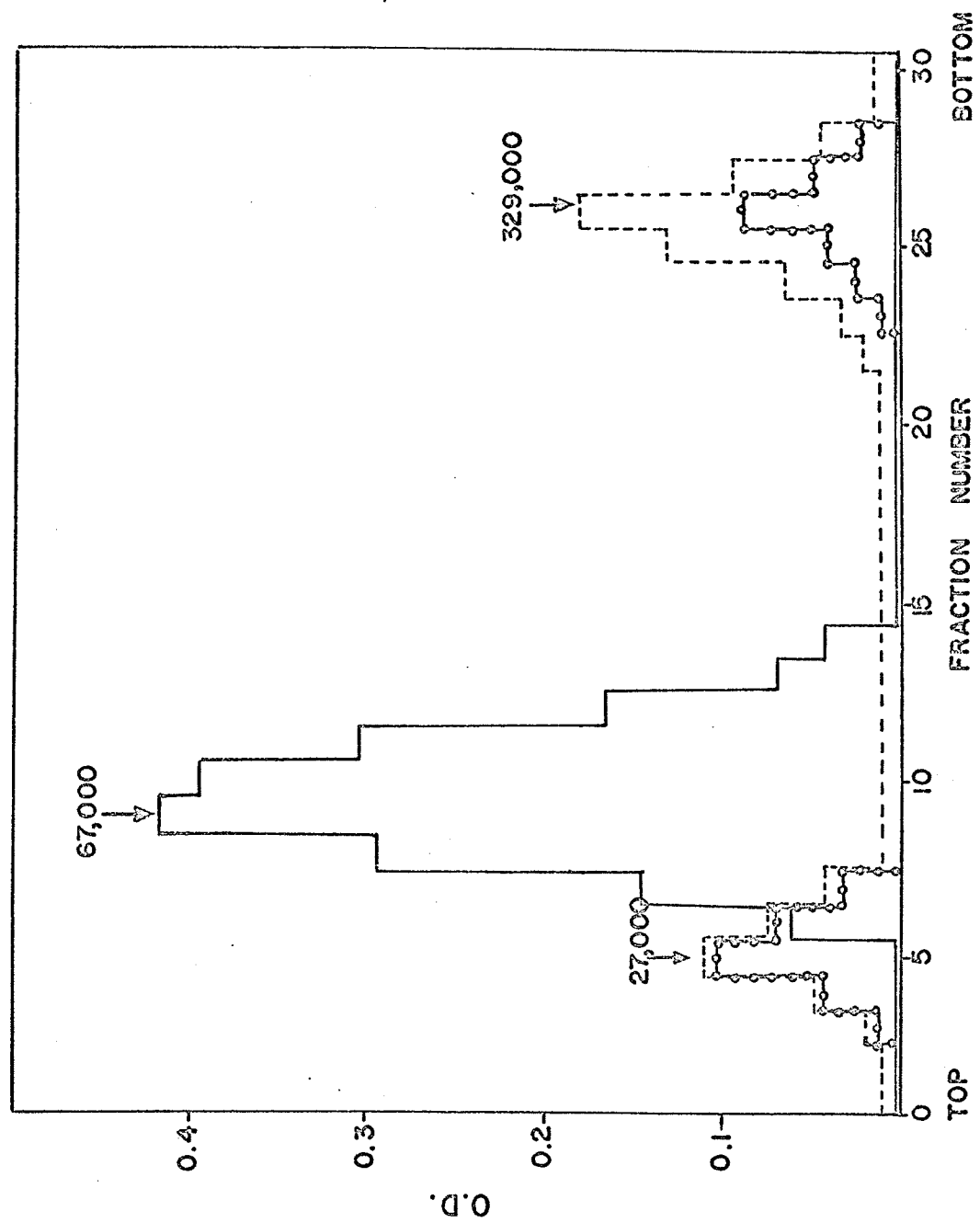


Fig. 20. Molecular weight determination of Fe^{++} -cyt. c reductase subunits using hemoglobin as marker.

260 m μ — — —

280 m μ —○—○—

407 m μ —————



exhibited a fluorescence peak at 350 mμ. Enzyme fluorescence was quenched by both substrates and end-products: Fe^{++} , Fe^{+++} , cyt. c, and reduced cyt. c.

The quenching of enzyme fluorescence was specific and was not evident when tryptophan was titrated with equal quantities of the substrates or end-products used to quench the enzyme. The decrease in fluorescence of tryptophan by substrates or end-products was equal to the dilution effect evident when enzyme or tryptophan was titrated with equal volumes of buffer (same volume as substrates or end-products). The quenching of fluorescence by both substrates and both end-products suggested a mechanism in which the enzyme is bound in a random order, or the existence of two enzyme forms. Kinetic studies were required to differentiate which of these mechanisms was present.

Kinetic studies and reaction mechanism

The presence of non-heme iron in Fe^{++} -cyt. c reductase suggested its possible involvement in the reduction of cytochrome c by ferrous iron. Detailed kinetic studies were carried out in order to elucidate the exact role of non-heme iron and the reaction mechanism.

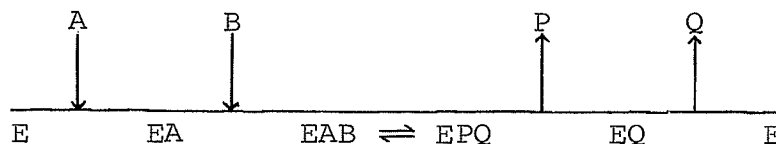
All initial velocity data was plotted in the double reciprocal form ($1/(\text{velocity})$ versus $1/(\text{substrate conc.})$) according to Lineweaver and Burk (1934). The nomenclature of reaction mechanisms and definition of kinetic constants are those proposed by Cleland (1963a:b:c).

Ferrous iron-cytochrome c reductase catalyses the following reaction:



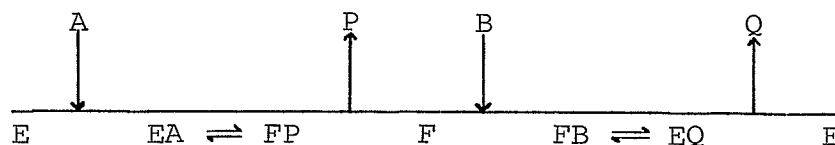
which can be explained by either of the two following mechanisms:

a) Ordered Bi Bi mechanism (compulsory sequential addition of two substrates, A and B, and sequential release of two products, P and Q)



where E is the enzyme and EA, EAB, EPQ, and EQ are the enzyme-substrate or -product complexes.

b) Ping Pong Bi Bi mechanism (release of one product before the addition of the second substrate with two stable forms of enzyme existing)



where E and F are the two stable forms of the enzyme and EA, FP, FB, and EQ are the enzyme-substrate or -product complexes.

Cleland (1963a) derived the following steady state rate equation for an Ordered Bi Bi mechanism:

$$v = \frac{V_1 V_2 (AB - \frac{PQ}{K_{eq}})}{K_{ia} K_b V_2 + K_b V_2 A + K_a V_2 B + V_2 AB + \frac{K_q V_1 P}{K_{eq}} + \frac{K_p V_1 Q}{K_{eq}} + \frac{V_1 PQ}{K_{eq}} + \frac{K_q V_1 AP}{K_{ia} K_{eq}} + \frac{K_a V_1 BQ}{K_{iq}} + \frac{V_2 ABP}{K_{ip}} + \frac{V_1 BPQ}{K_{ib} K_{eq}}} \quad (1)$$

where V_1 and V_2 are the maximum velocities for the forward and reverse directions and the K values are various constants. When P and Q are equal to zero equation (1) becomes

$$v = \frac{V_1 AB}{K_{ia} K_b + K_b A + K_a B + AB} \quad (2)$$

which in the double reciprocal form is transposed to

$$1/v = 1/V_1 + K_a/V_1(1/A) + K_b/V_1(1/B) + (K_{ia}K_b)/V_1(1/(AB)) \quad (3)$$

where v is the initial velocity, V_1 is the maximum velocity, A and B are the respective concentrations of the substrates, K_a and K_b are the Michaelis constants for A and B , and K_{ia} is the dissociation constant for the EA complex.

For a Ping Pong Bi Bi reaction mechanism the steady state rate equation is:

$$v = \frac{V_1 V_2 (AB - \frac{PQ}{K_{eq}})}{K_b V_2 A + K_a V_2 B + V_2 AB + \frac{K_q V_1 P}{K_{eq}} + \frac{K_p V_1 Q}{K_{eq}} + \frac{V_1 PQ}{K_{eq}} + \frac{K_q V_1 AP}{K_{ia} K_{eq}} + \frac{K_a V_2 BQ}{K_{iq}}} \quad (4)$$

When P and Q are zero equation (4) becomes

$$v = \frac{V_1 AB}{K_b A + K_a B + AB} \quad (5)$$

which in the double reciprocal form is transposed to

$$1/v = 1/V_1 + K_a/V_1(1/A) + K_b/V_1(1/B) \quad (6)$$

Determination of Michaelis constants (K_m)

The K_m values are defined as the concentration of substrate giving one-half maximum velocity (V_{max}) in initial velocity studies when the second substrate is in saturating concentrations. K_m values were determined by measuring initial velocities when one substrate was varied and the second substrate was fixed at several concentrations. The data was plotted in the double reciprocal form ($1/v$ versus $1/(\text{varied substrate})$). A replot of the reciprocal of $\text{app}V_{max}$ (intercept at the vertical axis) for each concentration of fixed substrate against the reciprocal of the fixed substrate concentration was made (Florini and Vestling, 1957). Since $\text{app}V_{max}$ equals $(1 + K_a/A)/V_1$ or $(1 + K_b/B)/V_1$ in equations (3) and (6), the replot intercept on the horizontal axis corresponds to $-1/K_m$.

The results of initial velocity studies and their replots are shown in Figs. 21-24. The K_m values calculated from the replot analyses (Figs. 22 and 24) were $K_{Fe++} = 0.59 \text{ mM}$ and $K_{\text{cyt. } \underline{c}} = 0.085 \text{ mM}$. These results compared favourably with the apparent K_m values ($K'_{Fe++} = 1.0 \text{ mM}$ and $K'_{\text{cyt. } \underline{c}} = 0.095 \text{ mM}$) previously calculated (see Figs. 13 and

Fig. 21. Double reciprocal plot of $(1/v)$ versus $(1/\text{Fe}^{++})$ at fixed concentrations of cyt. c.

The reaction mixture contained: ferrous iron as indicated, $24 \mu\text{moles}$ Na barbital-acetic acid buffer (pH 7.0), oxidized cyt. c, $36 \mu\text{g}$ protein, and H_2O in a final volume of 3.0 ml .

v = difference in $\text{OD}_{550 \text{ m}\mu}/\text{min}$.

A = $10 \times 10^{-5} \text{ M}$ oxidized cyt. c

B = $5 \times 10^{-5} \text{ M}$ oxidized cyt. c

C = $3.33 \times 10^{-5} \text{ M}$ oxidized cyt. c

D = $2 \times 10^{-5} \text{ M}$ oxidized cyt. c

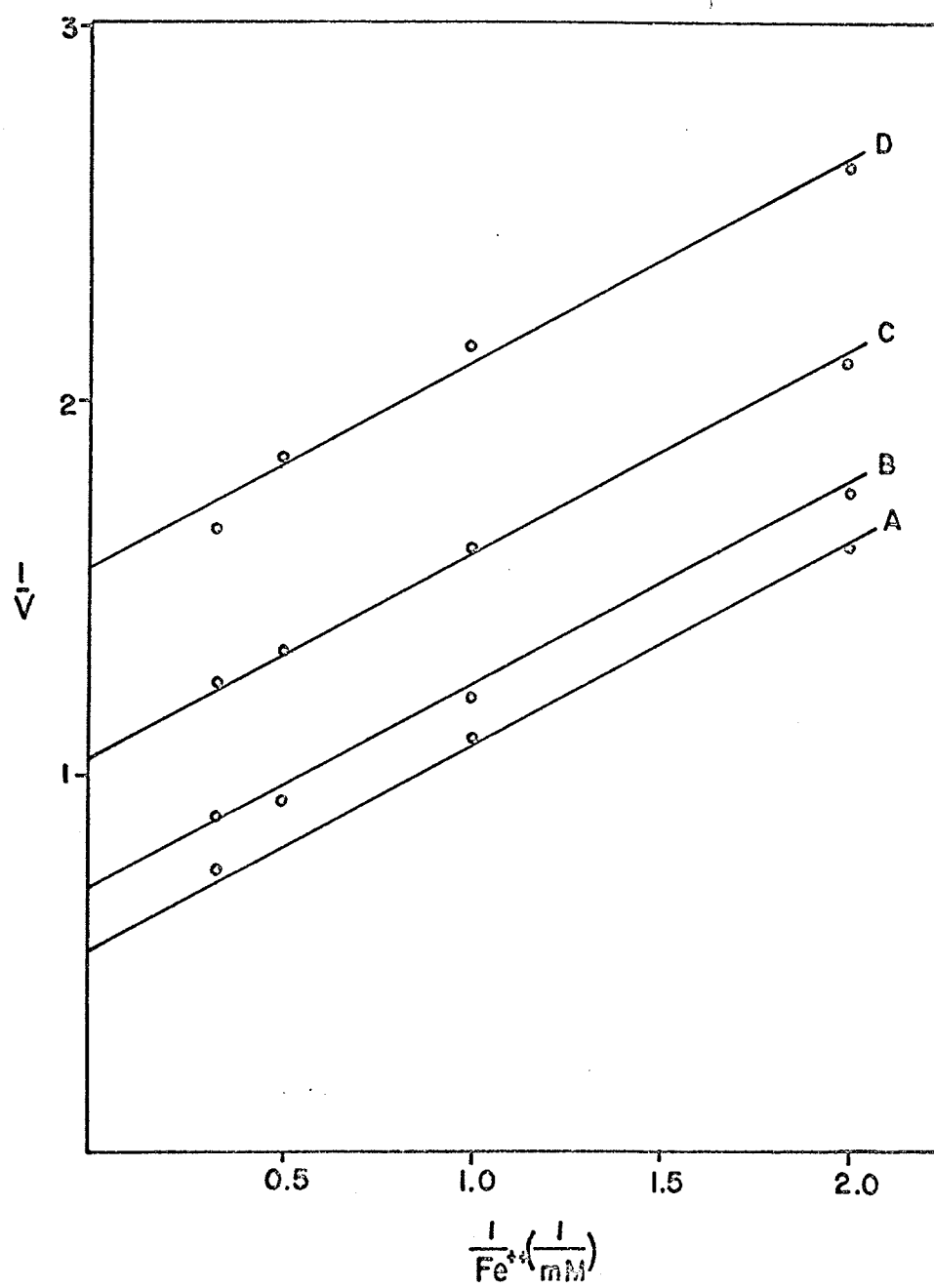


Fig. 22. Replot of Fig. 21, intercept ($1/(\text{appV}_{\text{max}})$) versus ($1/\text{cyt. } \underline{c}$).

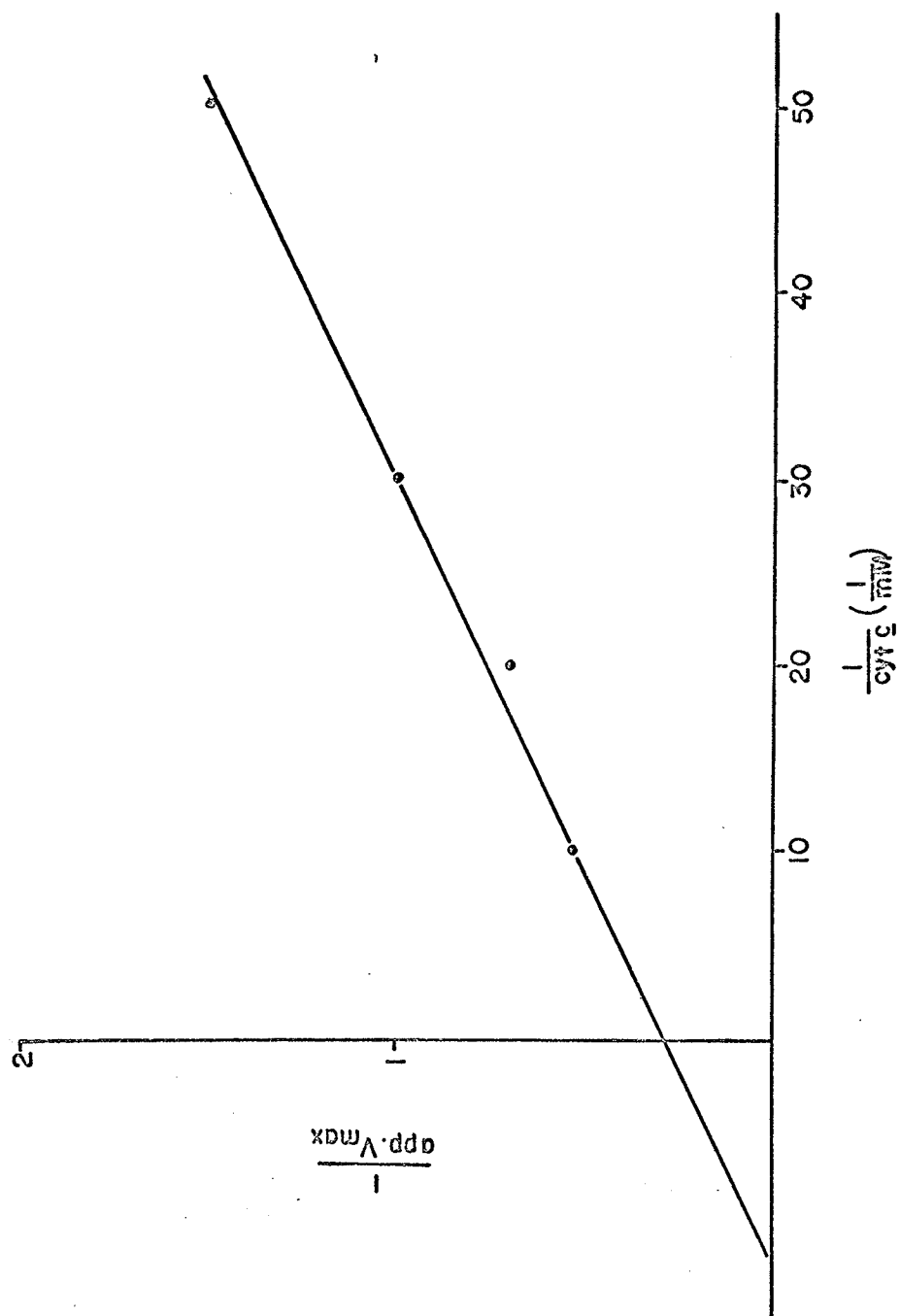


Fig. 23. Double reciprocal plot of $(1/v)$ versus $(1/\text{cyt. } \underline{c})$ at fixed concentrations of Fe^{++} .

The reaction mixture contained: oxidized cyt. c as indicated, $24 \mu\text{moles}$ Na barbital-acetic acid buffer (pH 7.0), ferrous iron, $36 \mu\text{g}$ protein, and H_2O in a final volume of 3.0 ml.

v = difference in $\text{OD}_{550 \text{ m}\mu}/\text{min.}$

$A = 3 \times 10^{-4} \text{ M Fe}^{++}$

$B = 2 \times 10^{-4} \text{ M Fe}^{++}$

$C = 1 \times 10^{-4} \text{ M Fe}^{++}$

$D = 0.5 \times 10^{-4} \text{ M Fe}^{++}$

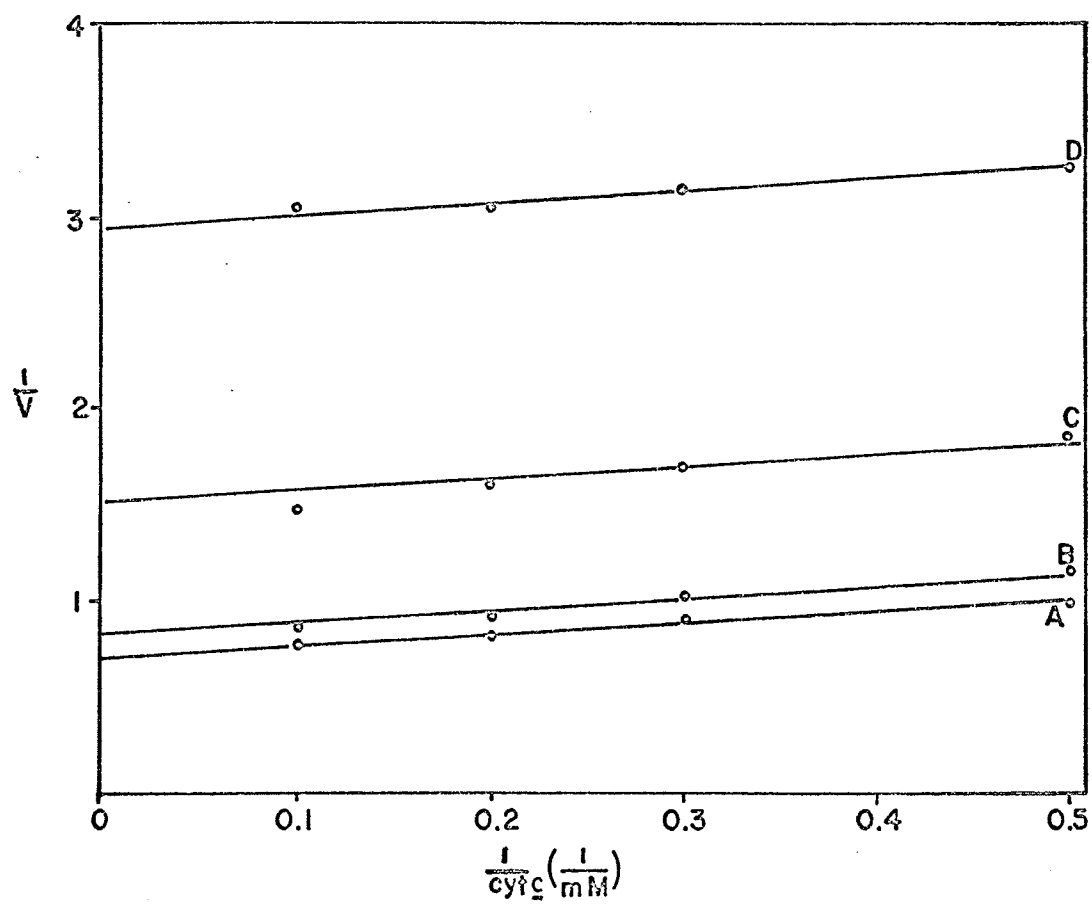
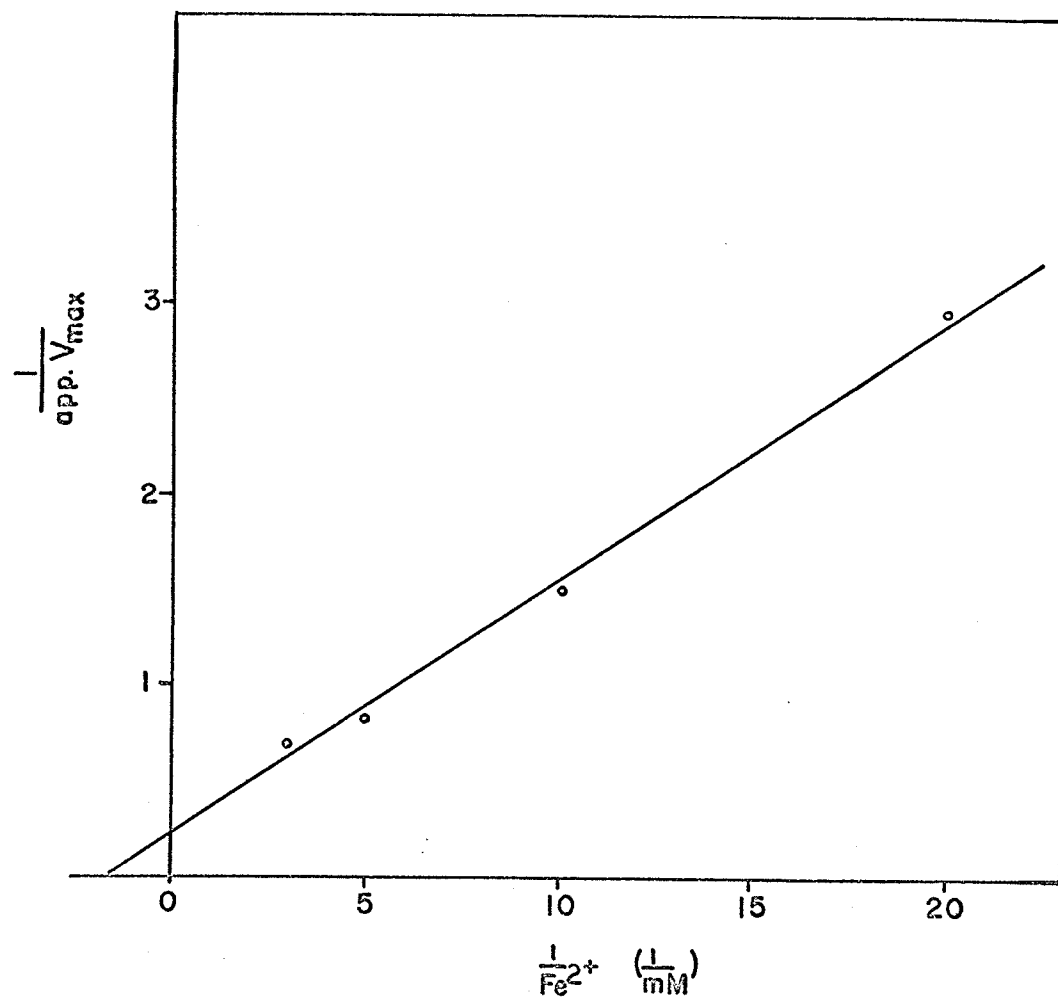


Fig. 24. Replot of Fig. 23, intercept $(1/(\text{app}V_{\text{max}}))$ versus $(1/\text{Fe}^{++})$.



15). The parallel lines obtained indicate that the reaction mechanism is Ping Pong Bi Bi rather than Ordered Bi Bi.

Initial velocity studies using a constant ratio of substrate concentrations

Cleland (1963a) described a method to differentiate a Ping Pong Bi Bi mechanism from Ordered and Rapid Equilibrium Random Bi Bi mechanisms. The latter mechanism assumes the random addition of substrates A and B, and the random release of products P and Q; and that all steps are very rapid except the interconversion of the central ternary complexes, EAB and EPQ. The rate equation is identical to that of an Ordered Bi Bi mechanism.

When initial velocities are measured at various concentrations of one substrate, keeping a constant ratio of the two substrate concentrations, a double reciprocal plot of a Ping Pong Bi Bi reaction is the only mechanism that results in a straight line relationship. When A/B is constant, equation (3) becomes

$$1/v = 1/V_1 + \text{constant}(1/A) + \text{constant}(1/A)^2 \quad (7)$$

and equation (6) becomes

$$1/v = 1/V_1 + \text{constant}(1/A) \quad (8)$$

Maintaining a constant ratio of $\text{Fe}^{++}:\text{cyt. c}$ concentrations (3:1), double reciprocal plots of $\text{Fe}^{++}\text{-cyt. c}$ reductase activity resulted in a linear relationship as shown in Figs. 25 and 26. Thus the mechanism of $\text{Fe}^{++}\text{-cyt. c}$ reductase is Ping Pong Bi Bi.

End-product inhibition studies

Kinetic studies on the inhibition of enzyme activity by end-product(s) can supply valuable information required to establish a particular reaction mechanism (Cleland, 1963b;c). Equations (1) and (4) indicate that when $Q = 0$ and P is used as the inhibitor, $1/v$ versus $1/B$ plots should result in a family of linear lines intersecting at the same point on the vertical axis (competitive inhibition) for Ping Pong Bi Bi mechanisms, but not for Ordered Bi Bi mechanisms. When $P = 0$ and Q is used as the inhibitor, $1/v$ versus $1/A$ plots for both mechanisms should result in competitive inhibition plots. Inhibition constants are obtained by replotting the slopes ($\text{app}K_m/\text{app}V_{\text{max}}$) against the concentrations of inhibitor. The intercept at the horizontal axis gives the

Fig. 25. Double reciprocal plot ($1/v$ versus $1/\text{Fe}^{++}$) maintaining a constant ratio of the substrate concentrations ($\text{Fe}^{++}:\text{cyt. c}$, 3:1).

The reaction mixture contained: 24 μmoles Na barbital-acetic acid buffer (pH 7.0), ferrous iron and oxidized cyt. c as indicated, 9 μg protein, and H_2O in a final volume of 3.0 ml.

v = difference in $\text{OD}_{550 \text{ m}\mu}/\text{min.}$

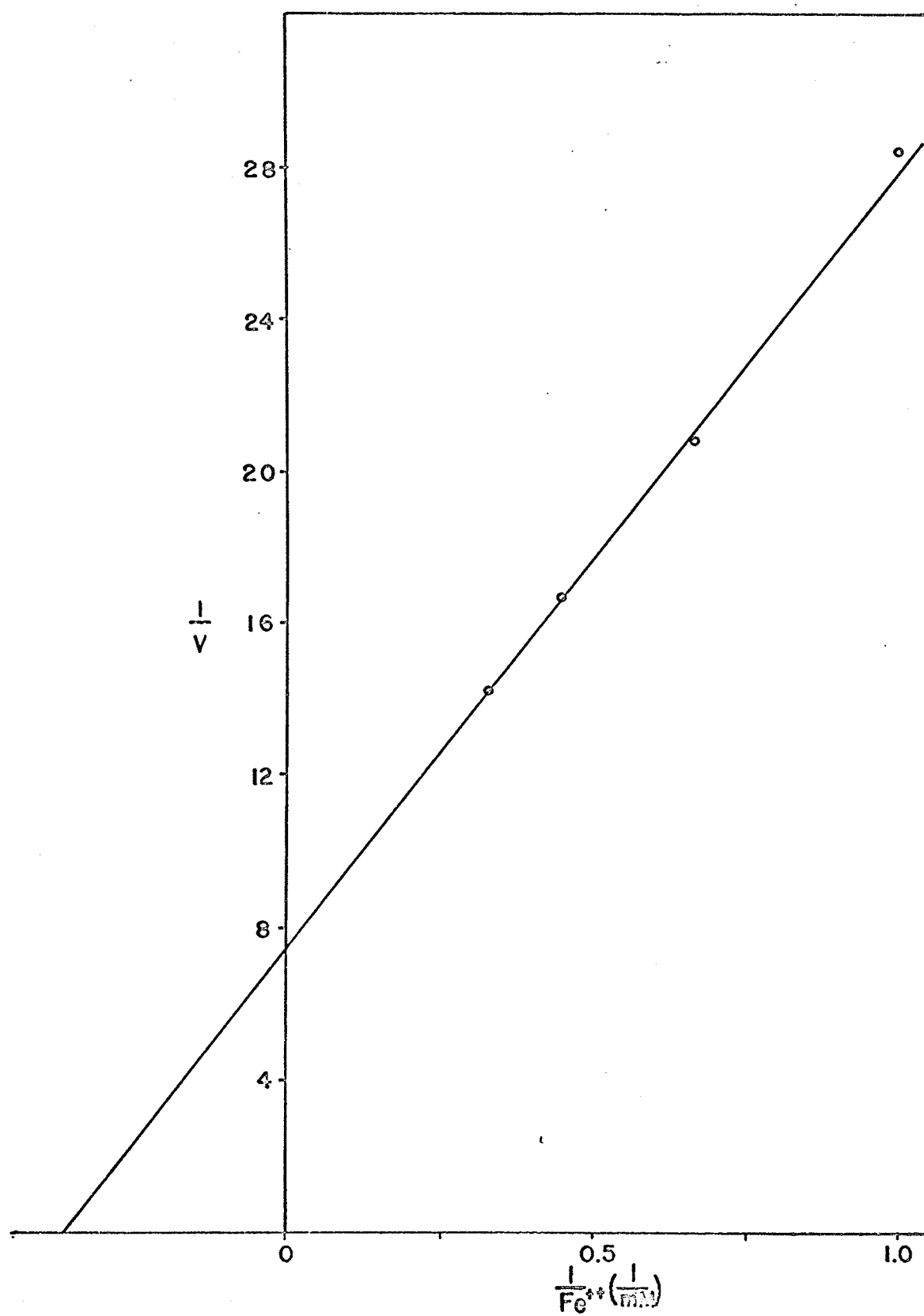
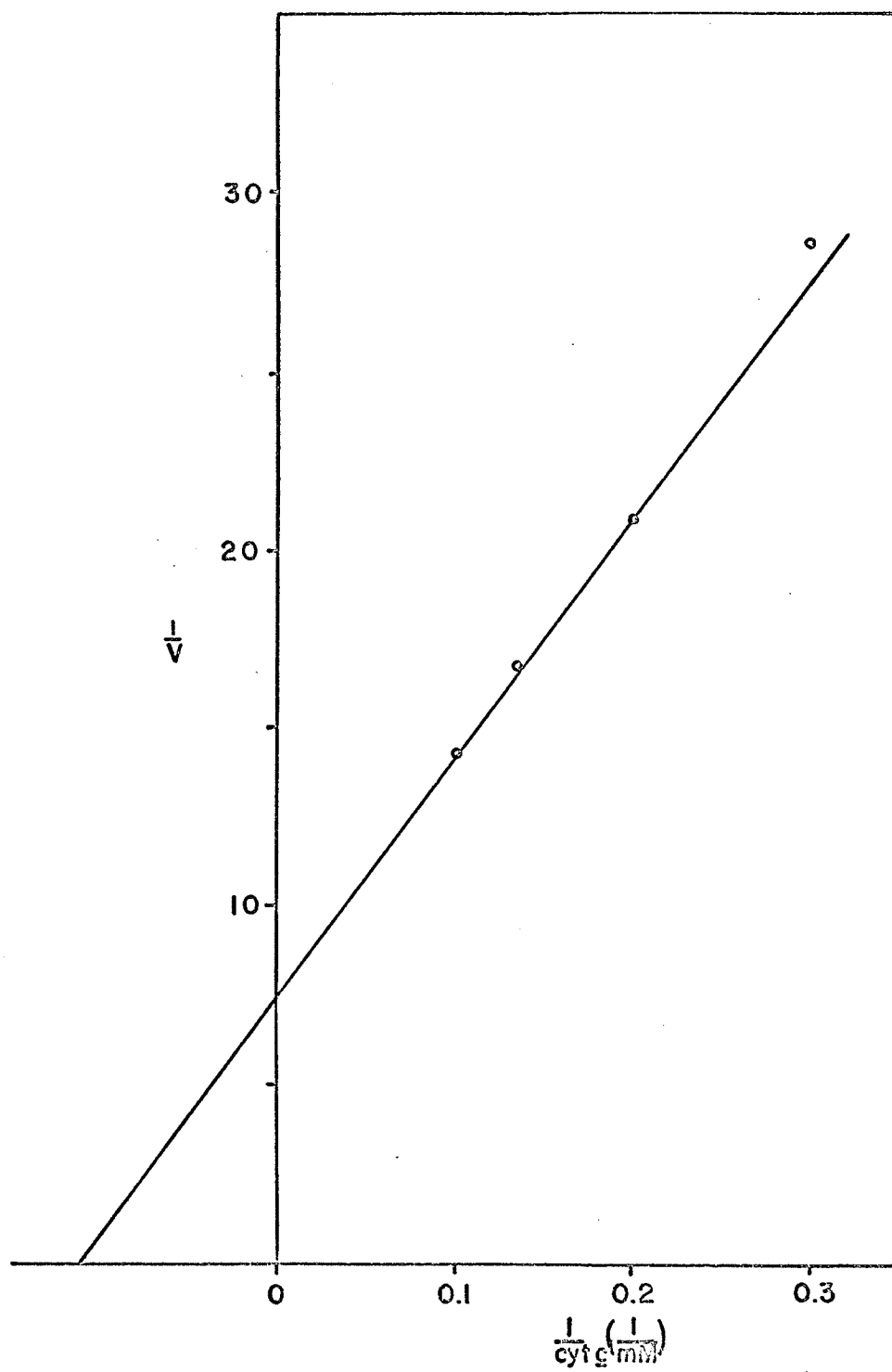


Fig. 26. Double reciprocal plot ($1/v$ versus $1/\text{cyt. } \underline{c}$) maintaining a constant ratio of the substrate concentrations ($\text{Fe}^{++}:\text{cyt. } \underline{c}$, 3:1).

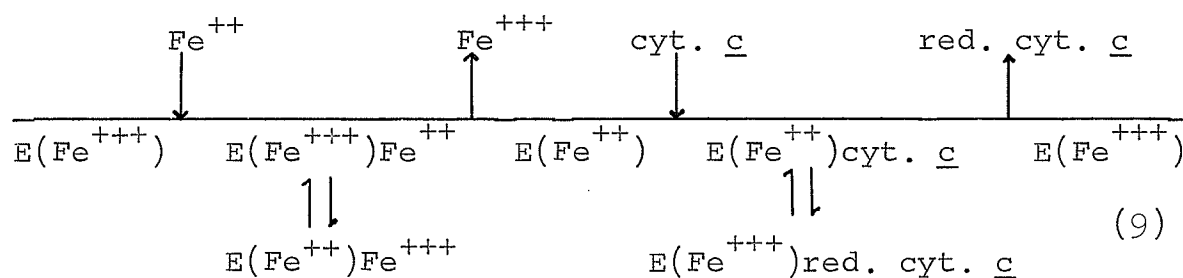
The conditions were the same as those outlined in

Fig. 25.



value of the inhibitor constant.

End-product inhibitor studies showed that ferric iron competitively inhibited the binding of oxidized cytochrome c (Fig. 27) and reduced cytochrome c competitively inhibited the binding of ferrous iron (Fig. 29) to Fe^{++} -cyt. c reductase. These results are compatible with a Ping Pong Bi Bi mechanism,



The inhibition constants for Fe^{+++} and reduced cyt. c are 0.137 mM and 0.0135 mM respectively (Figs. 28 and 30). The competitive inhibition by the Fe^{++} -red. cyt. c and Fe^{+++} -cyt. c pairs shows that there are probably two enzyme forms, $\text{E(Fe}^{+++}\text{)}$ and $\text{E(Fe}^{++}\text{)}$, and the reaction mechanism is Ping Pong Bi Bi.

Inhibition by sodium chloride

Inhibition studies were carried out using sodium chloride. Initial velocities were measured when ferrous iron concentration was varied and cytochrome c concentration

Fig. 27. Product inhibition of Fe^{++} -cyt. c reductase by ferric iron.

The reaction mixture contained: 12 μmoles Fe^{++} , 24 μmoles Na barbital-acetic acid buffer (pH 7.0), oxidized cyt. c as indicated, 16 μg protein, H_2O , and ferric iron in a final volume of 3.0 ml.

v = difference in $\text{OD}_{550 \text{ m}\mu}/\text{min.}$

A = 0 Fe^{+++}

B = 2.5×10^{-5} M Fe^{+++}

C = 5×10^{-5} M Fe^{+++}

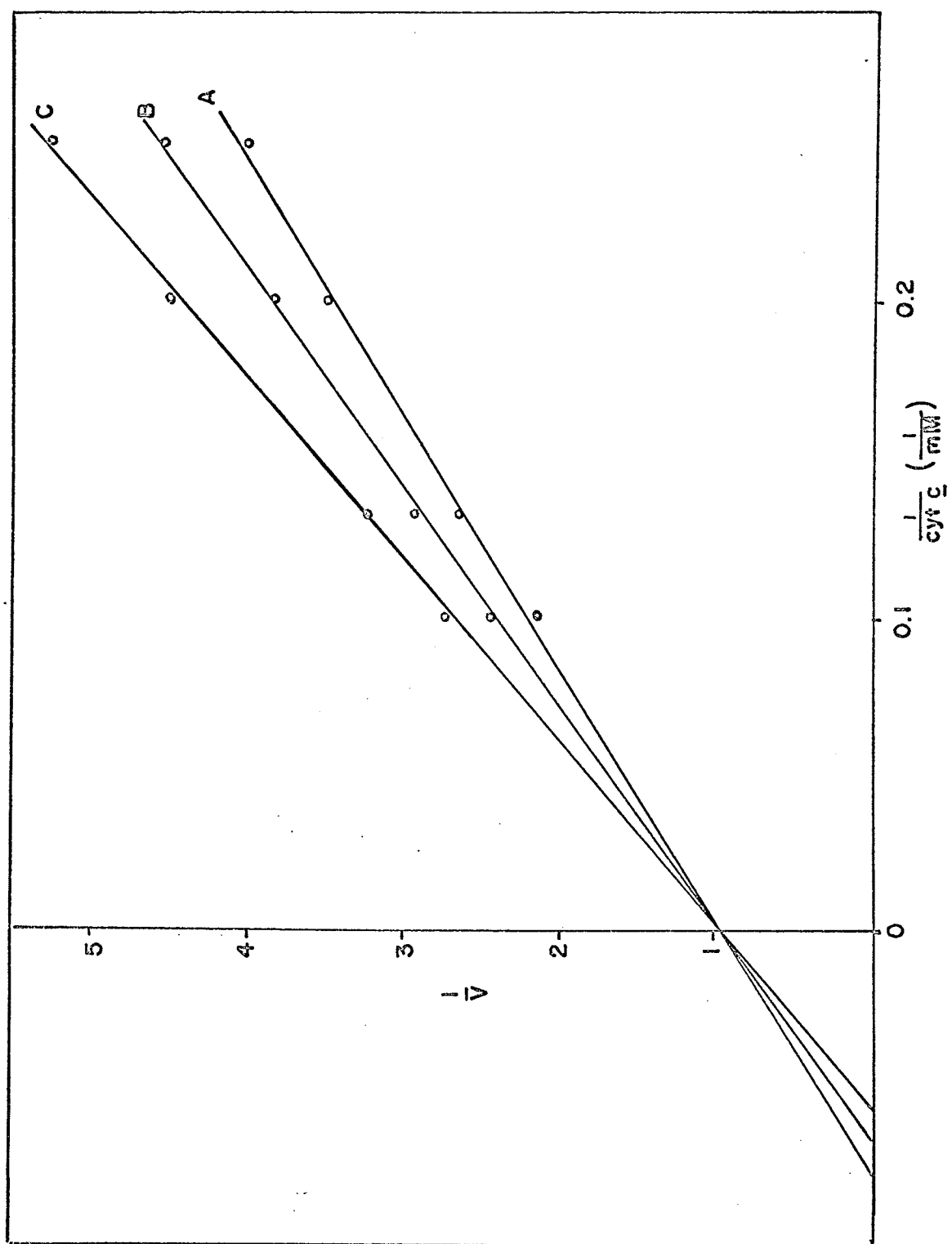


Fig. 28. Replot of Fig. 27, slope ($\text{appK}_m/\text{appV}_{\text{max}}$) versus ferric iron concentration.

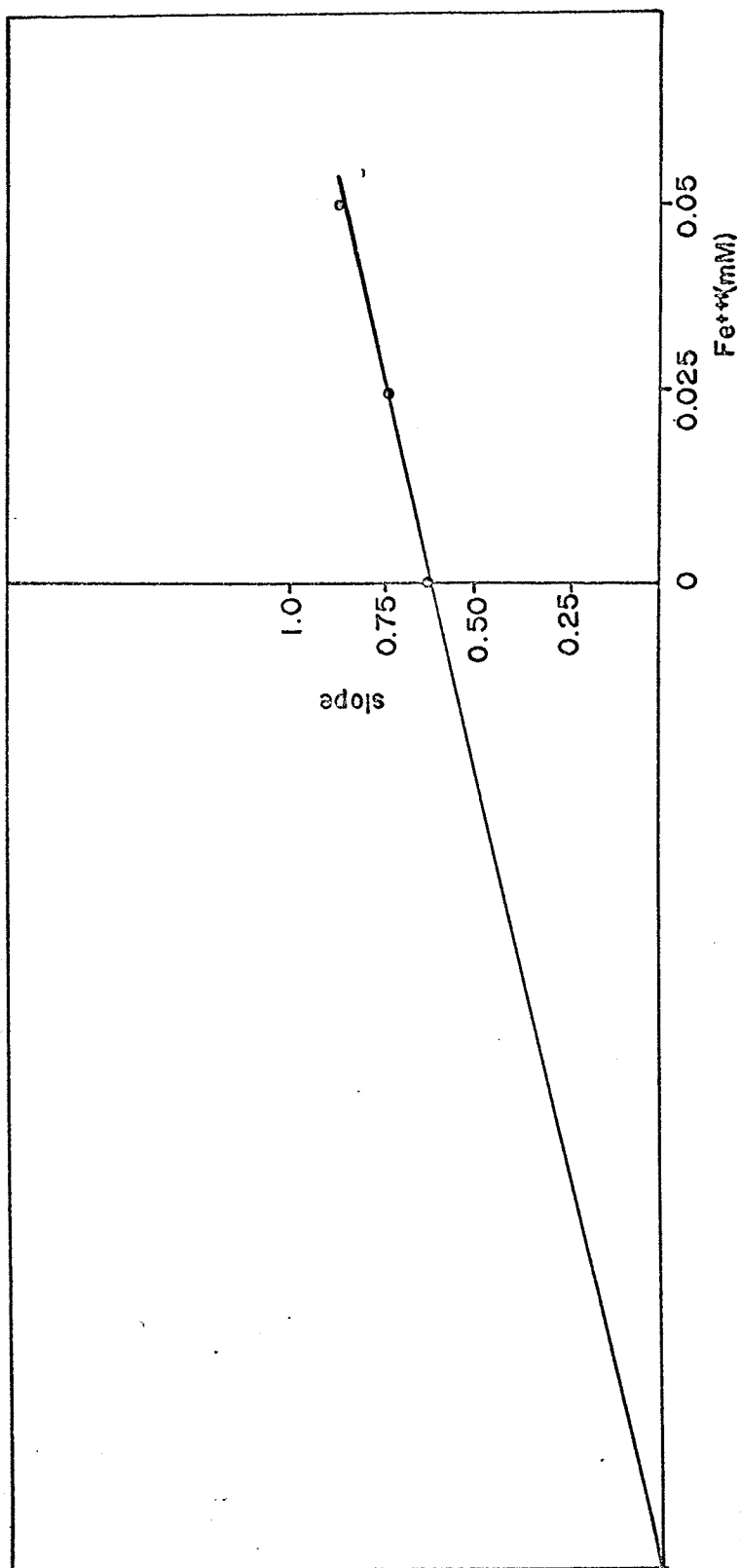


Fig. 29. Product inhibition of Fe^{++} -cyt. c reductase by reduced cyt. c.

The reaction mixture contained: 0.45 μ moles oxidized cyt. c, 24 μ moles Na barbital-acetic acid buffer (pH 7.0), ferrous iron as indicated, 19.2 μ g protein, reduced cyt. c, and H_2O in a final volume of 3.0 ml.

v = difference in $\text{OD}_{550 \text{ m}\mu}$ /min.

A = 0 reduced cyt. c

B = 1.9×10^{-5} M reduced cyt. c

C = 2.85×10^{-5} M reduced cyt. c

D = 3.8×10^{-5} M reduced cyt. c

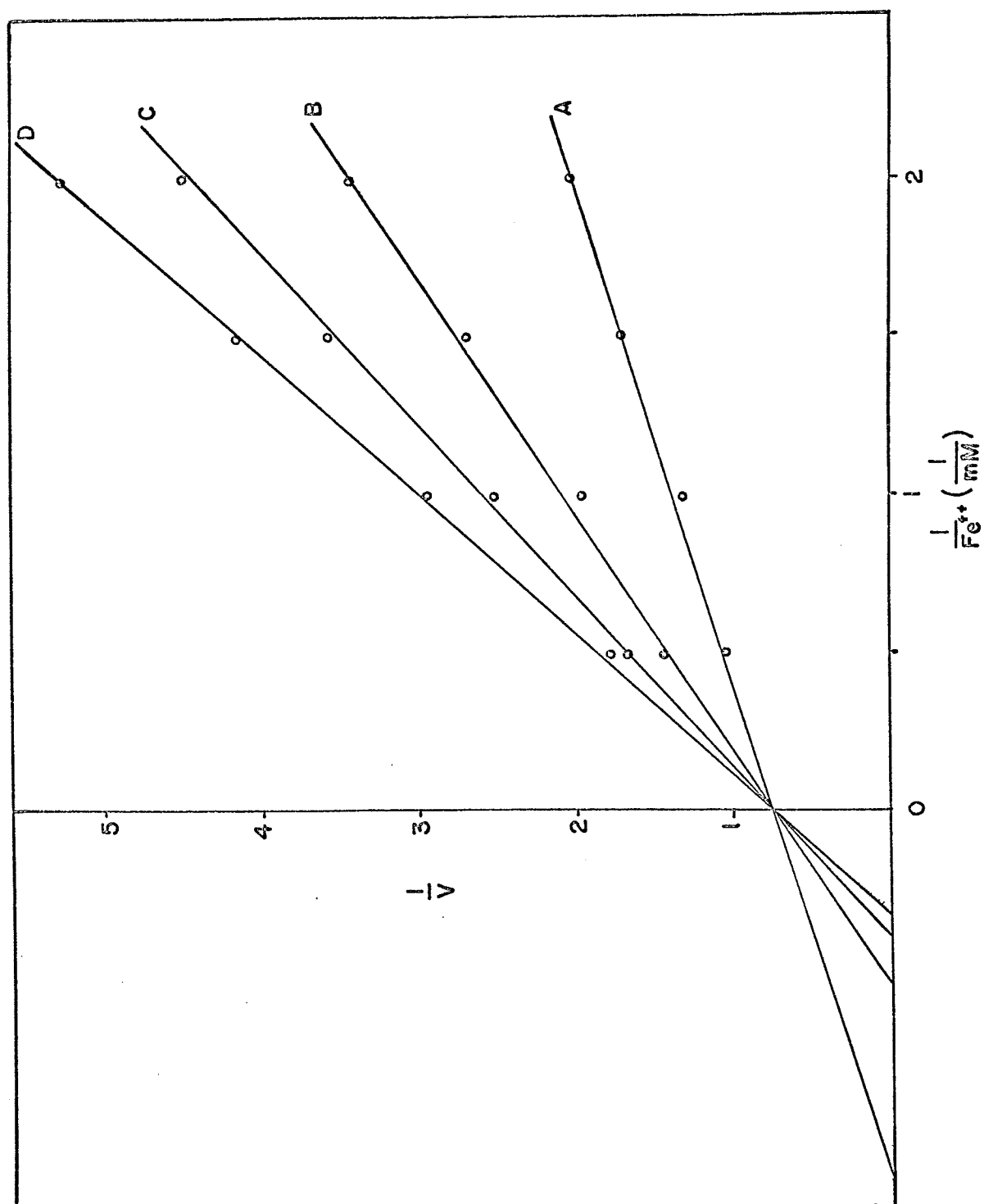
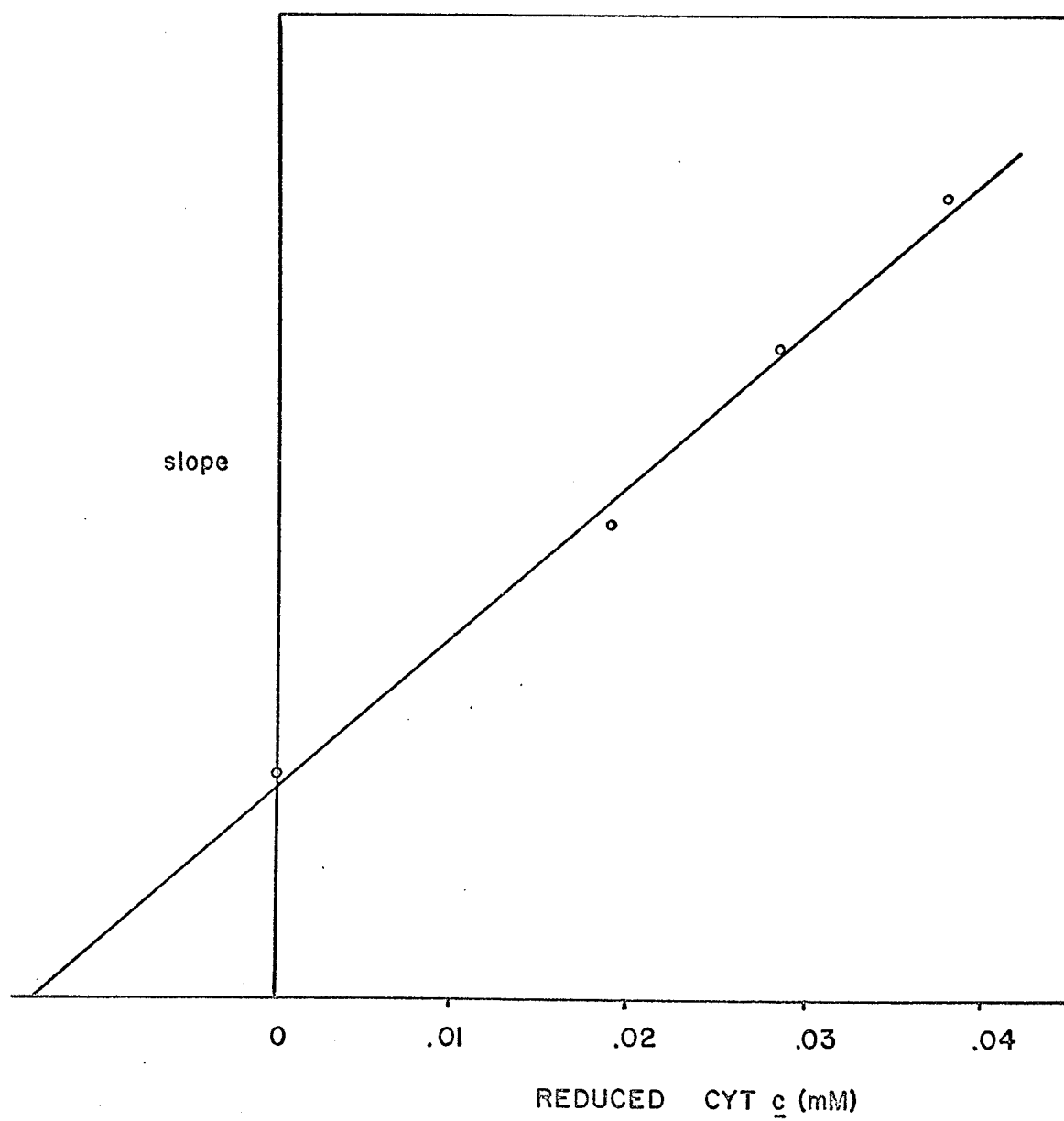


Fig. 30. Replot of Fig. 29, slope ($\text{appK}_m/\text{appV}_{\text{max}}$) versus reduced cyt. c concentration.



was kept constant at three concentrations of NaCl. An uncompetitive pattern of inhibition (Fig. 31) was evident from the $1/v$ versus $1/\text{Fe}^{++}$ plots. Replots of this data, when $1/\text{app}V_{\text{max}}$ was plotted against inhibitor concentration, resulted in an inhibition constant of 5.8 mM (Fig. 32). The uncompetitive inhibition by the Fe^{++} -NaCl pair indicated that the sodium chloride acted as a dead-end inhibitor, binding the ferrous form of the enzyme, $\text{E}(\text{Fe}^{++})$, but not the ferric form, $\text{E}(\text{Fe}^{+++})$ (see (9)) (Cleland, 1963c).

Reaction of cyt. $\underline{c}$ with Fe^{++} -cyt. $\underline{c}$ reductase

Spectrophotofluorometric and kinetic analyses indicated the reaction mechanism of Fe^{++} -cyt. $\underline{c}$ reductase is possibly a Ping Pong Bi Bi (Cleland, 1963a). This mechanism is accepted for transaminases where both forms of the enzyme are stable and easily interconvertible; both enzyme forms are capable of carrying out their respective half reactions. The ability of Fe^{++} -cyt. $\underline{c}$ reductase to carry out a half reaction was examined. Fe^{++} -cyt. $\underline{c}$ reductase and cyt. $\underline{c}$ were allowed to interact in the absence of ferrous iron. The amount of enzyme non-heme iron oxidized and the amount of cyt. $\underline{c}$ reduced were then measured.

Fig. 31. Inhibition of Fe^{++} -cyt. c reductase by NaCl.

The reaction mixture contained: 0.45 μ moles oxidized cyt. c, 24 μ moles Na barbital-acetic acid buffer (pH 7.0), ferrous iron as indicated, 41 μ g protein, NaCl, and H_2O in a final volume of 3.0 ml.

v = difference in $\text{OD}_{550 \text{ m}\mu}$ /min.

A = 0 NaCl

B = 1×10^{-3} M NaCl

C = 2×10^{-3} M NaCl

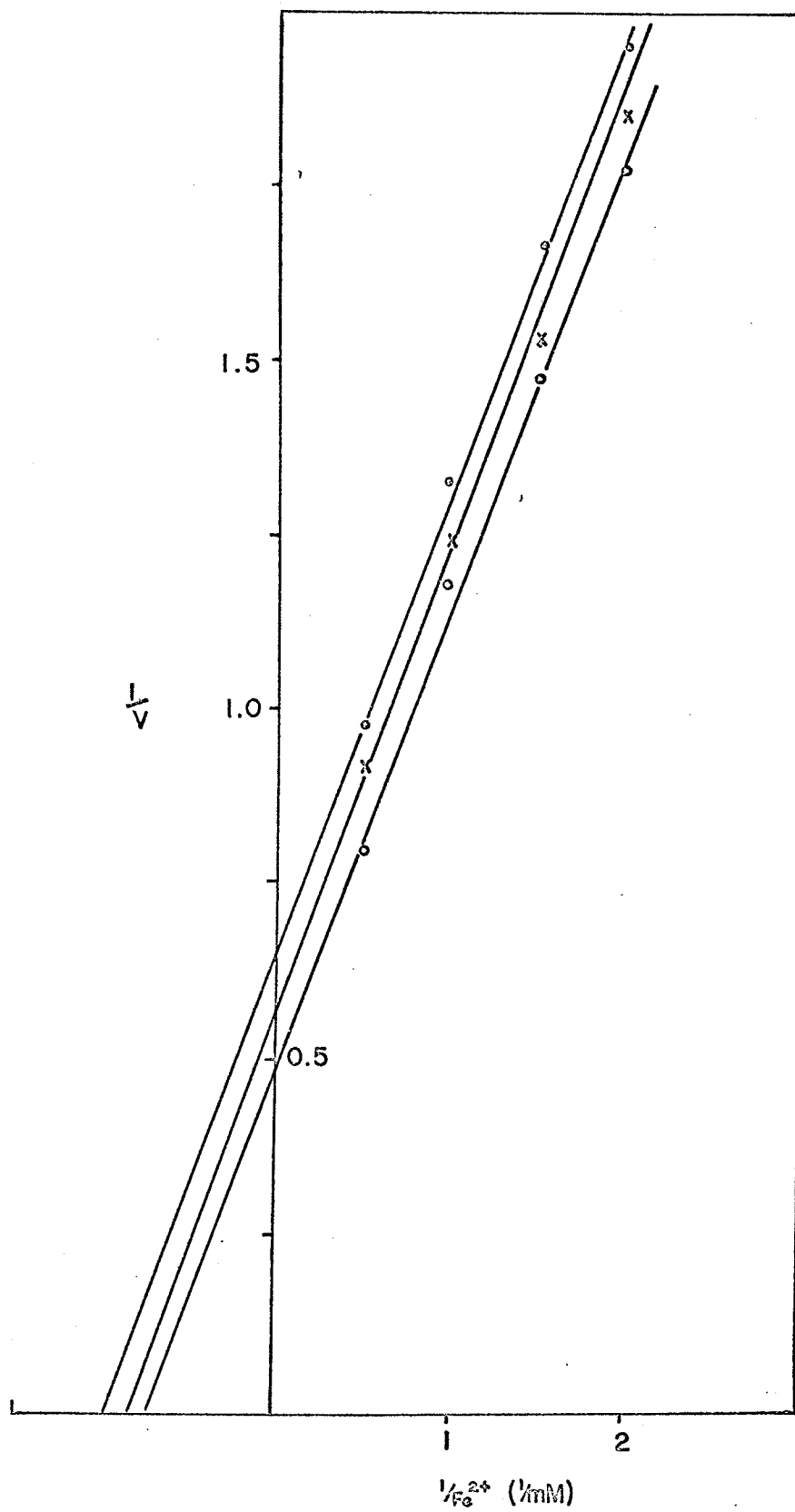
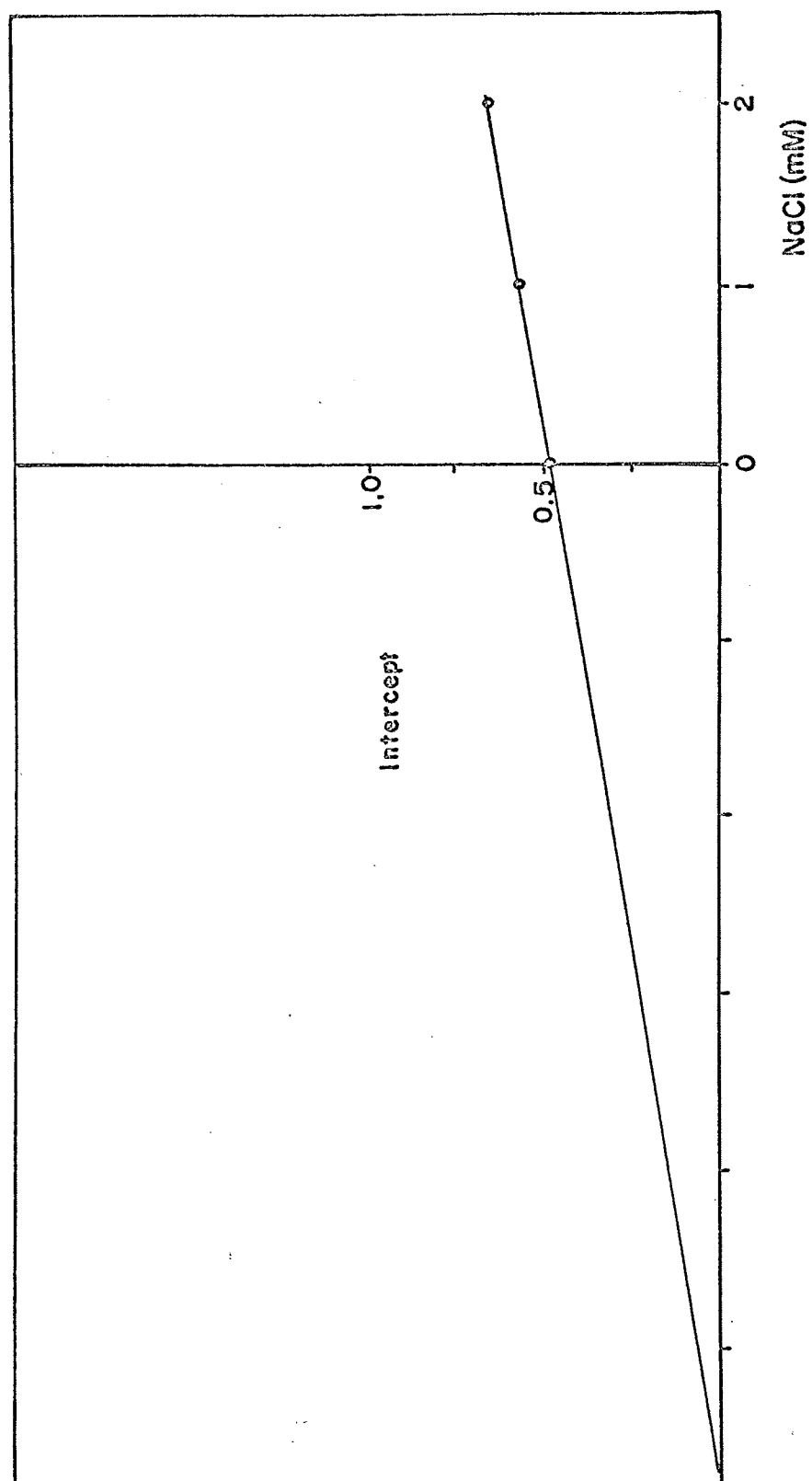


Fig. 32. Replot of Fig. 31, intercept ($1/\text{app}V_{\text{max}}$) versus NaCl concentration.



Fe^{++} -cyt. c reductase was treated with excess $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. After incubation for one hour the enzyme was dialysed against two changes of 0.01 M acetate-acetic acid buffer (pH 5.7), then centrifuged for 2 hours at $150,000 \times g$. The amount of ferrous and ferric iron on the enzyme was assayed before incubation with cyt. c. The reduction of cyt. c was followed spectrophotometrically and after completion of the reaction the enzyme was separated from cyt. c by DEAE-cellulose chromatography. The amount of ferrous and ferric iron was assayed again and compared with the amounts assayed prior to interaction with cyt. c.

The results of the reaction between Fe^{++} -cyt. c reductase and cyt. c are illustrated in Table XVI. The total iron associated with the enzyme before and after interaction with cyt. c was constant but the ferrous iron content decreased. Concomitantly, the cyt. c was reduced during the reaction. The mole ratio of iron oxidized:cyt. c reduced was 0.8:1.0. This result fortified the belief that Fe^{++} -cyt. c reductase is an enzyme that occurs in two stable forms and is capable of carrying out half reactions. This result illustrated that the reduction and oxidation of non-heme iron is responsible for the two enzyme forms,

TABLE XVI

Cytochrome c reduction by Fe⁺⁺-cyt. c reductase

Fraction	Protein ^a μg	Fe ⁺⁺ moles	Total iron moles	Iron: protein	Cyt. <u>c</u> reduced ^b moles	Fe ⁺⁺ after cyt. <u>c</u> reduction	Total iron after cyt. <u>c</u> reduction	Fe ⁺⁺ oxid.: cyt. <u>c</u> reduced
Holo-enzyme	300	1×10^{-8}	4.6×10^{-8}	1.1×10^{-7}	11:1	1×10^{-8}	3.8×10^{-8}	1.1×10^{-7} 0.8:1

^aBased on a molecular weight of 30,000 for the protein moiety (see Figs. 17 and 18).^bAssayed spectrophotometrically using a molar extinction coefficient of 45.5×10^3 at 417 mμ ($\Delta OD_{417} = 0.15$), (Margoliash and Frohwirt, 1959).

similar to the pyridoxal phosphate-pyridoxamine phosphate
interconversion found with transaminases.

DISCUSSION

DISCUSSION

I. Carbon Dioxide Fixation

Cell-free extracts, in the presence of certain essential intermediates, and metabolizing intact cells of Ferrobacillus ferrooxidans are capable of fixing carbon dioxide. Studies with intact cells revealed two classes of compounds as the primary intermediates, phosphorylated carbohydrates and amino acids formed from Krebs cycle intermediates. Maciag and Lundgren (1964) using intact cells of Ferrobacillus ferrooxidans showed that after 5 minutes in the presence of Fe^{++} and C^{14}O_2 the same two classes of labelled compounds appeared (sugars and phosphorylated carbohydrates; Krebs cycle intermediates and amino acids). This overall picture is consistent with results previously obtained with plants by Calvin and his co-workers, and with other chemoautotrophic bacteria (Elsden, 1962). The primary compounds indicate that the autotrophic "Calvin-Bassham cycle" (Mahler and Cordes, 1966) and the heterotrophic Wood-Werkman ($\text{C}_3 + \text{C}_1$ condensation) reaction

are the two major pathways of CO₂ fixation.

The presence of a high percentage of labelled 3-PGA in whole cell fixation studies suggested the presence of phosphoribulokinase and carboxydismutase. These enzymes have been investigated in the CO₂ fixation of other chemo-autotrophs: the thiobacilli (Trudinger, 1955; 1956; Aubert et al., 1957a; 1957b; Santer and Vishniac, 1955; Suzuki and Werkman, 1958c; Aleem and Huang, 1965; Gale and Beck, 1966), the hydrogen bacteria (Bergman et al., 1958) and the nitrifiers (Aleem, 1965). The results indicate that the primary CO₂ fixation reaction in Ferrobacillus is the carboxylation of RuDP which in turn feeds intermediates into pathways leading to hexose synthesis, the tricarboxylic acid cycle, and regeneration of R-5-P essential for the cyclic autotrophic mechanism to continue (Suzuki and Werkman, 1958a,c).

Cell-free extracts exhibited the following enzyme activities: phosphoriboisomerase, phosphoribulokinase, carboxydismutase, phosphoglycerokinase, and triose phosphate dehydrogenase necessary to form GA-3P from R-5-P. The other enzymes necessary for the conversion of GA-3P to R-5-P are ubiquitous and their activity was not assayed (Elsden, 1962). The triose phosphate dehydrogenase activity, similar to the

enzyme was present in crude extracts, dialysed extracts were lacking this activity. The enzyme could be similar to the E. coli enzyme (Kornberg, 1965) favouring the conversion of C_4 compounds to C_3 compounds when there is an excess of Krebs cycle intermediates and a paucity of C_3 compounds.

Although the presence of other carboxylation schemes is not excluded, the results implicate only the initial carboxylation of RuDP by carboxydismutase and the carboxylation by PEP carboxylase. The presence of the malic enzyme activity could not be demonstrated, similar to the finding of Suzuki (1958) in Thiobacillus thiooxidans.

Kornberg (1965) postulated that only the PEP carboxylase of Enterobacteriaceae serves the "anaplerotic" function and is the only major enzyme responsible for C_4 synthesis from C_3 compounds leading to tricarboxylic acid cycle intermediates. The results in Ferrobacillus ferrooxidans lead to the same conclusion.

The metabolic control of PEP carboxylase in Ferrobacillus mimics the control of the "heterotrophic" enzyme in the Enterobacteriaceae. Acetyl CoA and aceto-acetyl CoA activate the enzyme's activity, similar to results found in E. coli (Cánovas and Kornberg, 1965; Nishikido et al.,

1965) and S. typhimurium (Sanwal and Maeba, 1965). The enzyme activity is inhibited by aspartate (Nishikido et al., 1965; Sanwal and Maeba, 1965), thus implicating "allosteric" control of its "anaplerotic" function. The similarity in results between the heterotrophic Enterobacteriaceae and the chemoautotroph Ferrobacillus supports the contention that "heterotrophic" CO₂ fixation is subjected to the same control mechanisms.

CO₂ fixation studies in cell-free extracts provide evidence to support the findings in cell-free iron oxidation. The inability of cell-free extracts to oxidize Fe⁺⁺ is reflected in the inability of these extracts to fix CO₂ without the addition of necessary intermediates (PEP, R-5-P) and an energy source (ATP). Also, the requirement of cell-free extracts for exogenous NADH in order to reduce 1,3-DPGA exhibits the same inability. On the other hand, intact cells must be able to synthesize ATP, NADH, and necessary intermediates for CO₂ fixation by the oxidation of Fe⁺⁺ alone, thus substantiating the chemoautotrophic nature of this bacterium.

II. Iron Oxidation

Optimal iron oxidation by intact cells of Ferrobacillus ferrooxidans was found to occur at pH 3.0 ($\text{H}_2\text{O}-\text{H}_2\text{SO}_4$) in the presence of 250 μmoles $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (in 2.8 ml). The optimal pH for iron oxidation coincided with the results of Silverman and Lundgren (1959b) using Ferrobacillus ferrooxidans but differed from those of Beck and Elsdon (1958) and Landesman, Duncan and Walden (1966) using Thiobacillus ferrooxidans. Thiobacillus ferrooxidans seems to prefer a lower pH, while Ferrobacillus ferrooxidans prefers a lower Fe^{++} concentration. In other respects the oxidation mechanism in these two bacteria is the same.

The inhibition of iron oxidation in intact cells by azide points to the involvement of a cytochrome oxidase in the oxidation mechanism. Vernon, Mangum, Beck, and Shafia (1960) and Blaylock and Nason (1963) postulated that iron oxidation is accomplished by means of cyt. c and cyt. a. The results of studies with intact cells and cell-free extracts lead to the same conclusion. Cellular cyt. c under anaerobic conditions in the presence of Fe^{++} , ascorbate, or sodium dithionite is rapidly reduced. Under aerobic

conditions this same reduction takes place but there is a rapid reoxidation of the cellular cytochrome c. The presence of both cyt. c and cyt. a in Ferrobacillus ferrooxidans indicates the involvement of both these enzymes in the oxidation mechanism.

The inability to isolate cell-free extracts capable of oxidizing iron and the loss of iron oxidase activity in frozen cells indicate that the electron transport chain is disrupted between the initial reduction of cellular cyt. c and the subsequent reoxidation of cyt. c. The presence of both enzymes, Fe^{++} -cyt. c reductase and cytochrome oxidase, in extracts of inactive frozen cells shows that there is no loss of activity in the enzymes involved, only an inability of the electrons to pass from one electron carrier to the other. This disruption of the cytochrome chain between cyt. c and cyt. a is borne out by experiments with frozen intact cells and cell-free extracts. Although the reason for the disruption of the electron transport chain is known, the cause of the disruption is not understood.

FAD, FMN, and quinone were unable to repair the disruption in the electron transport chain. The breakage in the electron transport chain may be due to: (1) a

physical disruption of the electron transport system separating cyt. c and cyt. a in the membrane so that electron flow terminates at cyt. c; or (2) a loss of necessary cofactors bridging the cyt. c - cyt. a gap. The former seems the most probable. The loss of oxidizing ability by such a mild treatment as freezing points to the extreme lability of the system. In order to isolate cell-free extracts capable of oxidizing iron, milder techniques (e.g. protoplast lysis) might be employed rather than the harsh techniques of sonic oscillation, grinding, or other gross physical methods.

III. Ferrous Iron-Cytochrome c Reductase

The results presented here indicate that the properties of ferrous iron-cyt. c reductase are similar to those reported by Blaylock and Nason (1963) for the same enzyme. Ferrous iron-cyt. c reductase appears to be the initial enzyme in the iron-oxidizing system of Ferrobacillus ferrooxidans. The compactness and close association of the system to the cell (membrane) necessitated the use of harsh treatment to solubilize the enzyme. Once the enzyme was solubilized, it displayed remarkable stability for a protein;

this stability appeared to be attributable to its close association with nucleic acid (Blaylock and Nason, 1963).

In acetate buffer (Figs. 8 and 9) there was no pH optimum of catalytic activity evident (Blaylock and Nason, 1963). In 8 mM sodium barbital-acetic acid buffer the enzyme displayed an activity optimum at pH 7.0 (Fig. 10). However, when the enzyme was assayed anaerobically in 8 mM barbital no pH optimum was evident and under these conditions the actual pH optimum may be higher than 7.5. Since the oxygen tension in the environment of the enzyme is unknown, no definite statement can be made as to the actual pH optimum of the enzyme in vivo. Aerobically, in vitro, a pH optimum at 7.0 in barbital buffer was reproducible, but the non-enzymatic reaction above pH 7.0 increased rapidly to more than 50% of the total reaction (enzymatic plus non-enzymatic) and measurement of the reaction became extremely difficult.

Barbital buffer was found to be much better than acetate buffer in maintaining a constant pH of the reaction mixture. This increased buffering action was especially required when varying concentrations of cyt. c were used to find the effect of cyt. c concentration on enzyme activity.

The use of different buffers with varied buffering capabilities could account for the discrepancies in the concentrations of substrates saturating the enzyme and the $\text{app}K_m$ values with those previously reported by Blaylock and Nason (1963). In barbital buffer, the concentrations of substrate saturating the enzyme and the $\text{app}K_m$ values were: 4 mM and 1 mM Fe^{++} , 0.15 mM and 0.095 mM cyt. c respectively. Using acetate buffer (pH 5.7), Blaylock and Nason (1963) reported saturating substrate concentrations of 3 mM Fe^{++} and 0.18 mM cyt. c, and apparent K_m 's of 0.15 mM Fe^{++} and 0.08 mM cyt. c.

Reaction mechanism

The kinetic analyses of initial velocity studies with Fe^{++} -cyt. c reductase indicated that the enzyme adhered to a Ping Pong Bi Bi mechanism. The existence of this mechanism was substantiated by three criteria. When one substrate was varied at several fixed concentrations of the second substrate, double reciprocal plots of $1/v$ versus $1/(\text{varied substrate concentration})$ resulted in a family of linear parallel lines (Figs. 21 and 23). It was evident that equation (6) conforms with the following equation,

$$y = mx + b$$

where $y = 1/v$, $m = K_a/V_1$, $x = 1/A$, and $b = 1/V_1(1 + K_b/B)$ when A was the varied substrate. Thus at any fixed concentration of B the line has the same slope K_a/V_1 ; at fixed values of B, when A is varied, parallel lines should arise in double reciprocal plots (Bulos and Handler, 1965; Ray and Roscelli, 1964). In contrast, neither an Ordered or Rapid Equilibrium Random Bi Bi mechanism produces parallel lines under the same conditions (equation (3)); both the slopes and the intercepts of the lines would change with a change in the concentration of substrate B.

When a constant ratio of substrates ($\text{Fe}^{++}:\text{cyt. } \underline{c} = 3:1$) was used in initial velocity studies, a linear relation was evident in double reciprocal plots of $1/v$ versus $1/(\text{substrate conc.})$ (Figs. 25 and 26). Ordered and Rapid Equilibrium Random Bi Bi mechanisms would produce a parabola in the same circumstances (see equations (7) and (8)); thus it is evident that equations (2) and (3) do not describe the results of initial velocity studies of Fe^{++} -cyt. $\underline{c}$ reductase.

Cleland (1963b;c) demonstrated that a reaction mechanism could be identified in some cases by end-product inhibition studies. Fe^{++} -reduced cyt. $\underline{c}$ and Fe^{+++} -cyt. $\underline{c}$

pairs exhibited competitive inhibition, which is consistent with a Ping Pong Bi Bi or a Random Bi Bi mechanism. However, a Random Bi Bi mechanism can be ruled out because of the linear double reciprocal plots obtained when $1/v$ is plotted against $1/S$ where S is either substrate. A Random Bi Bi mechanism would yield a non-linear relationship (Cleland, 1963a). The Random Bi Bi mechanism can also be discounted because of the linear double reciprocal plots obtained when both substrates were varied but the ratio of one substrate to the other was constant.

The results are consistent with a Ping Pong Bi Bi mechanism (as depicted in (9)), where there are two stable forms of the enzyme, $E(Fe^{+++})$ and $E(Fe^{++})$. In this type of mechanism each enzyme form binds only one substrate (Fe^{++} or cyt. c). When the end-product (Fe^{+++} or reduced cyt. c) is released, the enzyme changes its form ($E(Fe^{+++})$ to $E(Fe^{++})$ to $E(Fe^{+++})$). The enzyme oscillates between the two forms, acting as a shuttle mechanism carrying some reactant from the first to the second substrate (in this case an electron).

The ability of the enzyme to carry out a half reaction was measured and the results fortified the belief that the factor responsible for the existence of two enzyme forms was

the non-heme iron tightly bound to the enzyme (only one mole of non-heme iron is tightly bound to the enzyme, as demonstrated in Table XIV). This ability of the enzyme to reduce cyt. c in the absence of externally added ferrous iron is consistent with a Ping Pong Bi Bi mechanism.

Composition of iron-cytochrome c reductase

All active preparations of the enzyme contained RNA, a fact consistent with the report of Blaylock and Nason (1963). Although the enzyme could be dissociated into protein and RNA subunits, the resultant protein subunit was devoid of catalytic activity. This unique phenomenon has not previously been reported, although several enzymes have been isolated associated with either DNA or RNA (Barth and Hele, 1964; Hele, 1964; Symons, 1965; Hofer and Pette, 1965a,b; Hele and Barth, 1966).

The inability of the RNA-free enzyme to catalyse the reaction can be explained only by a change in conformation of the protein (Koshland, 1964). The RNA of the enzyme must play some role in binding the enzyme to the cell (membrane) in the proper position and in maintaining the enzyme in the proper conformation for catalysis. Once the enzyme is

released from the RNA, the effect of the release on the protein conformation may be substantial indeed.

In both yeast cytochrome b_2 (lactate-cyt. c oxidoreductase) and Fe^{++} -cyt. c reductase the DNA- and RNA-components appear to be specific and are found in all fractions containing catalytic activity (see Fig. 16), but the yeast cytochrome b_2 catalytic activity is unaffected when the DNA-component is removed (Mahler and Pereira, 1962; Armstrong *et al.*, 1963; Symons, 1965). Hofer and Pette (1965b) reported that rabbit muscle phosphofructokinase activity is modulated by the nucleic acid associated with the enzyme. The presence of nucleic acid on phosphofructokinase alters the ability of the enzyme to catalyze the reverse reaction and results in a lowered forward:backward reaction ratio. Other cases of modulation of catalytic activity by nucleic acids have also been reported by Hele and Barth (1966) and Allende *et al.* (1966) with aminoacyl ribonucleic acid synthetases. Although the catalytic activity of these enzymes is modified by RNA, the presence of RNA is not obligatory, in contrast to Fe^{++} -cyt. c reductase.

Associated with the inability of the RNA-free protein

to catalyze the reaction is the failure of attempts to reconstitute the holoenzyme from the protein and RNA subunits. This failure to reconstitute the catalytic activity can be explained by the change in the RNA portion of the enzyme (Armstrong et al., 1963; Jackson et al., 1965). Yeast cyt. b₂ (Mahler and Pereira, 1962) contains about 33 nucleotide residues in each molecule of protein, which indicates a molecular weight of approximately 10,000 for the DNA portion of the enzyme. When the DNA portion of the enzyme is separated by chromatography (Symons, 1965), the DNA molecular weight increases to 100 - 120,000 (Armstrong et al., 1963; Jackson et al., 1965). This increase in molecular weight seems to be due to the DNA portion of the enzyme aggregating to form a new structure of higher molecular weight. The same type of problem exists with Fe^{++} -cyt. c reductase. As shown in Figs. 17 and 18, the holoenzyme exhibits the sedimentation velocity of a particle with molecular weight of 100 - 110,000. Upon dissociation, the enzyme subunits have molecular weights of 30,000 for the protein portion and 300 - 320,000 for the RNA portion (Figs. 19 and 20). If the weight of the RNA portion of the enzyme is 65,000 (Table XV), then the protein portion:RNA portion

ratio in the holoenzyme is 1:1 since the protein subunit (portion) has a molecular weight of 27-30,000 (Figs. 17 and 18). The appearance of an RNA molecule weighing 300 - 320,000 after dissociation can be explained by assuming that there is an aggregation of 5-6 RNA molecules to form the RNA subunit. The failure to reconstitute the holoenzyme from its subunits also hinged upon the failure of the RNA subunit to segregate into the 5-6 RNA molecules required to reunite with the protein subunit.

The presence of iron on the enzyme is consistent with the belief that the reaction mechanism is Ping Pong Bi Bi. Since the enzyme adheres to a Ping Pong Bi Bi mechanism, the two forms of enzyme must differ from one another in that one form carries an electron from the first substrate (Fe^{++}) to the second substrate (cyt. c). Since iron is capable of oxidation-reduction, a non-heme iron atom incorporated in the enzyme structure would be consistent with this premise. Non-heme iron analysis revealed 9-10 iron atoms attached to the holoenzyme, but only one non-heme iron atom was tightly bound and found with the protein subunit (Table XIV). The presence of the other iron atoms on the enzyme can not be explained unless one assumes that

the other atoms are bound non-specifically to the RNA portion of the holoenzyme. Attempts to show that the binding of iron to an iron-free enzyme is time-dependent failed and the results indicated that the incorporation of the non-heme iron was instantaneous. Analyses carried out to detect the presence of labile sulfide and flavin(s) were also negative. Thus the sole factor necessary for catalysis, in addition to the enzyme, was non-heme iron.

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