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MICROBIAL CORROSION OF CRUDE OIL PIPELINE

by



CHRISTIAN OKECHUKWU OBUEKWE

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH  
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## ABSTRACT

Comparison of some physico-chemical characteristics of crude oil samples from Pembina and Rainbow oil fields of North Central Alberta shows some differences. The generally higher Bottom Sediment and Water content of Rainbow crude oil, as against the lower values obtained for Pembina crude oil did not correlate with the higher incidence of pipeline failure from corrosion reported for the pipeline system serving the Pembina oil field. This indicates that factor(s) other than, or in addition to, pure electrochemical attack is responsible for the observed frequencies of pipe failure reported in the two pipeline systems. The marked absence of bacteria in Rainbow crude oil and the invariable occurrence of bacteria in Pembina oil samples are pointers to the probable contribution of bacteria to the high incidence of corrosion failure observed in Pembina pipeline system.

A variety of bacteria, including the anaerobic sulphate-reducers and aerobic/facultative bacteria occur in Pembina crude oil samples and produced water. Some of these bacteria isolated acted in concert to produce  $S^{2-}$  giving rise to the cascade system of  $S^{2-}$  generation.

A *Pseudomonas* sp. isolated from Pembina crude oil and designated Isolate #200 is capable of reducing sulphite, thiosulphate, and elemental sulphur to sulphide, in addition to reducing ferric iron  $[Fe(III)]$  to ferrous form  $[Fe(II)]$ . The capability of Isolate #200 to reduce  $Fe(III)$  to  $Fe(II)$  is iron-inducible and is associated with the cytochrome content of the cells. The ability to reduce  $Fe(III)$  to  $Fe(II)$  increases with cytochrome content and is inhibited by electron transport

inhibitors. Whole cells and spheroplast preparations are capable of reducing Fe(III) but isolated cell components lack any significant ability to reduce Fe(III) to Fe(II).

Polarization characteristics of mild steel in cultures of Isolate #200 show that the organism causes anodic depolarization. Both anodic and cathodic depolarization, however, may occur under certain cultural conditions. Photomicrographs associate anodic depolarization of the mild steel with the prevention of the formation of surface coat on the steel specimens. Intense anodic depolarization of mild steel passivated by nitrite treatment show that the depolarization is due to the removal of protective ferric film during the reduction to the soluble Fe(II) forms. Exposure of the mild steel coupons in cultures of Isolate #200 causes weight losses and may cause pit formation, as well.

Isolate #200, or any microorganism with similar physiological characteristics, possesses the capability to significantly modify its environment to cause corrosion of iron and steel and is, therefore, considered a corrosion organism; its corrosive activities stem from the combined ability to reduce Fe(III) to Fe(II) and to produce  $S^{2-}$ .

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## ABBREVIATIONS, SYMBOLS AND SYNONYMS

The symbols Fe(III) and Fe(II) have been used to denote iron in the oxidation states 3 (ferric) and 2 (ferrous), respectively. These symbols were used instead of  $\text{Fe}^{3+}$  and  $\text{Fe}^{2+}$ , in recognition of the fact that iron in iron compounds present in the oxidation state 3 or 2 may not necessarily exist as the dissociated species.

GLC = Gas Liquid Chromatography

DNA = Deoxyribonucleic acid

$e^-$  = electron (or reducing power)

synthetic medium = chemically defined medium

## INTRODUCTION

### The Problem

Very frequent failure of the crude oil pipeline system serving the Pembina oilfield of North Central Alberta has been observed. Associated with this observation has been a constant occurrence of a variety of bacterial populations in the crude oil being transported in the pipeline system and in the crusts of the corrosion products. In addition to anaerobes, a thriving aerobic-facultative anaerobic bacterial population also exists in Pembina crude oil.

Often, primary-producing oilfields (e.g. Rainbow Oilfield of Northwestern Alberta) do not have this load of bacteria. Although the Rainbow field crude oil has similar chemical characteristics as the Pembina oil, not as much pipe failure has been reported in the Rainbow oil-carrying system. Pembina field is a secondary-producing field and depends on the injection of water to maintain production. The conspicuous difference between these two oil samples is the marked absence of detectable microorganisms in the Rainbow crude oil. This is circumstantial evidence for a possible role of these microorganisms in the corrosion process. Moreover, the corrosion product in Pembina lines contains ferrous sulphide which is considered indicative of anaerobic microbial corrosion.

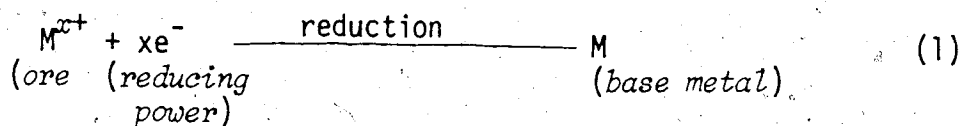
Some of the aerobic bacterial contaminants of Pembina oil show extensive biochemical capabilities but their role, if any, in corrosion process is not known. It was believed, however, in this laboratory that the corrosion of the pipeline system was engendered by the

activities of all these contaminant bacteria. It was in the attempt to understand the role of these organisms in the corrosion process that this research was undertaken.

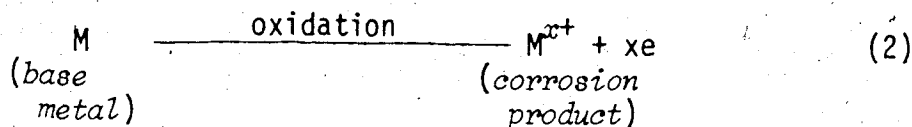
### Theoretical basis for microbial corrosion

It is difficult to visualize how microorganisms can cause corrosion of metals. A general consideration of the thermodynamic changes involved in winning metals from their ores is a good basis for understanding the potential role of microorganisms in the corrosion of metals.

Most metals occur in nature, not as the element, but as oxides, sulphides (von Fraunhofer, 1974) or other compounds, *i.e.* as ores, in which the metals occur in higher oxidation states than are observed when they are in the elemental forms. Extractive metallurgy includes the reduction of these oxidized states to the free metals. This reductive process involves input of energy, *i.e.* 'work' is done on the ores (equation 1).



This is essentially the reverse of corrosion:



As a result of the reductive process, free metals possess higher internal energy relative to their ores. In keeping with basic thermodynamic principles, metal will tend to lose their excess energy and be transformed into their more stable oxidized states. These changes are

illustrated in fig. 1.

Although structural metals are stable, their stability is finite and is determined by the metals' tendency to lose the extra energy (*i.e.* electrons) acquired during the extractive (reductive process). As shown in figure 1, it is evident that for a base metal, like iron, transformation to an oxidized state is thermodynamically favoured. However, although the reaction is thermodynamically feasible it still may not proceed fast enough to be an industrial problem.

Corrosion has been defined as the deterioration of a metal because of its reaction with its environment (Fontana and Green, 1967). The environment in which a metal is placed may contribute to the corrosion process by accelerating the oxidation reaction (equation 2). Microorganisms by virtue of their biochemical activities can modify their environment. It is by this process that microorganisms accelerate the de-energizing of metals (*i.e.* corrosion), a reaction which is favoured thermodynamically, as illustrated in fig. 1. Therefore, corrosion problems encountered in any environment are actually not a thermodynamic one but a kinetic one.

On the basis of this discussion, it is evident that a rational approach in understanding the role of microorganisms in the corrosion of metals is to study the processes by which the organisms modify the environment and how these environmental modifications can affect the structural integrity of the metal.


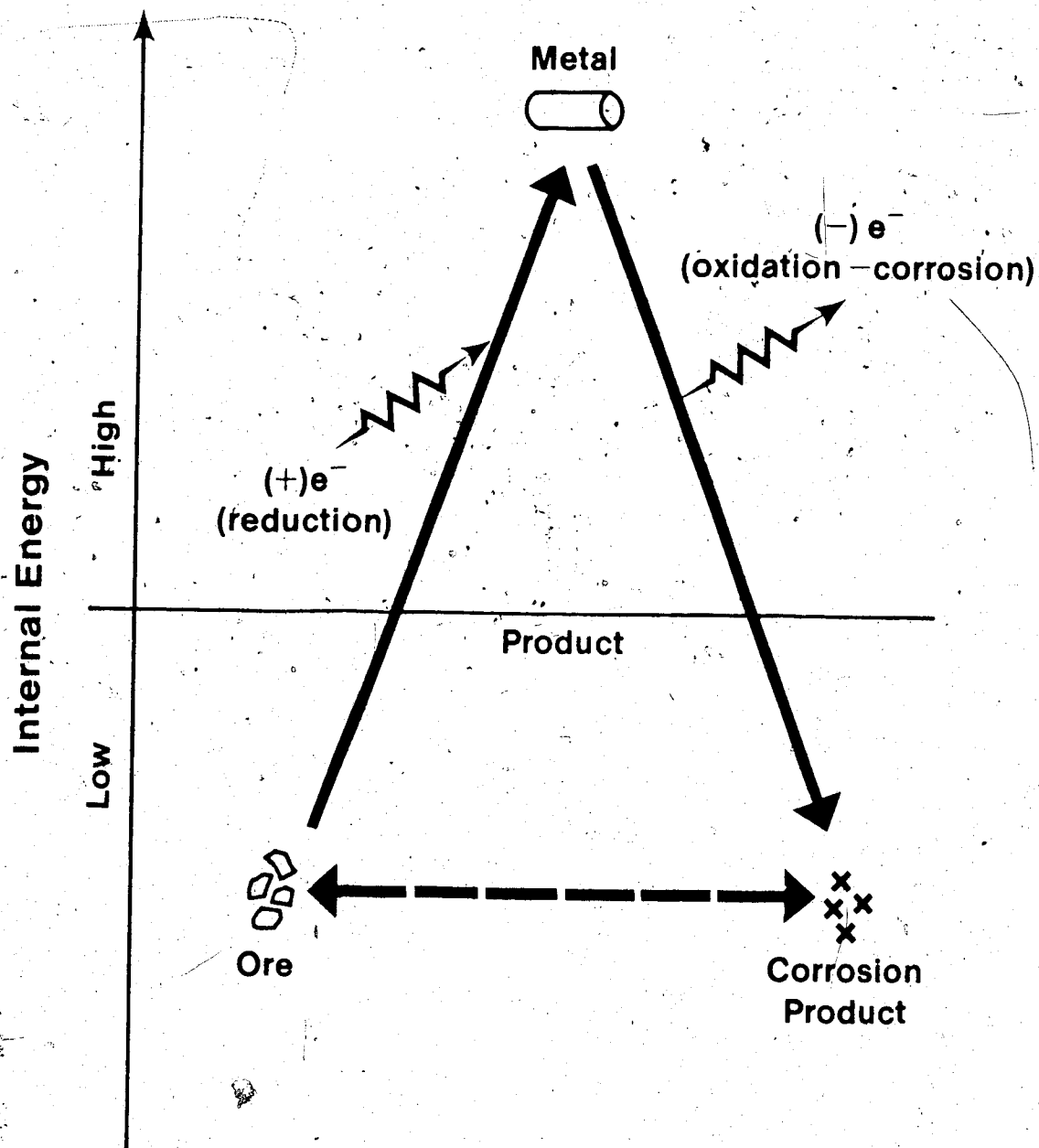


Fig. 1. Diagrammatic representation of the changes in the internal energy content of products of metal ore-metal-corrosion product transformation. The broken line between the metal ore and corrosion product indicates that these substances may or may not be of the same chemical nature.



## LITERATURE REVIEW

### Microbial Corrosion of Metals

Corrosion occurs as a result of physico-chemical interaction of metal with its environment. Microbes can cause and accelerate corrosion because of their ability to affect changes in the environment. If microorganisms are to alter their environment, there must be adequate sources of carbon and energy for growth and metabolism. Soil and water environments contain varied amounts of organic and inorganic materials that may serve as energy sources for chemo-organotrophic and chemo-lithotrophic organisms, respectively. Chemo-organotrophic organisms are those that utilize organic compounds as energy sources, while chemo-lithotrophs obtain their energy by oxidation of inorganic compounds. Moreover, the environment may encompass a range of physical conditions (like pH,  $E_h$ , and aeration) that supports different forms of microbial life. The degree of aeration plays a prominent role in the diversity of microbial life. Under aerobic conditions only organisms which need  $O_2$  as terminal electron acceptors (aerobes) and facultative anaerobes (organisms that can grow in the presence or absence of  $O_2$ ) thrive. Environments devoid of  $O_2$  support only the growth of anaerobes (organisms that do not use  $O_2$  as terminal electron acceptor and may be killed by traces of  $O_2$ ) and facultative anaerobes. It is apparent, therefore, that microbial corrosion has to be considered as a problem when metals are introduced to aquatic and/or soil environments.

The role of microorganisms in corrosion of metals has been discussed by Hughes (1963), Baumgartner (1973) and Westlake and Cook (1979).

However, the diversity of microbial population in any natural environment renders any exact classification of type of corrosion according to specific organisms difficult (Updergraff, 1955)

Corrosion of metals by microorganisms has been associated with two genera of bacteria, the aerobic *Thiobacillus* and the anaerobic *Desulfovibrio* spp. Their corrosive activities stem from their interactions with sulphur and iron compounds. Under aerobic conditions *Thiobacillus* spp oxidize reduced sulphur compounds to sulphuric acid which reacts with the metal. On the other hand, *Desulfovibrio* spp. act, under anaerobic conditions, reducing sulphate to sulphide which reacts with the metal to form the characteristic corrosion product like ferrous sulphide (FeS) when Fe(II) is present. Hadley (1948) noted that microorganisms which cause corrosion do so by one or more factors which depend on the physiology of the organisms. These factors include:

- (i) direct influence on the rate of the anodic or cathodic reaction;
- (ii) change of surface film resistance by their metabolic activities;
- (iii) creation of corrosive environment;
- (iv) establishment of barrier by growth and multiplication so as to create electrolytic concentration cells.

#### Anaerobic Corrosion of Metals

Ever since the early observations of Bengough and May (1924), and von Wolzogen Kuhr and van der Vlugt (1934), much has been reported on anaerobic corrosion of metals, mainly ferrous metals. This form of corrosion occurs in wet, neutral soil devoid of  $O_2$ ; conditions previously



thought to be ideal for the prevention of corrosion. Anaerobic corrosion is largely due to the production of hydrogen sulphides by sulphate-reducing bacteria and is characterized by pit formation and formation of insoluble corrosion product, ferrous sulphide. Sulphate-reducing bacteria, most important being *Desulfovibrio* spp., have been the most studied bacterial agent of corrosion. Moreover, *Desulfovibrio* spp. are ubiquitous, having been found in muddy water and soils all over the world (Miller, 1970), in rumen (Coleman, 1960) and in oil and gas storage systems (Pankhurst, 1967). To some extent, anaerobic spore-forming *Clostridium nigrificans*, now known to be *Desulfotomaculum nigrificans* (Postgate, 1979), has been implicated but its occurrence is rare in oilfields where much corrosion damage has been reported (NACE TPC Publication, No. 3).

Hadley (1939) considered microbial anaerobic corrosion of metals to be the most important corrosion process in nature. This conclusion stemmed from the inferred occurrence of pipeline failure due to the activity of bacterial sulphate-reducers. The importance of sulphate-reducing bacteria in the anaerobic corrosion of pipelines which carry sewage, and oil and gas was reiterated by Beckwith (1941). Bastin (1926) has earlier demonstrated the existence of sulphate-reducing bacteria in oil and water samples from a depth of 518.2 metres (1700 ft.) underground. Both the chemical characteristics of the water and the sulphur content of crude oil were affected by the presence of these organisms. The work of Kulman (1949) had shown the presence of sulphate-reducing bacteria in soils where corrosion of pipeline occurred. Although seasonal variations in aeration and soil moisture could affect the type of corrosion process, anaerobic bacteria were associated with

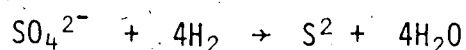
underground corrosion cycle (Kilman, 1949; Horvath, 1962). Butlin, Vernon and Whiskin (1952) reported that sulphate-reducing bacteria were associated with most of studied cases of corroded water mains. The extent of anaerobic underground corrosion in the United States was reviewed by Logan (1949); Deuber (1953) and more recently in Britain by Miller (1970).

The early studies of anaerobic corrosion was by burial of metal strips in soils. The result of these studies was complicated by the fact that neither the exact soil environment could be easily determined, nor was the soil uniform or stable. The variations in soil characteristics, chemical or physical, would exert some effect on the corrosion process independent of bacterial activity. As an example, since the conductivity of the soil is electrolytic, the amount and type of salt present would determine the conductivity, and corrosivity of the soil will increase with salt content. More recently, studies of microbial anaerobic corrosion involved the use of pure cultures of organisms. Such studies have enabled workers to investigate and evaluate mechanisms by which anaerobic organisms cause corrosion and have been extensively discussed by Miller (1970). Moreover, the use of pure cultures enables investigators to study the effects of specific environmental factors on metal corrosion. Willingham and Quinby (1971) studied the effects of hydrostatic pressure on anaerobic corrosion by three marine sulphate-reducing bacteria, while Booth *et al.* (1966, 1967) investigated the effects of ferrous ion concentration on anaerobic corrosion by *Desulfovibrio* spp.

## Mechanisms of Anaerobic Corrosion

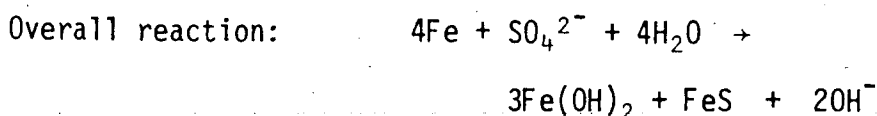
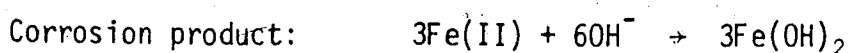
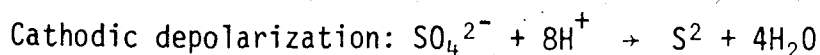
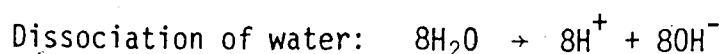
### Cathodic Depolarization

Most of recent work on microbial corrosion has been aimed at confirming or disproving the mechanism of anaerobic corrosion of iron and steel as proposed by von Wolzogen Kuhr and van der Vlugt (1934) and Wolzogen Kuhr (1961). These workers had proposed that sulphate-reducing bacteria are able to remove molecular hydrogen from polarized cathodes, oxidizing it to protons and electrons. The reducing power of the electrons is then utilized by sulphate-reducing bacteria for the dissimilatory reduction of sulphate to sulphide according to the equation:



This proposal is supported by an earlier observation (Stephenson and Strickland, 1931) that sulphate-reducing bacteria possess the enzyme, hydrogenase, that utilizes hydrogen for the reduction of sulphate and by the later works of Starkey and Wright (1945) and Butlin and Vernon (1952). The detailed mechanism proposed by von Wolzogen Kuhr and van der Vlugt

was:



This mechanism suggests that the ratio of metal corroded to the sulphide produced is 4:1, however, ratios much different from this have been known (Miller, 1970).

Working with pure cultures of sulphate-reducing bacteria, Horvath and Solti (1959) and Booth and Tiller (1960) demonstrated that these organisms actually brought about cathodic depolarization. With *Desulfovibrio vulgaris* strain Hildenborough, depolarization of the cathodic process was evident only during the active growth. It was also observed that cathodic depolarization occurred only with hydrogenase-positive organisms. Thus, no depolarization was observed with *Desulfotomaculum orientis* which is hydrogenase-negative. Because there is a linear relationship between corrosion rate and hydrogenase activity, Booth and Wormwell (1961) concluded that cathodic depolarization was a primary factor in corrosion by sulphate-reducers. Further studies by Booth and Tiller (1962) showed very interesting results. In halophilic *Desulfovibrio salexigens* California 43:63, no protective ferrous film was found. In addition, although this strain showed no hydrogenase activity with  $\text{SO}_4^{2-}$  electron acceptor, a very vigorous cathodic depolarization occurred. This depolarization correlated better with the hydrogenase activity obtained with benzyl viologen as the electron acceptor than with sulphate. It appears then that to cause cathodic depolarization hydrogenase activity does not have to be coupled to sulphate reduction. With thermophilic strains of *Desulfotomaculum nigrificans* that were hydrogenase-negative to sulphate, Tiller and Booth (1962) reported two contrasting results: the one which showed hydrogenase activity with benzyl viologen as the electron acceptor depolarized the cathode, while the other strain that was negative to

the dye did not.

In as much as the results of the polarization studies of Booth and Tiller (1962) and Tiller and Booth (1962) in batch cultures confirmed that cathodic depolarization is an important mechanism for corrosion of metals by sulphate-reducers, the corrosion rates obtained were low compared to those known to occur in buried metals. This difference could be due to interactions amongst the different organisms in the soil to increase corrosion or to substrate limitation to bacterial activity in batch cultures. The latter reason was considered more likely. It was thought that with continuous and semi-continuous cultures the bacteria would be kept in more active state for long periods. In semi-continuous systems, Booth *et al.* (1964) reported that no correlation between hydrogenase activity and the corrosion rate was observed, although hydrogenase-positive strains were more corrosive than the hydrogenase-negative organisms. Also, although there was an initial protective ferrous sulphide film formed on the coupons, this soon ruptured after about twenty weeks, resulting in a very high corrosion rate up to six times the initial one. The time interval before film detachment varied with the strain of organism used.

During anaerobic corrosion, not only the biochemical and physiological characteristics of the organism involved, but also the chemical composition of the culture medium, affect the corrosion rate. Using continuous culture techniques, Mara (1970, Ph.D. Thesis, as reported by Miller) observed that the breakdown of the protective sulphide film was dependent on the growth rate of the organism and the structure of the film. In low iron concentrations, the corrosion rate was dependent on the growth rate but not so in iron-rich medium. Earlier, Booth,

Cooper and Wakerley (1966) reported that the corrosion rates observed in continuous and semi-continuous cultures<sup>3</sup> were similar when the medium contained large amounts of iron. The low corrosion rate previously observed with batch cultures was thought to be the accumulation of free hydrogen sulphide which reacted with the metal surface to form a hard adherent film. In media sufficiently rich in iron, the sulphide is precipitated as ferrous sulphide and suppress the formation of the protective sulphide film on the specimen. During studies with five strains of *D. vulgaris* and one of *D. sulfuricans*, Booth, Cooper and Cooper (1967) found that large amounts of iron in solution indeed prevented the formation of protective sulphide film on mild steel coupons. Very high corrosion rate of up to  $221 \text{ mg dm}^{-2} \text{ day}^{-1}$  was obtained and the loose non-adherent covering of the coupon was found by X-ray analysis to be a mixture of  $\text{FeS}$  and  $\text{FeCO}_3$ . A similar observation was made by King *et al.* (1976). These workers found that the rate of breakdown of protective film was proportional to iron concentration of the medium. Film breakdown was associated with transformation from markinawite (protective) to smythite and pyrrhotite. Ferrous sulphide, either formed by the activity of sulphate-reducers or as a pure chemical product has been shown to effect cathodic depolarization. Once  $\text{FeS}$  has been formed in culture, depolarization activity can continue after changing to low-iron medium (Booth, Robb and Wakerley, 1967) or even in the absence of bacteria (Booth, Elford and Wakerley, 1968).

#### Stimulation of anodic dissolution

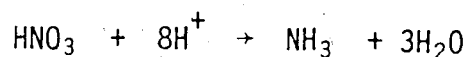
Mechanisms other than cathodic depolarization have been advanced by other workers to explain anaerobic corrosion by sulphate-reducers.

Wanklyn and Spruit (1952) from their studies of potential changes in iron in bacterial culture reported that a high ratio of iron corroded to ferrous sulphide product was obtained. They concluded that microbial corrosion by sulphate-reducers was, due to stimulation of anodic dissolution of iron by sulphide produced. Hoar and Farrer (1961) reported an increase in corrosion current when culture media and soil extracts contained some dissolved sulphide. The changes observed in the potential of submerged mild steel specimens (Wormwell and Farrer, 1952) would also support the theory of anodic stimulation. More recently, Sasaki *et al.* (1977) investigated the behaviour of mild steel in cultures of sulphate-reducing bacteria. The variations in electrode potential observed and the polarization characteristics of the steel specimen could only be explained by variations in  $H_2S$  concentration. There was no evidence that hydrogen depolarization by the bacteria occurred. These authors concluded that it seemed more reasonable that the corrosion process was catalyzed by  $H_2S$ , not the bacteria *per se*. The results of the work of Booth and Tiller (1960) though supporting the cathodic depolarization theory, showed that there was actually an initial stimulation of the anodic reaction followed by an inhibition.

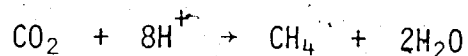
#### Anaerobic Corrosion by Other Microorganisms

Most afore-mentioned evidence implicates hydrogenase activity in the ability of microorganisms to depolarize the cathode. Understandably, many workers have investigated the ability of other hydrogenase-positive organisms to effect cathodic depolarization. From purely theoretical considerations, von Wolzogen Kuhr (1937, as reported by Hadley, 1948) suggested the possibility of involvement of nitrate-

reducing and methane bacteria in anaerobic corrosion. The overall equations for nitrate reduction and methane production given as



and



are envisioned as contributory to the cathodic depolarization process. Also, the common distribution of methane bacteria in waterlogged soils and their existence in association with sulphate-reducing bacteria in known extremely corrosive environments, further strengthens the probable contribution of methane bacteria to anaerobic corrosion process. Mara and Williams (1971) showed that nitrate-reducing bacteria were able to corrode mild steel if the organisms were hydrogenase-positive. The corrosion so observed was equivalent to the amount of  $\text{NO}_3^-$  reduced during the oxidation of cathodic hydrogen. These same workers (1971, 1972) in a separate set of experiments reported active depolarization of the cathode by hydrogenase-positive photosynthetic and non-photosynthetic bacteria and microalgae. However, Booth, Elford and Wakerley (1968) reported very poor cathodic depolarization by hydrogen and methane bacteria.

Ashton *et al.* (1973a) observed pitting corrosion of iron/carbon alloy behind adherent clumps of *Escherichia coli* cells in semi-continuous cultures of the organism after five weeks of exposure. However, no correlation between corrosion and nitrate reduced was observed, and a temporary protective film of  $\gamma\text{-Fe}_2\text{O}_3$  was initially formed. In further experiments involving batch cultures of *E. coli* and two different concentrations of nitrate, Ashton *et al.* (1973b) reported that the principal cause of corrosion observed with *E. coli* was the formation of



organic acids. Organic acids produced in the culture medium include fumaric, lactic, succinic,  $\alpha$ -ketoglutaric and acetic acids. The involvement of organic acid produced in metal corrosion was substantiated by a direct correlation initially between pH of solution and iron dissolution when nitrate was supplied at the rate of 1 g/litre. At a high concentration of nitrate (4 g  $\text{KNO}_3$ /litre) the initial rate of iron dissolution was the same as in low nitrate concentration. However, after a mere six hours, iron dissolution ceased and the soluble iron in solution disappeared. The onset of immunity was thought to be due to the formation of protective film of  $\gamma\text{-Fe}_2\text{O}_3$ . These authors also reported that although corrosion had ceased in nitrate-rich medium, nitrate was still present in the solution. They concluded that organic acid attack and not depolarization due to cathode reduction of  $\text{NO}_3^-$  by  $\text{H}_2$ , as suggested by Mara and Williams (1971), was the cause of corrosion.

Thus, it appears that anaerobic corrosion is effected by the ability of the organisms involved to utilize hydrogen and so depolarize the cathode, by the prevention of the formation of protective film in iron-rich environment, depolarization of the cathodic process by  $\text{FeS}$  formed and also by the stimulation of anodic dissolution of the metal by the sulphide produced. Strains of *Chromobacterium*, *Pseudomonas* and *Bacillus* isolated from soil had been shown to cause corrosion of iron in the presence of nitrate (Cook, 1961).

#### Corrosivity of Sulphides

Gosta Wranglén (1972) has attributed the corrosivity of sulphide to its high molar polarization. Anions of high molar polarization have a tendency to adsorb on metal surfaces, promote electron exchange

reactions and, consequently, catalyze corrosion reactions such as pitting and stress corrosion cracking. In solutions of sulphides, however, steel and other metals normally corrode quite uniformly with the formation of the metal sulphide (Dvoracek, 1976).

More localized attacks may occur with sulphide. Dvoracek (1976) reported the occurrence of pits with black iron sulphide corrosion product in sour oilfield brine. Moreover, under stress, steel and low alloy steel crack in sulphide solutions (Watkins *et al.*, 1976; Fraser and Treseder, 1952; Hoke, 1968; Snape, 1967). Sulphidation increases the susceptibility of high strength alloy to corrosion cracking. However, at concentrations less than 75 ppm no stress corrosion was observed with quenched and tempered API Grade P-110 steel (Dvoracek, 1970). Stress corrosion cracking is very common in petroleum industry which very often handles sour petroleum products.

Investigating the tendency of eleven anions to destroy iron passivity, Rostron (1979) noted that sulphide was one of the most effective. Also, the acceleration of corrosion of copper base alloys by sulphide was caused by the substitution of sulphide for the more protective hydroxide corrosion products that form in aerated sea water (LaQue, 1975). Bouet and Brenet (1963), using Pourbaix diagram, found that the region of passivity of ferrous metals in the presence of sulphide extend over a larger range of pH and potential compared to passivation by oxygen. Subsequently, Horvath and Novak (1964) reported that passivating sulphide films did not form as effective a barrier to corrosion as oxide films. The depression of immunity potential of steel in the presence of sulphides will make cathodic protection of such specimen more difficult since the applied cathodic potential will

have to be low enough to confer immunity (ibid).

Mara and Williams (1972) investigated the corrosivity of iron sulphide on pure iron. All sulphide materials caused both anodic and cathodic polarization. However, biogenic sulphide minerals were less effective depolarizers. This observation was thought to be due to impurities, like dead bacteria, present. A quantitative relationship between ferrous sulphide concentration and amount of iron corroded was reported by King and Wakerley (1973). Approximately 10 mg of iron was corroded by 88 mg of FeS added at pH 7 and a high corrosion rate was maintained throughout the experimental period when FeS was added semi-continuously. The corrosiveness of FeS was thought to be due to a cathodic depolarization of cathode by the absorption of polarizing agents in the crystal lattice and a possible galvanic Fe/FeS couple; sulphide films formed on iron are cathodic to the metal. The secondary effect of this is to promote pitting at discontinuities of the sulphide films (Que, 1975). Moreover, King, Miller and Smith (1973) reported the variation in the corrosiveness of chemically prepared sulphides with the sulphur content. Several workers (Meyer *et al.*, 1958; Shannon and Bogg, 1959; Battle, 1953; Elkins, 1953) have reported high corrosion rates in ferrous metals handling water that contained sulphide ions. The structure and composition of various sulphides and their corrosivity on ferrous metals have been extensively reviewed by Smith and Miller (1975).

Sulphides are not always known to be corrosive to metals. Mild steel exposed to sulphide solution often acquires a protective FeS film. However, the integrity of the film often deteriorates and accelerates corrosion results (Booth and Tiller, 1960; Booth, Shinn and

Wakerley, 1968). In a system containing oil, brine and sulphide, Shannon and Bogg (1959) observed the formation of hard, adherent sulphide scale at sulphide concentrations above 515 ppm. At lower concentrations of sulphide, no protection was conferred to this specimen. Sardisco, Wright and Greco (1963) studying an  $H_2S-CO_2-H_2O-Fe$  system, reported the formation of a protective scale (composed of pyrrhotite and pyrite) at sulphide concentrations of 15 to 1700 ppm. However, at higher sulphide concentrations (1700 to 66,000 ppm) only the non-protective mackinawite film was formed. Matsuda *et al.* (1972) investigated the effect of five organic sulphide compounds and reported that all except thio-urea showed a strong inhibiting effect on the corrosion of iron in hydrochloric and sulphuric acids. He suggested that the sulphur atoms in the molecules formed the anchoring sites owing to their high electron negativity, while the large molecular volumes of the compounds investigated enhanced their strong inhibitory effect. A similar observation and conclusion have been made by Markov *et al.* (1978). In their work, these workers observed that the number of sulphur atoms in the organic compounds was related to their inhibitory activity in sulphuric acid. With alkyl sulphides, inhibition by sulphur atom was enhanced by the presence of aromatic rings. The aromatic rings were considered to help increase the ability of the molecules to form protective layers on the metal surface because of additional  $\pi - \pi$  electron interaction. In general, it appears that organic sulphides have less tendency to corrode metals than the inorganic sulphides.

The effect of pH on the protectiveness of sulphide films has been reported by many workers (Meyer *et al.*, 1958; Ewing, 1955; Sardisco and

Pitts, 1965). There was a preferential formation of mackinawite film when the initial pH was between 6.5 and 8.8 and the more protective film of pyrrhotite and pyrite formed outside this pH range (Sardisco and Pitts, 1965). The non-protectiveness of mackinawite scale was explained by Meyer *et al.* (1958) as being caused by the large numbers of defects in the crystalline structure, and the resultant increased electronic conductivity which allowed diffusion at the crystalline boundaries.

#### Microbial Corrosion Under Aerobic Conditions

Although much of the corrosion by microbes studied is due to the activities of organisms under anaerobic conditions, microorganisms are known to cause corrosion under aerobic conditions. Under this condition, corrosion by the agency of microbes involves a wide variety of organisms and environmental conditions. These environments including marine, estuarine, freshwater and soil have the common characteristic of being oxygenated, though to different degrees. Organisms involved include bacteria, fungi, algae and even macroorganisms like barnacles. In most cases the corrosive effect of these organisms is not due to specific biochemical activities, but to the indirect effect of their physical presence. However, in a few cases like the sulphur bacteria and some fungi, corrosion of metal arises from the direct effect of their acidic metabolic products.

Romanoff (1945) showed that certain soils on being aerated change pH from near neutrality to acidic, while Kulman (1953) reported an increase in the population of sulphide oxidizing bacteria. These chemolithotrophic organisms assimilate  $\text{CO}_2$  at the expense of energy generated

by the oxidation of reduced sulphur compounds ultimately to sulphate. The acidity produced by the oxidation of reduced sulphur compounds is responsible for the corrosion of metals in mine environments. *Thiobacillus thiooxidans*, not only tolerates acidity, but produces as much acidity to reduce the pH to 1. *T. thiooxidans* does not grow at pH 6.5 and hardly grew at 6.0 (Horvath, 1962). Considering this physiological characteristic, it appears this organism may not be active in most soils. However, Parker and Prisk (1953) and Starkey (1959) have described several species of *Thiobacillus* which will reduce soil pH to 6.0 from which *T. thiooxidans* can begin to be active. These organisms, which themselves do not produce much acid to attack the metals, reduce the pH of the environment low enough to support the growth of *T. thiooxidans*. This fact must be considered very essential in aerobic corrosion involving sulphur oxidation. Working with aerated cultures of *Thiobacillus*, Horvath (1962) observed much higher weight loss in the presence than in the absence of the organisms. The *Thiobacillus* sp. oxidized sulphide previously generated anaerobically. There was a decrease in pH of solution and the redox potential of the metal became more negative. The increased corrosion rate corresponded with the rapid fall in pH and the potential of the metal. The decrease in potential of metal was explained by Horvath as being caused by solubilization of the protective sulphide film formed previously under anaerobic conditions, while the increased acidity furnished protons for cathodic reduction and increase in the corrosion current. Sulphuric acid production in soil by the members of the genera *Thiobacillus* and *Ferrobacillus* may be as high as 10-12%. *Ferrobacillus* sp. has been specifically associated with acid production in pyritic deposits.

(Hughes, 1963) and has also been implicated in the corrosion of water mains (Frederick and Starkey, 1948), mining equipment and associated pollution problems (Hughes, 1963; Purkiss, 1970; Westlake and Cook, 1978). These authors have discussed exhaustively the role of combined action of sulphur bacteria in metallic corrosion. The ability of *T. denitrificans* to couple sulphur oxidation to  $\text{NO}_3^-$  reduction anaerobically has further extended the scope of corrosion by acid production. The problem of corrosion due to the combined action of sulphate-reducing and sulphur bacteria has also been observed in gas holders. Purkiss (1970) reported that sulphate-reducing bacteria have seldom been found more than seven feet from the bottom of gas holder tanks, while there is evidence that oxidation of sulphur compounds occur at the top layer. In the case of buried metals in areas subject to fluctuating aerated and anaerobic conditions, the author suggested the existence of "a sequence of phases of broadly cyclic nature taking place". This sequence of phases is such that only a portion of the sulphur cycle (oxidative/reductive) would be dominant, passing into a different phase when physical conditions are favourable. In addition to the direct dissolution of metals by acid produced by sulphur and iron bacteria, tuberculation formed by the deposition of Fe(III) by *Ferro-bacillus* will cause differential aeration and a resultant pitting corrosion.

Tuberculation during aerobic microbiological corrosion of water pipe was reported by Olsen and Szybalski (1950), Butlin, Adam and Thomas (1949). Active corrosion of metal beneath the tubercule eventually led to perforation. The combination of low pH within the tubercule and the existence of differential aeration cell was responsible for the rapid

metal dissolution. Microscopic examination of the tubercles by Olsen and Szybalski revealed amorphous ferric hydroxide, while cultures from the tubercle gave rise to the growth of *Leptothrix* sp., an iron-oxidizing bacterium. *Gallionella* and *Sphaerotilus* spp. have also been known to cause tubercle formation (Anon, TPC publication No. 3). Other indirect effects of microorganisms in aerobic corrosion arise by the formation of colony mass on the metallic structure. Algae, fungi and slime-forming bacteria form large masses of growth within which oxygen-deficient or even oxygen-free conditions might exist. This condition sets up oxygen concentration cells (Anon, TPC publication No. 3, 1976) whereby well aerated portions are cathodic to the area beneath microbial growth. Bacterial and algal masses have been implicated in the corrosion of heat exchangers, cooling towers and associated pipe work. Moreover, large microbial masses on the surface of heat exchanges would reduce heat transfer and create a thermal gradient which may lead to stress cracking.

In one of the studies to determine specifically the effect of aerobic microorganisms, Smith, Compton and Coley (1973) investigated the polarization behaviour of carbon steel in sea water in the presence and absence of aerobic organisms. No significant differences were observed in the corrosion rates in the presence or absence of the organism. Rogers (1949) reported the acceleration of corrosion of copper and copper alloys by bacteria. The existence of a reversible redox reaction involving the sulphur atom of cysteine was said to be responsible for the severe corrosion of copper alloys in the marine environment. Ailor (1974) exposed, in half-tide and full-tide immersions, seven high purity aluminum and aluminum alloys in sea water.



The heavy fouling of aluminum panels resulted in severe etching of the specimens. However, it was noted that fouling had little effect on the depth of pits. Zaikina and Duganova (1975) isolated several fungal species from metallic objects damaged by biological corrosion. The isolated include different species of *Trichoderma*, *Aspergillus* and *Penicillium*, being the most common. A common attribute of these isolates was the ability to produce organic acids whose corrosivity is well-known (Alba-Yaron and Semel, 1976). The corrosion of zinc in the presence of aerobic bacteria was recently studied by Gillaume *et al.* (1977a, 1977b). Pitting corrosion of zinc occurred in fresh and sea water but disappeared in the presence of oxygen-utilizing bacteria. However, subsequent death of the bacteria was accompanied by an increase in corrosion rate. This increase in corrosion was much more if the bacteria also produced complexing agents. From experiments in which either  $O_2$  or  $N_2$  was bubbled into cultures of the bacteria the authors concluded that bacteria which decreased corrosion under aerobic conditions had opposite effect under anaerobic conditions.

From this review, it is apparent that the literature is abounding with the activity of anaerobic bacteria especially sulphate-reducers in metal corrosion process. With the exception of *Thiobacillus* spp. not much information is available on the role of aerobic bacteria in the corrosion of metal. Even much less is known of the possible interaction of aerobes with anaerobes during the corrosion process.

## MATERIALS AND METHODS

### Sources of Organisms

All the organisms used in the work reported herein were isolated from crude oil samples from the Pembina oilfield of North Central Alberta, or from a corroded segment of pipeline from the South Saskatchewan Pipeline Company. Crude oil samples from the Pembina oilfield were obtained directly from the pipeline system serving this field, at the Edmonton Terminal of the Interprovincial Pipeline Company.

### Crude Oil Samples

The pump system which handled the Pembina crude oil did not function at all times. As a result, it was considered that the initial few hundred millilitres of oil that came out of the sample port were 'stale' and not representative of the oil in the line. Consequently, before each sampling, about the first 500 ml of crude oil was discarded. All oil samples from which isolations were made were obtained only after the initial bleeding. Before each sampling, the pump nozzle was wiped clean with a clean dish cloth (or tissue paper). Subsequently, 70% alcohol was poured liberally to soak the dish cloth wrapped around the outlet for about two minutes to achieve disinfection. Samples were collected in sterile 250 ml plastic sample bottles.

### Microbiological

#### Maintenance of Culture

All isolates, with the exception of sulphate-reducing bacteria

were maintained on plate count agar (PCA) slants. Transfers were made regularly to fresh medium every four months. Usually, after an initial growth period of twenty-four hours at 25°C following each transfer, the cultures were stored at 4°C.

With the sulphate-reducers, maintenance was in liquid Butlin's medium dispensed in 15 ml aliquots in Hungate-type tubes. Each culture tube contained three iron finishing nails to create anaerobic environment and poise the potential as suggested by Pankhurst (1971).

### Cultural Techniques

#### Isolation of organisms

*Non-sulphate-reducers:* The cultural and isolation techniques differed slightly depending on the type of specimen. Isolation from corroded pipe specimens was preceded by an initial enrichment. The corrosion product was carefully picked from the corroded area with a pair of sterile forceps and transferred into modified Butlin's (referred to subsequently as simply Butlin's medium) or Brewer's liquid media (Appendices 1b & 1d). The use of Brewer's medium was later discontinued because it tended to blacken in the presence of finishing nails, at the absence of inoculation. After a growth period of 4 - 7 days, loop-fulls of culture were streaked on solid agar media (PCA and B<sub>10</sub>, Appendix 1a) to obtain isolated colonies. Where enrichment cultures were employed, only single colonies representing the different colonial types were picked for storage and later work.

With crude oil specimens, 2 ml samples were used as inocula for enrichment cultures. Incubation was either in the McCoy Anaerobic Hood or aerobically. In direct plating of samples, 0.1 ml aliquots were

spread aseptically on PCA or B<sub>10</sub> agar plates. Duplicate plates were incubated in the anaerobic chamber or aerobically up to a period of 7 days. All colonies on plates were carefully picked and re-streaked on fresh solid medium for purification. Medium B<sub>10</sub> was employed to expedite the identification and isolation of ferric-iron reducers. Organisms of this type possess a characteristic concave colonial morphology on this medium. Moreover, this medium appears to be partially selective to this group of organisms. On B<sub>10</sub> plates, more iron-reducing bacteria were obtained relative to other bacteria.

*Sulphate-reducing bacteria:* Isolation of sulphate-reducing bacteria was undertaken from enrichment cultures of organisms present in crude oil samples. Enrichment was by inoculation of modified Butlin's medium with 2 ml of fresh crude oil samples. Each culture tube contained 13 ml of the medium and three iron finishing nails. The additions of 2 ml of oil sample almost completely filled the 15 ml screw-cap tube. Incubation was in a McCoy Chamber, at a temperature of 38°C. No further deaeration of the tubes was undertaken. Any residual O<sub>2</sub> would be removed by the poisoning nail and by the reaction with H<sub>2</sub> over palladium catalyst contained in the hood.

Following the blacking of the culture 3-21 days later, one or two drops of culture were transferred to fresh molten Butlin's agar medium, cooled to about 40°C, contained in capped 15 ml tubes. The inoculated molten medium was gently swirled to ensure mixing. Further incubation for about 3 days yielded discrete black colonies. The culture tubes were then broken and each pin-point colony was carefully transferred to fresh liquid medium. Alternating transfers between liquid and solid media were done three times to purify and ensure the isolation of

discrete colonies. The purity of the culture was checked as suggested by Postgate (1979) and by light microscopy.

#### Purification of Sulphide-producing Bacteria

Attempts at isolation of sulphide-generating organisms from crude oil or corrosion specimens involved the use of enrichment cultures. The enrichment technique was not selective and many organisms survived and grew. In this purification procedure, use was made of the toxicity of mercuric ion,  $\text{Hg}^{2+}$ . About 2 or 3 drops of 1% solution of  $\text{HgCl}_2$  was added to actively-growing cultures of mixed sulphide and non-sulphide producers. The culture medium contained potential sulphide sources like thiosulphate, sulphite or sulphate, in the case of sulphate reducers. The addition of  $\text{Hg}^{2+}$  was at the onset of the  $\text{S}^{2-}$ -generating stage, about 12-72 hours, depending on the growth rate of the organisms. Incubation was continued for at least another 24 hours before transfers were made to fresh liquid medium (Butlin's) that lacked any added  $\text{Hg}^{2+}$ . Altogether, three successive transfers were made to dilute out any residual  $\text{Hg}^{2+}$  before plating on solid PCA plate for isolated colonies. Incubation was at  $30^\circ\text{C}$ .

#### Enumeration of Total Number of Bacteria and Ferric

##### Iron-Reducing Bacteria

Differential counts for ferric iron-reducing bacteria and the total number of bacteria in crude oil samples were carried out by the Most Probable Number (MPN) technique. Several dilutions of oil samples for total bacterial counts were made in milk dilution bottles containing 0.1 M phosphate buffer, pH 7.2 and a few glass beads to aid the

dispersion of oil in the aqueous phase. Ten mililitre aliquots of each dilution were added into 5 ml of double-strength B<sub>10</sub> (Appendix 1a) and Butlin's media (Appendix 1b) contained in 18 x 150 mm tubes, while the single strength media were inoculated with 1.0 or 0.1 ml aliquots. Each tube of Butlin's medium contained two finishing nails to help poise the potential and create anaerobic conditions for the growth of sulphate-reducers. Estimate of the total viable bacteria present in the crude oil samples was obtained by scoring all tubes containing Butlin's medium where growth (turbidity) occurred after a maximum of 5 days incubation.

Differential counts for ferric-iron-reducing bacteria were undertaken by taking advantage of the change in the colour of B<sub>10</sub> medium from brown to green with Fe(III) reduction. All inoculated tubes of B<sub>10</sub> which had turned green after 5 days incubation were considered positive presumptive for the presence of iron-reducers. Confirmation of the growth of ferric iron-reducers involved the formation of bright orange-red coloration with the addition of 2 drops of 2% solution of 1,10-orthophenanthroline into the tubes which showed positive presumptive test.

#### Preparation of Resting Cells

All cultures employed in the investigations reported in this thesis were either grown in modified Butlin's medium, C<sub>4</sub>F<sub>1</sub> or synthetic medium containing lactate as the carbon/energy source. Medium C<sub>4</sub>F<sub>1</sub> (Appendix 1c) is a modification of that employed by Torriani and Rothman (1961). The organisms were grown aerobically on a New Brunswick shaker (Model G-2; 295 rpm and 1½ inches eccentricity) at 30°C for

12-14 hours. The cells were recovered by centrifugation and washed three times in cold 0.1 M phosphate buffer, pH 7.2. Final cell suspension in the cold phosphate was at a concentration of 1 g wet weight per 80 ml of buffer. Cell suspensions were stored in capped tubes at 4°C.

#### Reduction of Fe(III) by Resting Cells

Experiments on bacterial reduction of Fe(III) were conducted with resting cells, the only exception being the initial screening process for the ability of the isolates to reduce Fe(III) in B<sub>10</sub> medium. Medium B<sub>10</sub> supports an active growth of iron-reducing bacterial isolated. The subsequent use of resting cells for Fe(III) reduction was to avoid the complications arising from the simultaneous growth and Fe(III) reduction in the reaction mixture.

One-millilitre portions of the variously treated resting cells were used to inoculate Hungate-type tubes containing 2 ml of 2% ferric phosphate (pH 7.2) (City Chemical Corp., New York) and sodium lactate (30  $\mu$ moles/ml). The pH of ferric phosphate solution was adjusted to 7.2 with 10 M NaOH. The reaction mixture was made up to 10 ml with sterile, iron-free, distilled water. Incubation was at 30°C for designated periods. All reactions were carried out in triplicate unless otherwise stated.

#### Reduction of Sulphur and Sulphur Compounds

In experiments to determine the ability of isolates to produce sulphide, only synthetic medium (Appendix 1e) was employed. The medium lacked all forms of sulphur except the compound being tested.

In all experiments except the reduction of elemental sulphur, one

iron finishing nail was introduced into the medium to act as a poisoning agent and indicator of  $S^{2-}$  production by the formation of black  $FeS$ . No finishing nails were employed in reduction of elemental sulphur since it was observed that blacking ( $FeS$  formation) would occur even in the absence of the organism. However, when the iron supplied was in the form of soluble iron ( $FeCl_2$ ), no such reaction occurred in the absence of  $S^{2-}$ -generating bacteria.

The sulphur compounds were prepared separately as sterile 10% solutions and 0.1 ml aliquots were added into the culture tubes. Elemental sulphur was sterilized by boiling for 1 hour each day for 3 successive days. After the final boiling, the supernatant fluid was decanted and the sulphur sediment washed twice with sterile distilled water before it was finally resuspended in sterile water. Elemental sulphur was supplied to the cultures in 1 - 4 ml of the suspension. No successful attempt was made to standardize the actual amount of sulphur being supplied since the preparation was not homogeneous. However, appreciable amounts of sulphur was introduced to each culture.

Sulphide generation was considered to have occurred if the medium turned black or if the yellow deposit of sulphur blackened.

#### Fe-starvation of Cells

To investigate the influence of exposure of the organism to iron in the growth medium on its ability to reduce  $Fe(III)$ , cells were grown separately in rich media (Butlin's  $C_4F_1$ ) normally containing iron, and in synthetic medium with or without added iron. The cells grown in iron-free synthetic medium will be referred to as 'iron-free cells'.

Iron-free cells were grown by passage of the organism twice



through the medium. All glassware employed in the handling of iron-free media were scrupulously acid-cleaned and rinsed in iron-free double-distilled water to prevent contamination with iron.

#### Induction of Nitrate Reductase in Resting Cells

A culture was grown aerobically in  $C_4F_1$  medium in the absence of added nitrate (*i.e.* under nitrate reductase repressive conditions) and inoculum prepared as described previously.

Freshly-prepared inoculum was divided into two portions. One portion was left aerobic, while the other portion was incubated anaerobically for 90 min in a tube in the presence of potassium nitrate (10  $\mu$ moles/ml). Anaerobic environment was achieved by evacuating the tube 3 times at 10 psi gauge pressure in a McCoy vacuum chamber, and flushing with a gaseous mixture containing 5%  $H_2$ , 5%  $CO_2$  and 90%  $N_2$ . Residual oxygen in the tube would be removed by its reaction with  $H_2$  catalyzed by heated palladium contained in the hood. The cell suspension treated in this manner will be referred to as 'induced cells'. The induced cells were then centrifuged, washed 3 times in the cold phosphate buffer and resuspended in the initial cell concentration (1 g wet weight per 80 ml).

#### Denitrification

Tests for denitrification were conducted in medium of the following composition (g per litre): yeast extract, 3.0; glycerol, 10;  $KNO_3$ , 10; and 1 g Noble agar (Stanier *et al.*, 1966). Medium was dispensed in 8 ml aliquots in 15 ml test tubes. Stab inoculation of the still molten medium, but cooled to about 40°C, was made with finely drawn Pasteur's

pipette. Withdrawal of the inoculation pipette was accompanied by an immediate sealing of the hole made by stabbing so that no appreciable amount of air would penetrate. After cooling briefly in ice-cold water, the soft agar medium was overlain with 5 ml of the same medium but containing 10 g agar per litre. The later plug of agar medium acted as a gas trap and helped maintain anaerobic conditions.

Denitrification was considered to have occurred when there was gas production as indicated by the existence of fracturing in the soft agar medium or separation of the agar plug. The initial reading was after 2 days but observation was continued up to 7 days before recording a negative result.

#### Crude oil utilization

The crude oil degradation capability of isolates was tested in rolling tube cultures. The rolling tube culture technique was adopted in preference to shake flasks because of its smaller space requirements and general ease of handling.

Each culture tube (18x150 mm capped tube) contained 5 ml of synthetic medium (Appendix 1e), 0.2 ml Rainbow Lake crude oil and 0.2 ml of 14-hour cultures of organisms (OD 0.5) grown in Butlin's medium. Culture tubes were inclined at an angle of about 20° to facilitate aeration. Incubation was for 60 days at 20° and an agitation rate of 58 revolutions per minute. Uninoculated tube constituted the control.

Rainbow Lake crude oil was chosen because it contains easily degradable fractions and it is sterile since enrichment culture and direct plating on agar growth medium (PCA) failed to reveal the presence of contaminants.

### Antibiotic sensitivity test

Three millilitres of overnight pure cultures of isolates were used to flood plates of Penassay Medium 3 solidified with 1.5% Noble agar. The flooded plates were drained, dried at 30°C for 30 minutes and seeded with antibiotic discs (BBL, Becton, Dickinson Co., Mississauga, Ont.). All plates were incubated at 30°C for 24 hours before being scored for zone clearing. Organisms were considered sensitive (+) when there was a clear zone of inhibition around the discs and insensitive (-) in the absence of a clear zone of inhibition.

### Spore production

Selected isolates were tested for spore production. Streaks of representative organisms were made on soil extract agar and incubated at room temperature for seven days. Negative staining with nigrosin or malachite green counterstained with safranin staining techniques were employed. Spore formation was not observed after a week's incubation. Further incubation was carried out for three weeks.

## Analytical Methods

### Physico-Chemical Analyses

#### Bottom sediments and water

Bottom sediments and water contents of crude oil samples were determined according to American Standards for Testing Materials (Designation D1796-68; API standard 2548; IP 75/69). Industrial grade toluene containing Tretolite F-65 (0.1% v/v) was the solvent-demulsifier

used. The crude oil-solvent mixture was heated at 60°C for 10 minutes in a solid metal block bath and centrifuged at 1300 (1285 rpm of 21.25 cm diameter) to give a relative centrifugal force of 500 *g* for 10 minutes. The centrifuge chamber was maintained at a temperature of 51.7°C.

#### Recovery of crude oil from culture

Crude oil fractions remaining after the growth of isolates were extracted by the addition of 5 ml of n-pentane (spectrophotometric grade) to the acidified cultures. Acidification (0.5 ml conc. HCl per culture tube) lowered the pH and prevented emulsion formation. Pentane-treated cultures were stoppered with rubber bung previously extracted with n-pentane, mixed by shaking for 5 minutes and left to settle.

The recovery of the extracted oil fractions for gas chromatography was by a flotation technique as used by Westlake *et al.* (1978).

#### Determination of iron (Fe)

*Standard Fe stock solution:* Two hundred milligrams of high purity (89.9-100%) iron wire (J.T. Baker Chemicals, Phillipsburg, New Jersey) was dissolved in 20 ml concentrated HCl (containing 0.0001% Fe) in an acid-washed 1 litre volumetric flask. The solution was made up to the 1 litre mark with double-distilled, Fe-free water to give a final concentration of 200 µg/ml and stored at 4°C.

Appropriate aliquots or dilutions of the stock solution was employed to construct a standard curve for Fe in the range of 0-120 mg/100 ml final solution. The actual procedure for the determination of Fe(II) was a modification of the procedure of Krishna Murti *et al.*

(1966). The modification was the addition of 1 ml iron-free B<sub>10</sub> medium to compensate for colour imparted to the reaction mixture when assaying for Fe(II) in B<sub>10</sub> medium. However, no B<sub>10</sub> was needed for the construction of standard curve when working with resting cells.

*Ferrous iron in culture:* To determine the Fe(II) produced in culture, 0.1 ml portions from culture tubes, acidified by the addition of 0.1 ml concentrated HCl, was pipetted into reaction vessel containing buffered phenanthroline reagent. Readings were taken within 3 minutes of the addition of the sample, to avoid autoreduction of Fe(III). Stoppage of bacterial activity by acidification was not necessary as the quantity of Fe(II) determined was the same whether or not the culture was previously acidified.

*Nitrite (NO<sub>2</sub><sup>-</sup>) determination:* Nitrite was determined by the procedure of Montgomery and Dymock (1961), with the exception that N-(1-naphthyl)-ethylene diamine hydrochloride was used and all readings were taken in Spectronic 20 (Bausch and Lomb).

#### Iodometric determination of S<sup>2-</sup>, SO<sub>3</sub><sup>2-</sup> and S<sub>2</sub>O<sub>3</sub><sup>2-</sup>

Cells were grown in Butlin's medium contained in 15 ml capped tubes. Each tube contained 10 ml of medium, 10 mg Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> and 1 ml of appropriate bacterial inoculum. Where mixed cultures were employed, 0.5 ml of each of the component organisms were used. The Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> was prepared separately as a filter-sterilized 10% solution and 0.1 ml of this solution was injected aseptically into the culture tubes. Inoculation was also by injection.

After appropriate incubation period, 3 ml of 2 M zinc acetate was injected into the culture tubes to precipitate the sulphide as ZnS. The tubes were then centrifuged to recover the precipitate and

supernatant for analyses. The precipitated sulphide was washed twice in distilled water before determination.

*Reagents:* Standard reagents used were prepared as in Standard Methods for Examination of Water and Waste Water (1975). Stock solution (0.1 M) of  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  was preserved by the addition of 5 ml of chloroform and stock at 4°C. Titrands were prepared for immediate use by diluting 4 times (250 ml to 1 litre) with cooled boiled distilled water.

*Titration:* Determination of the total sulphur, sulphite and sulphide was by the procedure of Blasius *et al.* (1968). Excess iodine remaining after reaction with the sulphur compounds was titrated with 0.025 M  $\text{Na}_2\text{S}_2\text{O}_3$  in a 500 ml Erlenmeyer flask. Each flask contained 200 ml distilled water, 5 ml glacial acetic acid, 5-8 ml of iodine (0.0126 M) and the sample to be analyzed. Three to five millilitres of concentrated HCl were sometimes added to facilitate ZnS dissolution. Three ml of 40% formaldehyde solution was added where necessary to remove sulphite from the solution. Uninoculated medium constituted the control.

#### Chromatographic analyses

*Nitrous oxide:* A Varian Model 700 gas chromatograph equipped with a thermal detector was used to detect nitrous oxide. One-half mililitre volumes of gas from culture headspace was injected into an alluminum column (3.66 M x 0.48 cm) packed with molecular sieve 13 (40/60 mesh) and maintained at 200°C. The carrier gas used was helium flowing at the rate of 86 ml per minute. The presence of nitrous oxide was established by the presence of a peak which has the same

retention time as the pure nitrous oxide standard.

*Crude oil extract:* N-pentane-extracted crude oil components were separated on chromosorbW-AW-DMCS column (80/100 mesh) coated with 3% SE 30 ultraphase, and packed in 6.10 M x 0.32 cm stainless stress steel tube. Samples, 2.0  $\mu$ l, were injected into Varian Model 1740 chromatograph fitted with flame detectors under the following operational conditions:

Carrier gas ( $N_2$ )      15 ml per minute

$H_2$                       30 ml per minute

Air                      300 ml per minute

Injector temperature: 300°C

Programmed column temperature: 50-300° at 10° per minute

Detector temperature: 300°C

Evidence for crude oil utilization was obtained by changes in the GLC profiles for n-alkanes and isoprenoids of the crude oil samples.

### Biochemical Techniques

#### Preparation of periplasmic enzymes

Periplasmic enzymes of the organisms were prepared by a modification of the procedures of Nossal and Heppel (1966). Periplasmic proteins are located between the cell wall and cytoplasmic membrane (periplasmic space) of bacteria and are released by osmotic shock (Malamy and Horecker, 1964). Fourteen-hour cultures of isolate #200 grown in Butlin's and  $C_4F_1$  media were harvested by centrifugation and washed twice in 0.33 M Tris-HCl-0.03 M NaCl solution, pH 7.2. Washed cells were suspended to a final concentration of 1 g wet weight per 80 ml of the buffer and divided into two portions. One portion was

retained and will be subsequently referred to as 'whole cells', while the other portion was subjected to an osmotic shock procedure.

A 10 ml volume of the cell suspension was mixed with an equal volume of 40% sucrose-0.66 M Tris-HCl- $2 \times 10^{-4}$  M EDTA solution and stirred vigorously by shaking on a rotary shaker for 10 min. Mixing the cell suspension with an equal volume of sucrose-buffer-EDTA solution will halve the concentration of the buffer system to the desired final concentration of 20% sucrose-0.33 M Tris-HCl- $1 \times 10^{-4}$  M EDTA. The cells were then centrifuged at 16,300 *g* for 10 minutes and the sucrose solution decanted. The well-drained cell pellet was dispersed in 10 ml of  $5 \times 10^{-4}$  M  $MgCl_2$  and stirred for 10 minutes. Two ml aliquots of this preparation was removed and will be referred to as 'shocked cell plus shock fluid'. After further centrifugation, the supernatant fluid was carefully removed and will be referred to as the 'shock or periplasmic fluid', while the pelleted mass resuspended to its original volume in 0.1 M phosphate buffer and will be known as 'shocked cells'. Viability of the shocked cells was checked by plating a loopful of the shocked cells on PCA and incubating at 30°C.

*Escherichia coli* C<sub>4</sub>F<sub>1</sub>, constitutive for alkaline phosphatase, was also grown in the same manner and subjected to osmotic shock treatment at the same time as the experimental organism. The alkaline phosphatase released served as marker to indicate the successful release of periplasmic protein under the experimental condition.



### Spheroplast preparation

Culture of isolate #200 grown in  $C_4F_1$  and Butlin's media was washed and dispersed, as described previously, in 20% sucrose-0.33 M Tris-HCl  $2 \times 10^{-4}$  M EDTA solution, and agitated for 10 minutes. Lysozyme (Sigma, St. Louis, Mo.) was added to the cell suspension to a final concentration of 250  $\mu\text{g/ml}$  and incubated at  $30^\circ\text{C}$ . Spheroplasts are bacterial cells whose cell wall has been partially removed. Spheroplast formation was followed by observing the decrease in optical density at 600 nm accompanying the dilution (1:10) of 0.5 ml samples of lysozyme-treated cells in distilled water. Very low spheroplast formation was observed when lower concentration of lysozyme was employed. When no further decrease in O.D. was observed with dilution, the treated cells were centrifuged at 16,300  $g$  for 10 minutes, washed once in 20% sucrose- $2 \times 10^{-4}$  M  $\text{MgCl}_2$ -0.33 M Tris-HCl solution and resuspended to its original volume. Phosphate buffer (0.1 M) was later used in place of Tris-HCl. Phase contrast microscopy revealed amorphous shapes of the organisms in the preparation. No obvious spherical cells were observed. However, many cells still retained their regular shape but showed a greater tendency to clump together than untreated cells. This final preparation constituted the spheroplast preparation and will be subsequently referred to as such.

### Vesicle (membrane) preparation

A known volume of the washed spheroplast preparation was centrifuged at 7,000 for 20 minutes. The well-drained pellet was resuspended in  $4 \times 10^{-3}$  M  $\text{MgCl}_2$ - $10 \times 10^{-3}$  M Tris-HCl (Lascelles and Burke, 1978).

Phosphate buffer,  $1 \times 10^{-4}$  M, was later used in place of Tris-HCl

component. The cell suspension was then agitated for 5 minutes and incubated at 4°C for 2 hours to allow for complete lysis of the spheroplasts, before centrifuging at 1,000 *g* for 5 minutes to remove debris. The lysate was further centrifuged at 60,000 *g* for 60 minutes. The supernatant was carefully removed and will be subsequently referred to as the 'cytoplasmic content'. The resuspended pelleted material will be subsequently referred to as the 'membrane (vesicle) preparation'.

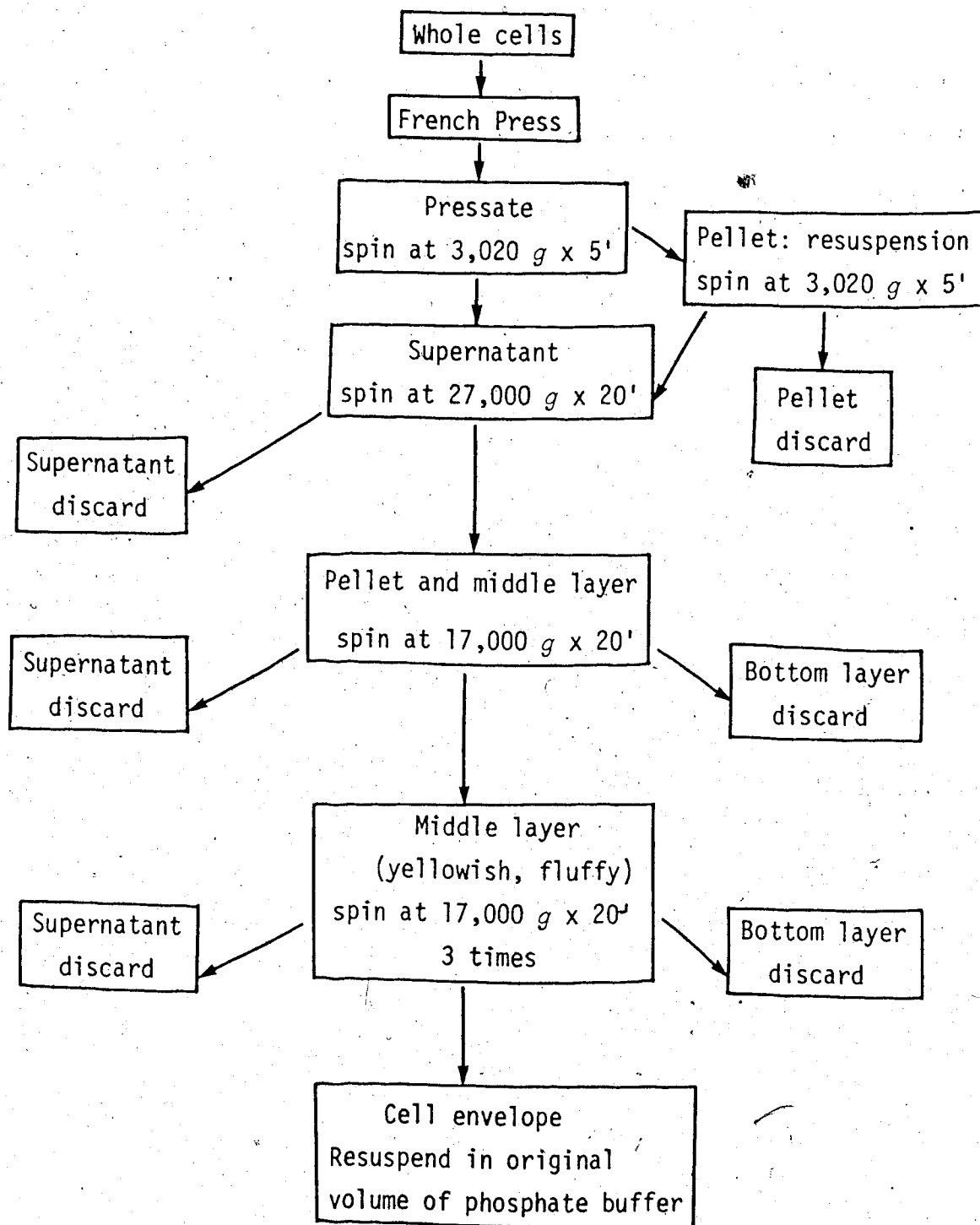
#### Cell envelope preparation

Fourteen-hour culture of isolate #200 in C<sub>4</sub>F<sub>1</sub> medium was washed once in 0.1 M phosphate buffer, pH 7.2 and resuspended in the same buffer in a final concentration of 1 g wet weight per 3 ml of buffer. The cells were passed twice through the French press to ensure maximum breakage. The broken cell material was differentially centrifuged according to the protocol (Fig. 2) to separate cell envelope from unbroken cells and cytoplasmic content. All washings were with 0.1 M phosphate buffer.

#### DNA preparation

DNA was prepared according to the method of Marmur (1961) except the product (DNA) was initially incubated with pronase at 30°C for 30 minutes to hasten deproteinization and to destroy DNase activity. The amount of DNA present in the preparation was measured at 260 nm using the relationship that 1 unit of absorbance is equivalent to 50 µg of DNA per millilitre.

Fig. 2. Procedure for preparation of cell envelope.



### Percent guanosine and cytosine (G+C)

Purified DNA samples were analyzed by bouyant density centrifugation in cesium chloride solution containing 130 g of CsCl in 70 ml of 0.02 M Tris buffer, pH 8.5. Two micrograms of *Escherichia coli* NCIB 8666 DNA was added to 2  $\mu$ g of DNA extracted from isolate #200 contained in 1 ml CsCl solution. Another CsCl-DNA sample containing only the *E. coli* DNA was prepared separately (reference) and centrifuged (at 44,000 rpm at 25°C) simultaneously with the mixed DNA sample. Centrifugation was for 24 hours in a Beckman Model E ultracentrifuge. Nucleic acid band distances were measured from densitometric tracings of developed films. Densitometric tracings were made in a Beckman Model RB Analytrol Densitometer and Integrator. From the measurements the bouyant density of the DNA from isolate #200 was determined using standard formula. Percent G+C content was calculated from the formula of deLey (1970).

### Assay for phosphatase activity in shock fluid

Indication of successful release of periplasmic proteins by the osmotic shock procedure adopted was demonstrated by the presence of alkaline phosphatase activity in the shock fluid. Phosphatase activity was used as a marker for the release of periplasmic enzymes (proteins).

The assay procedure involved the addition of 0.05 ml of shock fluid to tubes containing 0.9 ml 1 M Tris-HCl buffer (pH 8.0) and 0.05 ml of  $2 \times 10^{-2}$  p-nitrophenylphosphate. The reaction was stopped after 5 min by the addition of 4 ml of 0.1 M phosphate buffer, pH 10. The intensity of yellow color developed due to the release of p-nitrophenol was read in a Spectronic 20 spectrophotometer at 405 nm, the instrument being zeroed with reaction mixture without the shock fluid. All assays were

in triplicate.

#### Absorption spectra of whole cells

Absolute visible light absorption and cytochrome difference spectra of washed whole cell suspensions were determined in Pye Unicam recording spectrophotometer (Model SP8-100) by the procedures of Jones (1972). For the determination of absolute absorption spectra, the cell suspensions (1 g wet weight per 80 ml 0.1 M phosphate buffer) were blanked against 0.1 M phosphate buffer. Dithionite and potassium ferricyanide were employed as the reducing and oxidizing agents, respectively, prior to the determination of the reduced-minus-oxidized difference spectra. Treatment with carbonmonoxide was done by bubbling CO<sub>2</sub> into screw-cap tubes for 3 min and transferring to spectrometer tube for immediate scanning. The cell suspensions were scanned over a wavelength range of 300 nm, starting from 700 to 400 nm.

#### Microscopy

##### Epifluorescence microscopy

Previously immersed coupons were stained in acridine orange solution (10 mg/100 ml distilled water) for 3 minutes. The dye solution was initially filtered through Milipore membrane filter, pore diameter 2  $\mu$ , to remove any particulate matter-like undissolved dye crystals. The coupon samples were either rinsed in running distilled water (4 litres/min) first to remove non-adherent material before staining or were stained without rinsing. Stained samples were immersed in three changings of isopropanol to remove surface staining of the metal, and then air-dried. Specimens were then viewed under immersion oil on

standard Zeiss microscope fitted with epifluorescence illumination system, including a halogen lamp.

### Electron Microscopy

Electron microscopy studies employed transmitting and scanning techniques.

#### Transmission electron microscopy

Fourteen-hour old cultures were negatively stained with 2% (w/v) zirconium oxide solution containing 0.2% sucrose (w/v) as the wetting agent, and mounted on formvar-coated copper grids. The examination of preparations were either in a Phillip Model 200 or AEI Model electron microscope.

Cells for ultrastructural studies were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0), with or without 0.025% ruthenium red. Samples were then dehydrated in a regime of increasing concentration of acetone made up in distilled water. For details see Appendix 2. The acetone used was freshly distilled. The dehydrated cells were then fixed in osmic acid, with or without ruthenium red, washed in cacodylate buffer and embedded in a resin mixture (Appendix 2) and hardened by incubation at 60°C for 8 hours.

#### Scanning electron microscopy

immersed coupons were observed for changes in topography and microbial adherence in a Cambridge Stereoscan Model scanning electron microscope. Because of the possibility of loss of surface features with treatment, the specimens were treated

in different ways before observation in the microscope. Three treatment processes were tried.

1. Either the freshly withdrawn coupons were immediately air-dried or were gently rinsed in distilled water to remove loosely adherent materials before air-drying.
2. Subject the previously exposed coupons to a more gentle drying process by sequential immersion for 5 minutes per step in 30, 50, 70, and 95% ethanol before air-drying.
3. Fixing the specimen in 0.5% glutaraldehyde and drying at the critical point of freon 113 in a critical-point apparatus. In this process, the coupons were initially dried by passage through 30, 50, 70, 95 and 100% ethanol, and immediately transferring into another set of solutions containing 30, 50, 70, 95 and 100% freon 113. The ethanol solutions were made up in distilled water, while the freon solutions were made up in absolute ethanol. The specimen had a residence time of 5 minutes at each concentration of the dehydrating solution. The coupons so-treated were immediately transferred to a critical-point apparatus, and filled with freon 113. The temperature of the apparatus was slowly raised (about 90°C) until the freon went into the gaseous phase. Under this condition, the freon would not liquify whatever the pressure imposed. A slow release of the pressure led to a removal of trace water content of the specimens, so that drying was achieved solely at the gaseous phase of freon 113. Critical-point drying would prevent the collapse of biological structures, thus maintaining any biological



specimen intact.

The dried coupons were then coated with approximately 150 Å gold sputtered unto the surface in an Edmond's sputter chamber (Pirani Jennings, Model 4) and mounted on aluminum stubs by means of electrically-conducting silver emulsion. The microscope was operated at 20 KV accelerating voltage.

### Electrochemical Techniques

#### Polarography

Electroreduction of the contents of the culture medium before (control) and after growth of isolate #200 was carried out in 3-electrode cell compartment (Princeton Applied Research, P.A.R.) fitted with platinum flag auxiliary electrode, reference Saturated Calomel Electrode (S.C.E.) (Fisher Scientific Co., Pittsburg, Pa.) and P.A.R. Dropping Mercury Electrode (DME). The DME was set up with tripply distilled mercury and coupled to P.A.R. Model 174A Polarographic Analyser equipped with Model 174/70 Drop Timer.

Each run was preceeded by deaeration of the medium. Deaeration was achieved by bubbling O<sub>2</sub>-free N<sub>2</sub> (passed through 0.8% Vanadous chloride solution) through the medium for 5 minutes. Reduction currents were recorded using Houston Instrument Omnigraphic Recorder Model 2000. All measurements made at room temperature (25° ± 2°). The general operating conditions were as follows:

Modulation amplitude, 25 mv

Operating mode, D.C. (direct current)

Initial voltage, + 0.2 V

Voltage range, - 3.0 V

Current range, 2 mA

Mercury drop time, 2 sec.

Scan rate (recorder), 5 mV sec<sup>-1</sup>

Chart speed, X<sub>axis</sub> 25 mV cm<sup>-1</sup>

Y<sub>axis</sub> 0.5 V cm<sup>-1</sup>

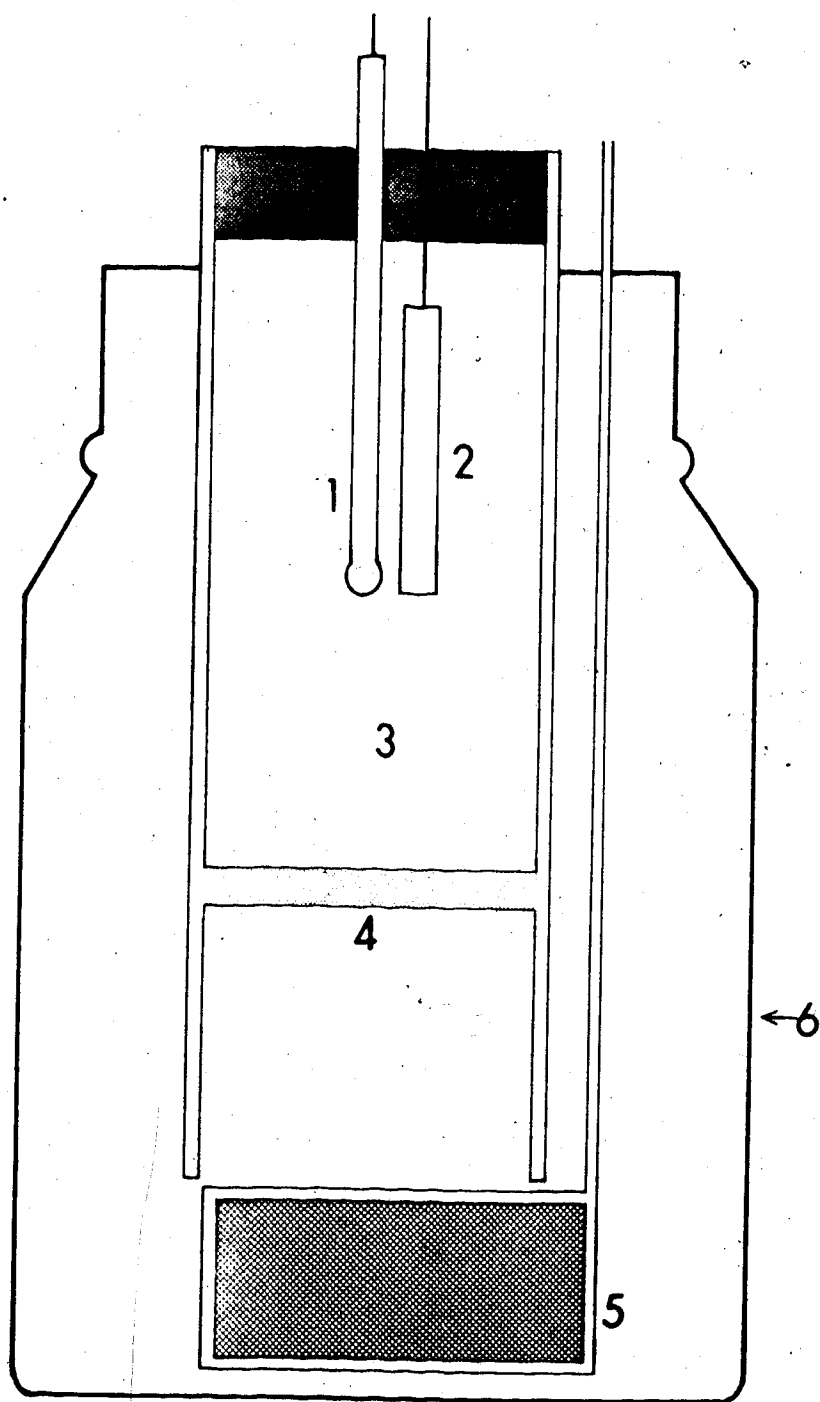
## Polarization

### Polarization cell

The polarization cell used consisted of a smaller corrosion pyrex glass chamber inserted into a larger chamber and separated from each other by about 0.4 cm-thick porous seal of pore dimensions 0.32-10  $\mu$  (Fig. 3). The corrosion chamber was constructed from 33 mm-diameter sealing tube (Ace Glass Inc., Vineland, New Jersey). The corrosion chamber housed the reference Saturated Calomel Electrode (SCE, Fisher Scientific Co., Pittsburg, Pa.) and the working electrode. The working electrode was an AISI 10-18 mild steel coupon of dimensions 5.0x1.2x0.1 cm (Caproco Corrosion Prevention Ltd., Edmonton, Alberta) fine glass-blasted to a smooth finish. The auxillary electrode (Platinum flag of large area) was contained outside the corrosion chamber but in the large surrounding chamber (auxillary electrode chamber) containing about 800 ml of sterile medium. By this arrangement, the auxillary electrode was desirably separated physically from the vicinity of the working electrode but electrically connected through the porous seal through which ionic migration could occur. Furthermore, the large auxillary electrode compartment containing the bulk of the medium,

Fig. 3. Polarization cell.

1, Reference electrode (SCE); 2, working electrode (mild steel); 3, corrosion chamber; 4, porous seal; 5, auxillary electrode (platinum); 6, auxillary electrode chamber (medium reservoir).



which slowly diffused into the corrosion chamber, acted as a substrate reservoir for more sustained bacterial growth and activity.

A major advantage of this cell is that it is simple to construct and assemble for use. The whole unit, minus the reference and working electrodes is autoclaved as one, reducing chances of bacterial contamination.

#### Operation of the polarization cell

The SCE was sterilized by wrapping it for 10 minutes in tissue paper soaked in 70% ethyl alcohol, while the working electrode was sterilized by immersion in 70% ethanol for 10 minutes and rinsed in 95% ethanol to degrease. All other components of the cell were assembled and autoclaved at 121°C and 15 pounds pressure per square inch (PSI) for 15 minutes.

Before each polarization run, the medium was deaerated by passing a stream of deoxygenated nitrogen gas ( $N_2$ ) for 5 minutes, and the cell immediately fitted with neoprene stopper carrying the working and reference electrodes. Deoxygenation was as described previously. No further deaeration of the polarization cell was attempted so that the experiments were conducted under microaerobic conditions. Unimmersed portion of the working electrode (coupon) was covered with teflon tape.

Immediately after inoculation (time, zero), the working electrode was polarized potentiodynamically over a range of 0.4 volts in the negative and positive directions (cathodically and anodically, respectively) of the open circuit potential. Uninoculated polarization cell was the control. All runs were conducted with fresh coupon samples.

The potentiostatic device employed was a Princeton Applied Research

(P.A.R.) Potentiostat/Galvanostat coupled to P.A.R. Model 175 Universal programmer which provided the desired potential (as measured by Electro-meter Probe Model 178) between the working electrode (coupon) and the reference SCE. The corresponding current output was recorded on Houston Instrument Omnigraphic Recorder Model 2000. Other operational conditions were as follows:

Current range (Model 176 current-voltage converter),

100  $\mu$ A-10 mA

Imposed voltage,  $\pm 0.4$  V (with respect to open circuit potential)

Scan rate, 2 mV  $\text{sec}^{-1}$

Chart speed, as in polarography

All measurements were carried out at room temperature ( $25^\circ\text{C} \pm 2^\circ$ ).

#### Coupon Corrosion Assembly

##### Continuous culture system

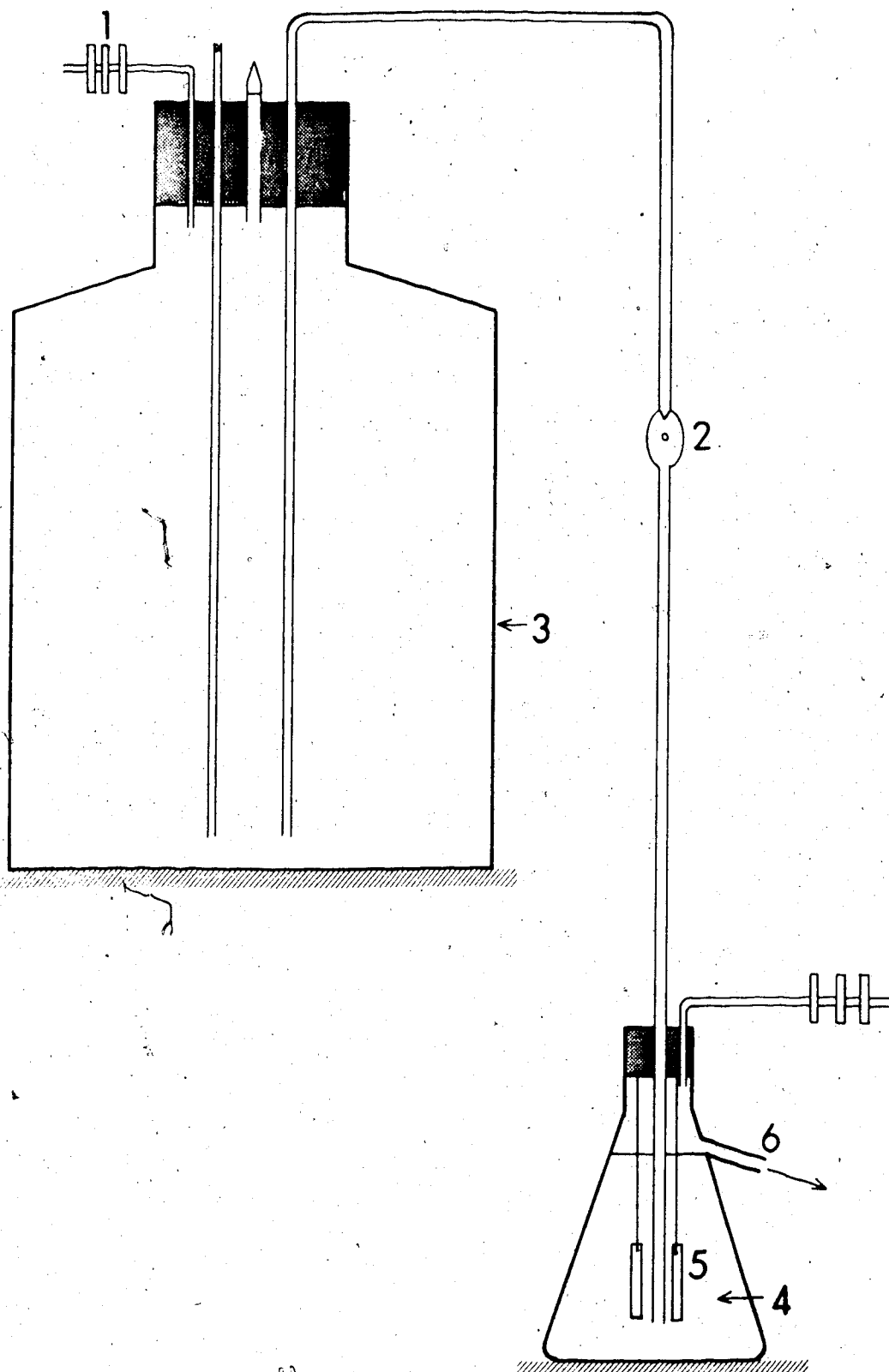
The continuous culture system employed consisted of two components; the medium reservoir and the corrosion chamber as in Fig. 4.

Each medium reservoir is a 44-litre container which can simultaneously feed five corrosion chambers. Each reservoir is additionally fitted with a medium supply port and a filter-fitted equilibration tube.

Each corrosion chamber is a 500 ml Erlenmeyer flask, fitted with a side arm and is essentially an overflow flask. Three coupons per flask were suspended by means of nylon thread from neoprene rubber stopper fitted with nichrome wire hooks.

Fig. 4. Apparatus for continuous cultivation of Isolate #200 during corrosion of mild steel coupons.

1, filter-tipped aspirator; 2, flow breaker; 3, medium reservoir; 4, corrosion chamber; 5, mild steel coupons; 6, effluent.





### Operation of the continuous system

Media in reservoir and corrosion chambers were autoclaved separately. The coupon-rubber bung assembly was sterilized by immersion for 20 minutes in 70% ethanol prepared with cooled boiled distilled water (to deaerate). The sterilized coupons were then immersed in 95% ethanol for about 10 minutes for degreasing and dried immediately in dry air-flow under ultraviolet light.

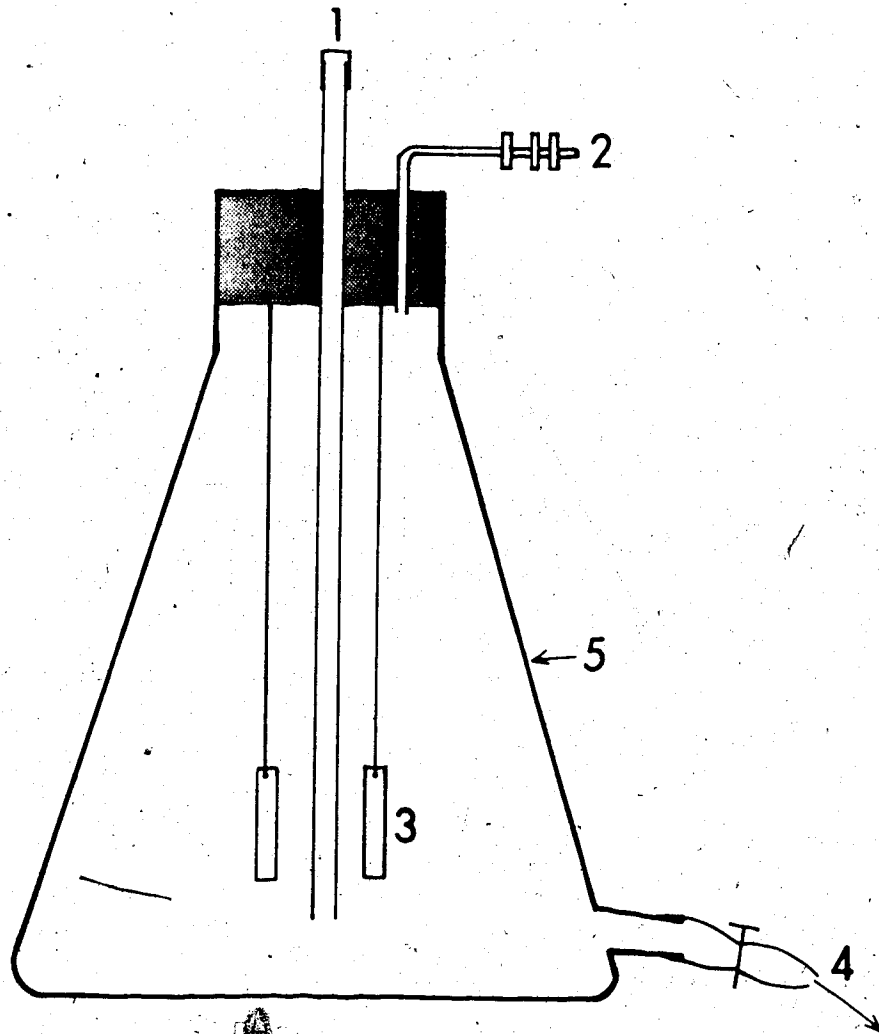
Each corrosion chamber, initially filled with 400 ml of medium was assembled under aseptic conditions (in a laminar flow hood) and inoculated. Cultures in the corrosion chamber were allowed to grow for 24 hours before being connected to the medium feedline. This procedure allowed the culture to establish and prevents washout. A medium flow breaker was inserted into each feedline to prevent ascending contamination of the reservoir from the corrosion chamber and to facilitate the regulation of medium flow rate by counting the number of drops as they fell through the flow breaker. The flow rate through each supply line was independently controlled by means of clamps. The dilution rate was  $0.012 \text{ hr}^{-1}$ . The medium dilution rate was chosen to simulate slow growth under natural conditions and higher flow rate caused backing up of medium in the flow line. One of the five corrosion chambers was left uninoculated and constituted the control flask.

### Semi-continuous culture operation

The semi-continuous culture system consisted of a 500 ml Erlenmeyer flask with a side arm at the bottom side of the flask (Fig. 5). Each flask is stoppered with a neoprene rubber bung carrying a medium delivery tube and filter-fitted equilibration tube. The top of each

Fig. 5. Semi-continuous culture system.

1, inoculation port (serum-capped); 2, filter-tipped aspirator; 3, mild steel coupon; 4, effluent; 5, corrosion chamber.



medium delivery tube was covered with serum rubber cap through which injections of inoculum and fresh medium could be made. Spent culture was run off by opening the clamp at the side arm and an equivalent volume of fresh medium injected through the serum cap, once every week.

### Static cultures

Static cultures were maintained in 500 ml Erlenmeyer flasks into which the coupons were suspended. Once inoculated and assembled, the static cultures were not tampered with until the end of the designated experimental period.

Sterilization of components of the semi-continuous and the static culture systems was as described previously for the continuous culture system.

### Cyclic Exposure of Coupons to Cultures of Isolate #200 and the Air

Five mild steel coupon specimens prepared as described previously were submerged in 500 ml static cultures of isolate #200 in Butlin's and B<sub>10</sub> media. After 3 days of immersion, these coupons were removed aseptically and transferred to sterile, empty 500 ml Erlenmeyer flasks with side-arm for 4 days. The side arm was fitted with Gelman filters to allow free diffusion of air and prevent bacterial contamination. During the atmospheric exposure, the surface oxidation of the coupon to Fe(III) form was supposed to occur.

After 4 days of atmospheric exposure, the coupons were then returned to fresh cultures of the organism. The above cycle was repeated for a total of 4 weeks (4 cycles). After the final exposure to the

atmosphere, the coupons were cleaned and weighed to determine weight loss. Two of the coupons were withdrawn from each culture flask for scanning electron microscopy.

#### Corrosion Test Specimens

The corrosion test specimens (coupons) were AISI 10-18 mild steel of the following dimensions: length, 5 cm; width, 1.2 cm; thickness, 0.1 cm. Each coupon had a hole (diameter 0.6 cm) punched 0.4 cm from one end. The coupons were obtained from Caproco Corrosion Prevention Ltd., Edmonton, a firm of corrosion consultants.

Each coupon was punched out on a die from sheared, cold-rolled sheets of the metal. A uniform bright finish was obtained by blasting with powdered glass. Coupons were finally packaged singly in protective paper sleeves and sealed in water-proof casing. The coupon containers were only opened to remove specimens for immediate use and unused samples were stored under vacuum.

## RESULTS AND DISCUSSION

### Problem Definition

#### Characteristics of the crude oil samples

In understanding the corrosion problem of the Pembina crude oil pipeline system it is pertinent to investigate the characteristics of the crude oil itself which might influence corrosion processes. Factors like inorganic matter and water content will decidedly influence the conductivity of oil samples and hence the intensity of direct electro-chemical attack of the pipeline. Moreover, the water content will influence the bacteria-carrying capacity of the oil samples. Organisms need water for their metabolic activities so that an oil sample which is free of water will not sustain any biological activity.

#### Physico-chemical characteristics

Crude oil samples from Pembina and Rainbow fields cannot be differentiated by appearance or smell. These two oil samples also contain microbially degradable components (Westlake and Cook, 1979).

#### Bottom sediment and water content and specific gravity

Tables 1 and 2 show the Bottom Sediments and Water (BS and W) content of Pembina and Rainbow crude oil samples, respectively. Although these samples were taken a year apart, they were sampled at the same season (winter), and the figures obtained were similar to all-year trends.

Pembina oil's BS and W content was constant (0.05%) throughout the

TABLE 1. Summary of bottom sediment and water (BS and W) and specific gravity of Pembina crude oil<sup>1</sup>.

Sampling date	BS and W	Gravity at $\approx 60$ F (15.6°)
13-1-78	0.05	0.601
16-1-78	0.05	0.611
17-1-78	0.05	0.611
18-1-78	0.05	0.608
19-1-78	0.05	0.611
20-1-78	0.05	0.614
23-1-78	0.05	0.610
24-1-78	0.05	0.610
25-1-78	0.05	0.618
26-1-78	0.05	0.614
27-1-78	0.05	0.616
30-1-78	0.05	0.598
31-1-78	0.05	0.626
1-2-78	0.05	0.619
2-2-78	0.05	0.608
20-1-80	0.05	n.d. <sup>2</sup>

<sup>1</sup>Provided by Interprovincial Pipe Line Co. Ltd., Edmonton.

<sup>2</sup>Not determined.

TABLE 2. Summary of some readings obtained for bottom sediment and water (BS and W) and specific gravity of Rainbow crude oil<sup>1</sup>

Sampling date	BS and W	Specific Gravity (15.6°)
23-1-78	0.10	0.616
24-1-79	0.05	0.614
25-1-79	0.05	0.622
26-1-79	0.05	0.616
27-1-79	0.05	0.619
28-1-79	0.05	n.d.
29-1-79	0.25	0.624
30-1-79	0.10	0.624
31-1-79	0.10	0.642
1-2-79	0.05	0.634
2-2-79	0.05	0.627
3-2-79	0.20	0.632
4-2-79	0.05	0.630
5-2-79	0.05	0.621
7-2-79	0.05	0.627
20-1-80	0.05	n.d. <sup>2</sup>

<sup>1</sup>Provided by Interprovincial Pipe Line Co. Ltd., Edmonton.

<sup>2</sup>Not determined.



sampling period. The specific gravity varied only very little, too. On the other hand, the BS and W value for Rainbow oil varied more and was on the average higher than Pembina's. Similarly, the specific gravity of the Rainbow oil varied more and was generally higher. The higher average specific gravity obtained in Rainbow oil would be a reflection of the higher contribution by water in the oil-water emulsion. If no factor other than pure electrochemical attack on the metal pipe were responsible for the corrosion observed in the lines, it would be expected that deterioration would be higher in Rainbow oil-carrying lines than in the Pembina system. This is because of the higher water-containing (BS and W) characteristic of Rainbow oil. The higher water content would dissolve and transport more inorganic salts from the earth and thus enhance the conductivity of Rainbow oil. However, more corrosion failure was reported in Pembina than in the Rainbow pipe line systems signifying that factor(s) other than, or in addition to, direct electrochemical attack was responsible for the corrosion observed. Since BS and W measures the total content of water, by volume, and water-soluble substances (organic and inorganic) it indirectly measures the potential microbe-carrying capacity of the oil samples since microorganisms need water, dissolved salts and carbon/energy contents of the oil-water emulsion for metabolic activities. Therefore, the constancy of occurrence of BS and W in all the crude oil samples assured the supply of water and water-solubles to any microorganism present and raised the problem of possible bacterial involvement in the corrosion process.

## Microbiological Characteristics

### Occurrence of bacteria in crude oil samples

The higher BS and W value for Rainbow crude oil samples did not correlate with the low frequency of pipe failure observed. This and the contrary situation in the Pembina line suggested factors other than direct electrochemical phenomenon were involved. Moreover, the invariant occurrence of BS and W pointed to an environmental condition which could support microbial life. These observations prompted the investigation of bacterial occurrence in the oil samples. The role of bacteria in metallic corrosion is well known, having been discussed in the Literature Review.

The data shown in Table 3 show the incidence of bacteria in Pembina and Rainbow oil samples over a period of one year. It is evident that the number of bacteria varied considerably in Pembina. In all cases, bacteria were always present. However, only a small part of the population was able to produce  $S^{2-}$  from  $SO_4^{2-}$  or  $SO_3^{2-}$ .

In a more extensive survey for a period of 3 years, bacterial organisms capable of producing  $S^{2-}$  from  $SO_4^{2-}$  and  $SO_3^{2-}$  were always detected (Westlake and Cook, unpublished data).

No bacteria were detected in Rainbow oil samples throughout the test period. The result of enrichment procedures observed failed to show the presence of any contaminant bacteria in the oil. It was unlikely, that any bacteria were present but failed to grow.

Since bacteria were found in Pembina but not Rainbow samples the possible contribution of bacteria could explain the high frequency of pipe failure in the Pembina system. This circumstantial evidence of

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TABLE 3. Summary of bacterial counts taken from Pembina and Rainbow crude oil samples over a period of a year.

Sampling date	Viable bacterial count/ml			
	Pembina crude oil		Rainbow crude oil	
	Plate counts <sup>1</sup>	S <sup>2-</sup> generators <sup>2</sup>	Plate counts	S <sup>2-</sup> generators
15/12/77	1020	n.d.	--	--
15/2/78	70	n.d.	--	--
17/4/78	294	220	--	--
22/6/78	594	175	--	--
27/7/78	2374	542	--	--
11/9/78	154	35	--	--
24/11/78	68	110	--	--

<sup>1</sup>Viable colony forming units

<sup>2</sup>MPN counts in modified Butlin's medium containing SO<sub>4</sub><sup>2-</sup> and SO<sub>3</sub><sup>2-</sup>.

n.d. = not determined.

-- = not detectable.

bacterial involvement is supported by the fact that among these bacteria were those whose activities would overtly change the chemical and physical characteristics of the interior of pipe environment. For example, the growth and activities of  $S^{2-}$ -generating bacteria might increase the  $S^{2-}$  in the environment.

#### Incidence of Ferric Iron-Reducing Bacteria

Estimation of the contribution of iron-reducing bacteria to the total bacteria in Pembina oil (Table 4) by the most probable number technique (Cochran, 1950) yielded extremely low values. However, in all samples tested there was always iron-reducers present. In many instances, plating the oil directly on  $B_{10}$  agar medium yielded counts in excess of 200 cells/ml. Crude oil is immiscible with water and so could not be transferred as a homogenous system during the serial dilution in MPN technique. The problematic transfer of organisms from the oil phase to the liquid phase in which growth would be initiated was thought to be responsible for low MPN counts. It was probable that most of the organisms were still associated with the oil phase and thus several viable cells (instead of a single cell) would yield only one positive result. The thin spread of the oil inoculum on the larger agar surface increased the medium/oil interface from which growth would be initiated. Very low medium/oil interface was the case in MPN counts where dilutions were made in test tubes which have very small cross-sectional area. Thus, while bacterial counts involving crude oil cannot be considered accurate, it is likely that counts obtained on  $B_{10}$  agar plates would be closer to that which exist in the oil-water system. Therefore, iron-reducing bacteria would be expected to be far more than

TABLE 4. MPN counts of iron-reducing bacteria and the total bacteria in Pembina crude oil samples.

Sampling date	Viable bacterial counts/ml	
	Total bacteria	Ferric iron-reducing bacteria
3/3/78	1300	79
8/8/78	310	1
10/8/78	110	3
14/8/78	24	1
18/8/78	1800	1

1-79 cells/ml as indicated by the MPN counts. The more important fact, though, is that this type of bacteria was always present. Much more interesting, however, was the realization that some of these organisms combine the ability to produce  $S^{2-}$  and reduce Fe(III) (Table 4). The concomittant production of  $S^{2-}$  and reduction of Fe(III) could reduce considerably the redox potential of the environment, a situation which is inimical to the maintenance of the integrity of buried ferrous metals (Miller, 1970).

#### Studies on Sulphide Production by Bacteria Present in Crude Oil Systems

The generation of sulphide is a recognized means by which bacterial activity can cause corrosion of ferrous metals. Gösta Wranglen (1972) ascribed the corrosivity of  $S^{2-}$  to its high molar polarization. Anions of high molar polarization have a tendency to adsorb on metal surfaces, promote electron exchange reactions and, as a result, catalyze corrosion reactions. Thus, sulphides may cause the stimulation of anodic dissolution of ferrous metals by direct chemical reaction with the metal (Wanklyn and Spruit, 1952). Furthermore, FeS, once produced, may stimulate further corrosion by cathodic depolarization. It was the realization of the important rôle of  $S^{2-}$  in the corrosion process that prompted the investigation of  $S^{2-}$  production by bacteria isolated from crude oil systems.

Blackening of medium due to FeS formation was always observed whenever Butlin's medium (with or without  $SO_3^{2-}$ ) was inoculated with Pembina crude oil samples or produced water from Pembina field. It was observed also that the addition of  $SO_3^{2-}$  to the medium always

decreased the time needed for visible production of  $S^{2-}$ . It would appear from this observation that either  $SO_3^{2-}$  enhanced the activity of sulphate-reducers already known to be present or that there was, in addition, a population of microorganisms which specifically reduced  $SO_3^{2-}$ .

In an investigation of the microbiology of a failed, corroded segment of a pipe from South Saskatchewan Pipeline Company, no sulphate-reducing bacterium was isolated or its presence demonstrated. However, culture of the corrosion products yielded sulphite-reducing bacteria. This result was corroborated in a separate study in Dr. Cook's laboratory. Thus, it appeared that a population of bacteria, other than the already-recognized sulphate-reducers, exist that contribute to overall  $S^{2-}$  generation in crude oil systems. These other bacteria, by virtue of their ability to produce  $S^{2-}$  would be involved in the corrosion process.

#### Sulphide Production in Pure Cultures

To investigate the hypothesis that other bacteria, in addition to sulphate-reducers contribute to the corrosion picture, a large number of bacteria were isolated from Pembina crude oil samples and their ability to reduce oxidized inorganic sulphur compounds to  $S^{2-}$  investigated. Sulphate, sulphite, thiosulphate and elemental sulphur were specifically chosen because they are potential intermediate products of dissimilatory sulphate reduction (Jobson, 1975; Findley and Akagi, 1970; Suh and Akagi, 1969), while sulphate is a common content of soil water which may be the produced-water. Thus,  $SO_3^{2-}$  and  $S_2O_3^{2-}$  would be expected to be present in Pembina oil where the presence of sulphate-

reducing bacteria has always been demonstrated. Elemental sulphur was included in the regime of test compounds because it is often a common content of many crude oils.

The result of the reduction of  $\text{SO}_3^{2-}$ ,  $\text{S}_2\text{O}_3^{2-}$  and  $\text{S}^0$  using lactate as electron donor by the non-sulphate-reducing isolates is shown in Table 5. The 21 isolates capable of reducing  $\text{SO}_4^{2-}$  were also able to reduce  $\text{SO}_3^{2-}$ ,  $\text{S}_2\text{O}_3^{2-}$  and  $\text{S}^0$  in Butlin's medium. The result shown in Table 5 indicates that over 80% of the isolates were able to reduce one form of sulphur or the other. Essentially 4 groups of bacteria, based on their ability to reduce the tested sulphur compounds of different oxidation states, existed in Pembina oil. Only a minority failed to reduce any form of sulphur. Sulphate could only be reduced under anaerobic conditions. Moreover, none of the aerobes was able to reduce  $\text{SO}_4^{2-}$  to  $\text{S}^{2-}$ . It was observed that a ceiling on the reductive capability of each group of bacteria appeared to be set by the oxidation state of the sulphur compounds. Thus, a particular group could not reduce a given compound with the sulphur higher than a certain oxidation state. However, other compounds whose sulphur groups were of lower oxidation states than the maximum were reduced by the given group. Since  $\text{SO}_3^{2-}$  and  $\text{S}_2\text{O}_3^{2-}$  are intermediates of  $\text{SO}_4^{2-}$  reduction and since the reduction of  $\text{SO}_3^{2-}$  can produce  $\text{S}_2\text{O}_3^{2-}$  or *vice versa* it is apparent that the reductive step of each group of bacteria would form a potential substrate for another group to act upon. The result of this interactive bacteria action will be a 'cascade of sulphide generation'.

The concept and functioning of the cascade system in Pembina oil is depicted in Fig. 6. Group A organisms are the sulphate-reducing bacteria which also reduced  $\text{SO}_3^{2-}$ ,  $\text{S}_2\text{O}_3^{2-}$  and  $\text{S}^0$ . Group B could reduce



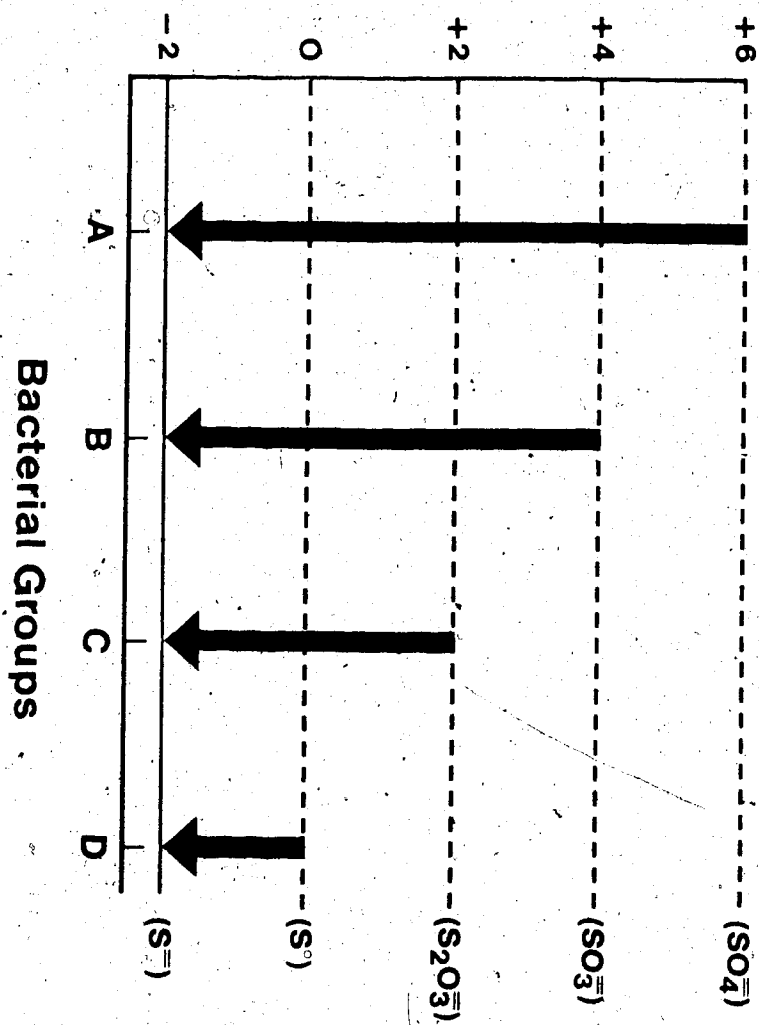
TABLE 5. Reduction of  $\text{SO}_4^{2-}$ ,  $\text{SO}_3^{2-}$ ,  $\text{S}_2\text{O}_3^{2-}$  and  $\text{S}^0$  by bacteria isolated from Pembina oil.

Bacterial Group	$\text{S}^{2-}$ formation from test compounds				No. of organisms <sup>1</sup> showing positive reduction
	$\text{SO}_4^{2-}$	$\text{SO}_3^{2-}$	$\text{S}_2\text{O}_3^{2-}$	$\text{S}^0$	
A	+	+	+	+	21
B	-	+	+	+	19
C	-	-	+	+	124
D	-	-	-	-	56
E	-	-	-	-	36

<sup>1</sup>256 isolates tested.

Fig. 6. Diagrammatic representation of the 'cascade system of  $S^{2-}$  generation' by bacteria in the Pembina crude oil.

# Oxidation States of Sulphur Compound



$\text{SO}_3^{2-}$ ,  $\text{S}_2\text{O}_3^{2-}$  and  $\text{S}^0$  but not  $\text{SO}_4^{2-}$ , while group C could reduce only  $\text{S}_2\text{O}_3^{2-}$  and  $\text{S}^0$  but not  $\text{SO}_4^{2-}$  and  $\text{SO}_3^{2-}$ . Group D organisms reduced only  $\text{S}^0$ . Thirty-six isolates tested did not reduce any of the test compounds. Thus, the intermediate products,  $\text{SO}_3^{2-}$  and  $\text{S}_2\text{O}_3^{2-}$ , which would have accumulated under poor growth conditions from the activity of sulphate-reducers would be further reduced by groups B and C organisms. Consequently, there would be an enhanced production of  $\text{S}^{2-}$  due to the interaction of these groups of bacteria. The enhanced generation of  $\text{S}^{2-}$  may increase the reaction with ferrous metal, causing an increased stimulation of anodic dissolution as was pointed out by Wanklyn and Spruit (1952). In addition, the increased generation of  $\text{S}^{2-}$  and the accompanying anodic dissolution would produce more FeS (corrosion product), whose cathodic depolarization characteristic is well documented (Booth *et al.*, 1967; Booth *et al.*, 1968).

It is apparent from the foregoing discussion that the interactive relationship among the different groups of the  $\text{S}^{2-}$ -generating bacteria would prevent the accumulation of toxic products. Butlin *et al.* (1949) noted that  $\text{SO}_3^{2-}$  in cultures of *Desulfovibrio* sp. could become rapidly toxic and induce morphological changes. Jobson (1975), albeit he did not consider  $\text{SO}_3^{2-}$  concentration at the level of 0.39  $\mu\text{moles/ml}$  in cultures of *Desulfovibrio* sp. toxic, associated a temporary decline in the synthesis of cell nucleic acids and protein with the  $\text{SO}_3^{2-}$  accumulation. Hence in Pembina oil system where the interactive  $\text{S}^{2-}$  production exists any repressive influence of the intermediate products of sulphur compounds' reduction would be prevented by prompt removal of the toxic substance by the different bacterial groups. The result would be a sustained, enhanced bacterial activity. It is very apparent that the

cascade system of  $S^{2-}$  generation which exists in the Pembina crude oil system would enhance corrosion in the corrosion process. The cascade system, therefore, is considered one very important mechanism by which the severe corrosion of Pembina crude oil pipeline system arises.

#### Substantiation of the Cascade System of $S^{2-}$ Production

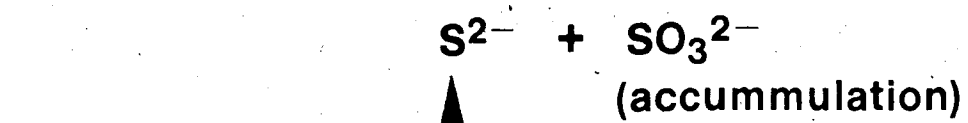
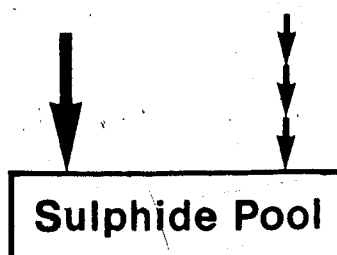
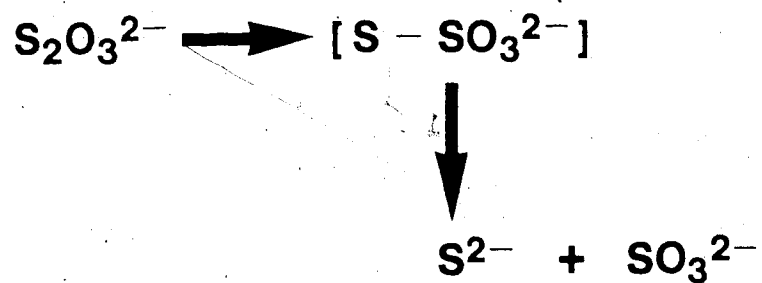
The proof of the existence of the 'cascade system of  $S^{2-}$  generation' necessarily involves the demonstration of increased  $S^{2-}$  production and prevention of accumulation of an intermediate product in mixed cultures of two groups of bacteria as depicted in Fig. 6. In this study, Isolate #230 (group B) and Isolate #213 (group C) were grown as pure or mixed cultures in Butlin's medium containing  $S_2O_3^{2-}$ . As was shown earlier Isolate #230 will reduce  $SO_3^{2-}$ ,  $S_2O_3^{2-}$  and  $S^0$ , while Isolate #213 will reduce  $S_2O_3^{2-}$  and  $S^0$  but not  $SO_3^{2-}$ . Since the structural difference between  $SO_3^{2-}$  and  $S_2O_3^{2-}$  is the presence of the sulphane sulphur in  $S_2O_3^{2-}$  it was thought that the reduction of  $S_2O_3^{2-}$  probably involved an initial cleavage and then reduction of the sulphane sulphur, with the formation of sulphonyl sulphur ( $-SO_3^{2-}$ ). This type of reaction has been known to occur in other bacteria (Roy and Trudinger, 1970; Leinweber and Monty, 1963). With pure culture of Isolate #213,  $SO_3^{2-}$  would be expected to accumulate with  $S^{2-}$  production. On the other hand, in a mixture culture of Isolate #213 and #230 no  $SO_3^{2-}$  would be expected to accumulate and more  $S^{2-}$  production would be also expected, too. This relationship is portrayed in Fig. 7.

The data in Table 6 show the result of  $S^{2-}$  and  $SO_3^{2-}$  formation from  $S_2O_3^{2-}$  in pure and mixed cultures of Isolates #230 and #213. More  $S^{2-}$  was formed in mixed culture than in pure cultures of #230 and #213.

Fig. 7. Model for  $S^{2-}$  and  $SO_3^{2-}$  formation from  $S_2O_3^{2-}$  by groups B and C.

$S^{2-}$ -producing bacteria from Pembina crude oil.

## Group B



## Group C

TABLE 6. Formation of  $S^{2-}$  and  $SO_3^{2-}$  from  $S_2O_3^{2-}$  in pure and mixed cultures of isolate no. 213 and 230.

Organism	Incubation time (hr)			
	3		10	
	$S^{2-}$ ( $\mu$ moles)	$SO_3^{2-}$ ( $\mu$ moles)	$S^{2-}$ ( $\mu$ moles)	$SO_3^{2-}$ ( $\mu$ moles)
#213 (Group C)	5.9	3.2	10.9	3.7
#213 + #230	7.9	4.6	11.5	0.7
#230 (Group B)	2.7	4.0	3.2	2.3



Very little residual  $\text{SO}_3^{2-}$  was found in the mixed culture after 10 hours.

With Isolate #213, there was an increase in residual  $\text{SO}_3^{2-}$  with incubation. This increase in  $\text{SO}_3^{2-}$  accumulation accompanied an increase in the total sulphide formation; a situation consistent with Fig. 7. However, the amount of  $\text{S}^{2-}$  formed after 10 hour incubation was approximately thrice as much as  $\text{SO}_3^{2-}$  formed. This would not be expected if the reduction process involved simply the reductive cleavage of the sulphane-S, with the  $\text{SO}_3^{2-}$  produced and accumulating (Kobayashi *et al.*, 1969; Leinweber and Monty, 1963). However, the non-stoichiometric accumulation of  $\text{SO}_3^{2-}$  might be due to instability of  $\text{SO}_3^{2-}$  or because other intermediates which were not analysed for were formed.

In cultures of Isolate #230, an increase in  $\text{S}^{2-}$  formation with incubation was accompanied by a decrease in  $\text{SO}_3^{2-}$  remaining in the culture medium. This observation is consistent with the ability of #230 to reduce both  $\text{SO}_3^{2-}$  and  $\text{S}_2\text{O}_3^{2-}$ .

After 3 hour incubation, there was a higher accumulation of residual  $\text{SO}_3^{2-}$  in the mixed culture (#213 + #230) than in either of the pure cultures. This observation is still consistent with the model presented in Fig. 7. As shown in the model, the initial step involves the cleavage and reduction of the sulphane sulphur with the formation of  $\text{SO}_3^{2-}$ . In the mixed culture, the independent formation of  $\text{SO}_3^{2-}$  by Isolates #213 and 230 at the initial stage of reaction would be expected to contribute more  $\text{SO}_3^{2-}$  to the reaction mixture than the single organisms. With longer incubation, the residual  $\text{SO}_3^{2-}$  in the mixed culture was much less than that in pure culture of Isolate #230. Thus, Isolates #213 and 230 acted in concert to produce more  $\text{S}^{2-}$  and prevented greater  $\text{SO}_3^{2-}$  accumulation than was the case when the organisms acted

singly after 10 hours of incubation. However, when the incubation was protracted (over 24 hours), the result was difficult to interpret. It is concluded that the result of  $S^{2-}$  and  $SO_3^{2-}$  formation from  $S_2O_3^{2-}$  by pure and mixed cultures of Isolates #213 and #230 is consistent with and duly supports the existence of 'cascade system of  $S^{2-}$  generation' in Pembina crude oil system.

A less direct evidence of the occurrence of the cascade system was furnished by the observation that enriched cultures of Pembina crude oil treated with mercuric chloride yielded almost exclusively  $S^{2-}$ -generating bacteria (F.D. Cook, personal communication). Amongst the surviving organisms were those which reduced only  $S_2O_3^{2-}$  although this compound was not added originally to the culture. The property of selective killing of non-sulphide-producers by  $Hg^{2+}$  was adopted in this work to isolate and purify cultures of  $S^{2-}$ -producing bacteria. The significance and the probable means by which  $S^{2-}$ -producing bacteria survive  $Hg^{2+}$  toxicity is discussed subsequently.

#### The Role of $Hg^{2+}$ in Isolation of $S^{2-}$ -producing Bacteria

Mercuric ion is a very potent poison. Its potency is associated with the avidity with which it binds and inactivates active sites of several enzymes. The binding is directed specifically to the sulphhydryl moiety. In the presence of  $S^{2-}$ , however,  $Hg^{2+}$  reacts to form an extremely insoluble  $HgS$  ( $K_{sp} 1 \times 10^{-52}$ ) which is precipitated from the solution. Thus, in the presence of  $S^{2-}$ ,  $Hg^{2+}$  and, therefore, its toxicity is reduced or eliminated.

In the purification of the enriched culture of Pembina oil containing reducible S-compounds the addition of  $Hg^{2+}$  killed off all (or most)

organisms except the active  $S^{2-}$  producers. Since both  $S^{2-}$ - and non-sulphide-producers were originally present in the enriched culture, the survival of only the  $S^{2-}$ -producers must be due to inability of  $Hg^{2+}$  to penetrate their cell envelopes. Conceivably,  $S^{2-}$  as it was being produced and diffusing from the organism reacted with  $Hg^{2+}$  to form the insoluble  $HgS$  which in turn formed an insoluble coat around the cells. The presence of the insoluble  $HgS$  prevented the ingress (diffusion) of  $Hg^{2+}$  into the cell cytoplasm and the organisms survived. With the non-sulphide-producers, no such protective  $HgS$  would be formed and  $Hg^{2+}$  was able to permeate the cells and kill them. This technique was also utilized to purify  $SO_4^{2-}$ -reducing bacteria.

However, it is not impossible that the  $S^{2-}$ -producing organisms are innately insensitive to  $Hg^{2+}$  toxicity. This suggestion is not supported by other pieces of evidence. It was observed that a fresh inoculum of  $S^{2-}$ -producing bacteria, introduced into Butlin's medium containing  $SO_3^{2-}$  and  $Hg^{2+}$ , failed to survive. However, when the same inoculum was pre-grown to the active stage the addition of  $Hg^{2+}$  failed to kill off the organisms. Therefore, active  $S^{2-}$  production is a prerequisite to survive  $Hg^{2+}$  toxicity and the  $S^{2-}$ -producing bacteria are not intrinsically resistant to  $Hg^{2+}$ .

This technique is considered a very sensitive and fast approach to the isolation of potential iron-corroding bacteria, the rationale being that the ability to produce  $S^{2-}$  is a corrosion hazard.

#### Concurrence of $SO_3^{2-}$ and Fe(III) reduction

Most isolates which were able to reduce  $SO_3^{2-}$  to  $S^{2-}$  also reduced Fe(III) to Fe(II). Nineteen isolates were able to reduce  $SO_3^{2-}$  and 18

of these reduced Fe(III), too. On the other hand, 16 of the 19 organisms which reduced Fe(III) also reduced  $\text{SO}_3^{2-}$ . Thus, of a total of 20 isolates only 3 did not possess the combined ability to reduce Fe(III) and  $\text{SO}_3^{2-}$ . From this observation there appears to be a very close relationship between the ability to reduce  $\text{SO}_3^{2-}$  and Fe(III). The author is not aware of any literature referring to this relationship. Possibly the genes for the enzymes are linked or that transfer of electrons to these substrates is through a similar pathway.

#### Scope of alternate electron donors for sulphite reduction to $\text{S}^{2-}$

In these studies, sulphide reduction was estimated visually noting the degree of blackening due to the formation of FeS. The controls were inoculated medium plus  $\text{SO}_3^{2-}$  but no substrate (endogenous control) and uninoculated tube containing the potential electron donor and  $\text{SO}_3^{2-}$ . The latter control measure was to check for  $\text{S}^{2-}$  production by direct chemical reduction of  $\text{SO}_3^{2-}$  without microbial participation.

It was earlier observed that only a fraction of the isolated bacterial population could reduce  $\text{SO}_3^{2-}$  to  $\text{S}^{2-}$  using lactate as the electron donor. However, it was not possible to attribute the lack of  $\text{S}^{2-}$  production to inability of the organisms to couple lactate dissimilation to  $\text{SO}_3^{2-}$  reduction or that the organisms completely lacked the capability to reduce  $\text{SO}_3^{2-}$ , the potential electron donor notwithstanding. But when isolates #117, 87 and 120, which were not able to reduce  $\text{SO}_3^{2-}$  in the presence of lactate, were incubated instead with other potential electron donors - glutamine, glutamic acid, L-histidine, DL-tryptophan, fumarate, malonate, malate tartarate, stearic acid or D-ribose - no  $\text{S}^{2-}$  was produced either. Based on this study, although

limited in the number of organisms and substrates employed, it can be inferred that these organisms did not produce  $S^{2-}$  because they did not have the capability to reduce  $SO_3^{2-}$  and not because lactate was an inappropriate substrate. Probably these organisms lacked the necessary enzymes to reduce  $SO_3^{2-}$ . Elemental sulphur and  $S_2O_3^{2-}$  were not tested.

For the isolates which were able to reduce  $SO_3^{2-}$  using lactate as the electron donor, further investigation showed that only a limited number of substrates could function as electron donors for  $SO_3^{2-}$  reduction. Table 7 shows that of the 9 amino acids tested only histidine served as electron donor for  $SO_3^{2-}$  reduction by majority of the organisms. It was only Isolate #200 that could utilize serine for the reductive process. This same organism (Isolate #200) also reduced  $SO_3^{2-}$  to  $S^{2-}$  (not shown in the Table) but failed to do so with DL-tryptophan. Blackening of the culture medium was observed with Isolates #233, 234, 235 and 218 when cysteine was employed as the substrate. Although the control tubes failed to show  $S^{2-}$  production, it was suspected that the  $S^{2-}$  observed in the culture probably arose, at least in part, from the sulphur of cysteine molecule. This was duly confirmed in that in the absence of added  $SO_3^{2-}$  there was still some blackening of the culture medium. However, it was not improbable that both the desulphyrylation and  $SO_3^{2-}$  reduction reactions could occur simultaneously. Table 8 shows that of the glycolytic and tricarboxylic acid cycle intermediates tested lactate and pyruvate were the most commonly utilized. Lactate was readily utilized by all the organisms but only about 50% of the organisms used pyruvate as the electron donor. All the organisms which utilized pyruvate as the electron donor also used lactate. This was not very surprising since pyruvate could be an intermediate in

TABLE 7. Reduction of  $\text{SO}_3^{2-}$  by bacteria isolated from Pembina oil using amino acids as electron donors!

Isolate	Incubation time (hr)											
	24				48				72			
	GLU	SER	HIS	CYS	GLU	SER	HIS	CYS	GLU	SER	HIS	CYS
77	-	-	-	-	-	-	-	-	-	-	-	-
78	-	-	-	-	-	-	-	-	-	-	-	-
134	-	-	-	-	-	-	-	-	-	-	-	-
167	-	-	-	-	-	-	-	-	-	-	-	-
169	-	-	-	-	-	-	-	-	-	-	-	-
180	-	-	-	-	-	-	-	-	-	-	-	-
181	-	-	-	-	-	-	-	-	-	-	-	-
198	-	-	-	-	-	-	-	-	-	-	-	-
200	-	-	-	-	-	-	-	-	-	-	-	-
201	-	-	-	-	-	-	-	-	-	-	-	-
202	-	-	-	-	-	-	-	-	-	-	-	-
216	-	-	-	-	-	-	-	-	-	-	-	-
217	-	-	-	-	-	-	-	-	-	-	-	-
218	-	-	-	-	-	-	-	-	-	-	-	-
230	-	-	-	-	-	-	-	-	-	-	-	-
232	-	-	-	-	-	-	-	-	-	-	-	-
233	-	-	-	-	-	-	-	-	-	-	-	-
234	-	-	-	-	-	-	-	-	-	-	-	-
235	-	-	-	-	-	-	-	-	-	-	-	-

<sup>1</sup> Concentration - 10  $\mu\text{mole/ml}$ . ( $\pm$ ) trace of blackening; (+) slightly black; (++) moderately black; (+++) intensely black. Other amino acids, glycine, alanine, methionine, asparagine and glutamine, were tested but were not utilized by any of the isolates as electron donors.

TABLE 8. Reduction of  $\text{SO}_3^{2-}$  by bacteria isolated from Pembina oil using low molecular weight organic acids as electron donors.

Isolate	Incubation time (hr)											
	20				29				48			
	LAC	PYR	MALO		LAC	PYR	MALO		LAC	PYR	MALO	
77	-	-	-		+	+	-		++	+++	+	
78	-	-	-		+	+	-		++	+++	-	
134	-	-	-		+	+	-		+++	+++	-	
167	-	-	-		++	+	-		+++	+++	+	
169	-	-	-		+	-	-		+++	+	-	
180	-	-	-		++	-	-		+++	-	-	
181	-	-	-		+	-	-		+++	-	-	
198	-	-	-		+	+	-		+++	+	-	
200	-	-	-		+	-	-		+++	-	-	
201	-	-	-		+	+	-		+++	+	-	
202	-	-	-		+	-	-		+++	-	-	
216	-	-	-		+	-	-		+++	-	-	
217	+	++	-		++	+	-		+++	+	-	
218	-	±	-		++	±	-		+++	+	-	
230	++	+++	-		+++	+	-		+++	+	-	
232	++	+++	-		+++	+	-		+++	+	-	
233	-	-	-		+	+	-		+++	+	-	
234	-	-	-		+	+	-		+++	+	-	
235	-	-	-		+	+	-		+++	+	-	

(±) trace of blackening; (+) slightly black; (++) moderately black; (+++) intense black.

Fumarate and malate were also tested but were not utilized by any of the isolates as electron donors.

lactate utilization (oxidation to acetate). What was surprising, however, was that not all organisms that utilized lactate were able to use pyruvate. It can only be inferred that in such organisms pyruvate is not an intermediate in lactate utilization or the organism may be impermeable to pyruvate.

Aliphatic acids like palmitic, stearic and propionic were also tested on Isolate #78 but this organism failed to reduce  $\text{SO}_3^{2-}$  to  $\text{S}^{2-}$  in the presence of these substrates. Other substrates tested were glycerate, succinate, tartarate, acetate, citrate, glucose-6-phosphate, D-ribose-5-phosphate and D-2-deoxyribose but no  $\text{SO}_3^{2-}$  reduction to  $\text{S}^{2-}$  was observed during the experimental period. Elemental sulphur and  $\text{S}_2\text{O}_3^{2-}$  were not tested as electron acceptors.

It was noteworthy that although most of these substrates could support the growth (evident from turbidity) of these bacteria, these carbon compounds could not serve as electron donors for  $\text{SO}_3^{2-}$  reduction. It was evident, therefore, that the growth of these organisms was not necessarily synonymous to  $\text{SO}_3^{2-}$  reduction as was thought to be the case with the sulphate-reducing *Desulfovibrio* sp. (Jobson, 1975; Jobson *et al.*, 1979). Instances where substrate dissimilation was not coupled to  $\text{S}^{2-}$  production is not uncommon. Several strains of *D. desulfuricans* and *D. gigas* could grow by fumarate dismutation (Miller and Wakerley, 1966) and so no  $\text{SO}_4^{2-}$  reduction was necessary.



Carbon and Energy Sources for Bacterial Isolates  
from Pembina Oil

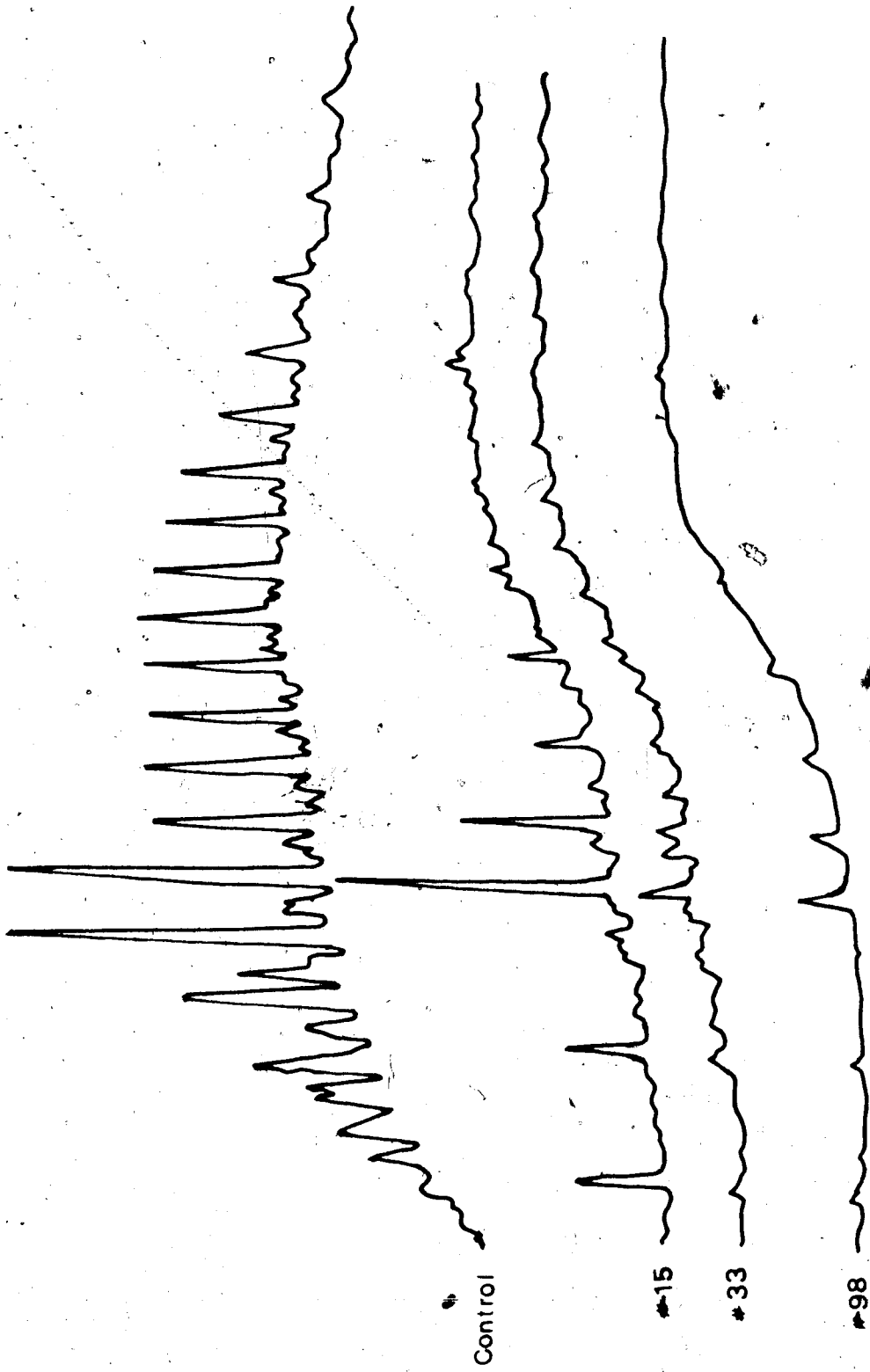
Crude oil degradation by the isolates from Pembina Oil

This study was conducted as a part of the overall physiology of the isolates with a view to understanding how these organisms can affect the composition of the crude oil and, consequently, the environment of the metal pipe which carries the oil. The understanding of the diversity of physiological capabilities among the members of the microbial population will help formulate the inter-relationship amongst the microflora. As was pointed out by Jobson (1975), non-hydrocarbon degraders in a mixed culture are not necessarily handicapped in their nutritional requirements. Products of hydrocarbon degradation by the hydrocarbon-utilizing component of the population serve the nutritional needs of the non-primary hydrocarbon utilizers. Dead cells may provide nitrogen and phosphate, also.

At the onset of the experiment, the crude oil samples floated on the medium surface. In many of the culture tubes it was found that after about 2-3 weeks of incubation the oil samples formed globules that settled to the bottom of the culture tubes. This observation was later shown to accompany bacterial degradation of the samples. This settling of the modified crude oil sample was shown to accompany an increase in oil density incident on bacterial modification of the oil sample (Jobson, 1975).

Of 128 bacteria tested, 32 demonstrated the ability to degrade crude oil as shown by a change in the gas-liquid chromatographic profile of saturate fraction of oil (Fig. 8). Although these organisms were

Fig. 8. GLC profile of crude oil samples extracted with pentane  
after incubation with bacteria for 60 days.



able to degrade Rainbow oil, they differed in the details of activity. For example, Isolate #33 showed a general tendency to utilize most of the n-alkane components more uniformly. On the other hand, with Isolate #15 there was a selective utilization such that the components selected against appeared not to be altered whatsoever. However, Isolate #98 although showing a general utilization of the N-alkanes, some of these components appeared to be less readily utilized than the others.

These bacteria tested were isolated at different periods, and some have, as a result, been maintained for longer periods on complex media. Iizuka and Komagata (1964) reported that Gram-negative bacteria which were originally able to degrade hydrocarbon soon irrevocably lost the oil-degrading capability after being maintained on nutrient agar slants. It was, therefore, possible that some of these organisms tested which showed negative results had lost their crude oil-degrading capability. Presumably, a greater proportion (than was demonstrated in this work) of the bacteria normally present in Pembina oil are in nature able to degrade petroleum.

#### Organic compounds as carbon and energy sources for growth

The utilization of an array of organic compounds for growth by the isolates was tested initially on synthetic agar medium. The substrates were employed at the level of 0.1% as recommended by Stanier *et al.* (1966). Growth was scored visually by comparing the growth on the substrate relative to that on the control plates (without substrates). Because of doubt arising from scanty growth on some plates, such results were corroborated by additional growth study in liquid media. However, the problem of doubtful growth still persisted in liquid

culture especially with some of the aromatic compounds which often became coloured on autoclaving or incubation. In view of this, marginal growth from impurities was not improbable.

In general, growth in liquid medium was better than on solid medium and so was not necessary, in most cases, to check for growth spectrophotometrically. These organisms showed the ability to grow on a wide variety of organic compounds including amino acids, hydroxy acids, fatty acids and miscellaneous organic acids (Table 9). Of the amino acids tested arginine and tryptophan were the least utilized. Only Isolates #120, 230 and the reference organisms included for comparison (*Pseudomonas fluorescens* ATCC 17397, *P. putida* and *P. aeruginosa* ATCC 9027) were able to grow on arginine. In a similar manner, only three organisms, Isolates #2, 200 and 230 could grow on DL-tryptophan.

Dicarboxylic acids like fumarate and succinate, the hydroxy acids DL- $\beta$ -hydroxybutyrate and tartarate, and other organic acids like citrate were generally utilized. The utilization of tartarate was, however, limited. Only Isolates #2, 200 and 213 were able to grow on tartaric acid; none of the reference *Pseudomonas* spp. grew on this substrate. Although not shown in the Table, malate, pyruvate and lactate were readily utilized by such versatile organism as #200. These oxyacids and organic acids were considered core substrates for members of aerobic Pseudomonads (Stanier *et al.*, 1966) and should be considered in the taxonomy of Pseudomonads. It is interesting to note that these compounds are intermediate products of major metabolic pathway like the tricarboxylic acid cycle. The significance of this is that the organisms would be able to scavenge on these products from other organisms.

TABLE 9. Characteristics of bacterial isolates from Pembina crude oil. Utilization of organic compounds as sole carbon and energy source.

Organic compound	Growth Organism										
	2	103	117	120	200	213	218	224	230	<i>P. fluorescens</i>	<i>P. putida</i> <i>P. aeruginosa</i>
<i>Amino acids</i>											
Histidine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Arginine	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++
L-isoleucine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
L-valine	+	+	+	+	+++	+++	+++	+++	+++	+++	+++
L-glutamine	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
-alanine	-	-	++	+	+++	+++	+++	+++	+++	+++	+++
L-tyrosine	+++	+++	-	+++	+++	+++	+++	+++	+++	+++	+++
DL-tryptophan	+++	-	-	-	+++	+++	+++	+++	+++	+++	+++
L-phenylalanine	-	+++	+++	+	+++	+++	+++	+++	+++	+++	+++
DL-serine	+++	+++	-	-	+++	+++	+++	+++	+++	+++	+++
<i>Organic acids</i>											
Citrate	+++	+++	-	+++	+++	+++	+++	+++	+++	+++	+++
Tartrate	+++	-	-	-	+++	+++	+++	+++	+++	+++	+++
Acetate	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Succinate	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Fumarate	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
DL-β-hydroxybutyric acid	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
γ-hydroxybutyric acid	+++	-	+++	++	+	+++	+++	+++	+++	+++	+++
<i>Aromatics</i>											
Benzoic acid	±	±	+	-	++	±	±	±	+++	+++	+++
P-hydroxybenzoic acid	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++



This view is re-enforced by the wide utilization of amino acids which could be easily available from autolysed dead cells, while the other organic acids would be readily formed from microbial oxidation of hydrocarbons (Davis, 1967).

Aldoses were very widely utilized; glucose being the most preferred by the isolates. With xylose, galactose, mannose and ribose only with one exception in each case were these not utilized by all the bacteria.

The non-nitrogenous aromatic compounds proved recalcitrant to most of the organisms. In many cases, like cathecol and p-phenyl phenol it was difficult to determine growth because of colour development. Even in cases where growth was thought to have occurred, they were doubtful and, at the best, poor growth took place. However, it is well known that even with many organisms that grow on aromatic compounds utilization is very slow and consequently growth is poor.

An overview of the scope of carbon/energy sources for growth clearly indicate that these organisms have a marked capability to grow on a wide variety of organic compounds which could result from metabolic activities of other organisms. Isolate #200 showed a versatility which compared favourably or even tended to surpass the proverbial versatile *Pseudomonas* spp. used for comparison. It is apparent that the number of organic compounds tested is rather limited compared to what would be recommended on the basis of Stanier and co-workers' work. Nonetheless, this investigation has served to point to the nutritional versatility of these environmental organisms.



### Miscellaneous

All isolates from Pembina oil tested (Table 10) reduced  $\text{NO}_3^-$  to  $\text{NO}_2^-$ . However, vigorous denitrification, as indicated by gas production, was observed only in Isolates #117 and 120. With Isolates #200, 218 and 224, only tiny disruptions (indicating gas formation) occurred in the agar column. In a separate test in liquid medium only small amounts of  $\text{N}_2\text{O}$  were detectable in each culture. Therefore, true denitrification may be said not to occur in these three organisms. Other physiological characteristics of the isolates are also shown in Table 10.

The trend in sensitivity of the tested organisms to various antibiotics (Table 11) was very similar to that observed with marker organisms - *P. aeruginosa*, *P. putida* and *P. fluorescens*. All the isolates tested were sensitive to chloramphenicol and tetracycline and, with the exception of Isolates #2 and 200, to streptomycin. On the contrary, all isolates were sensitive to erythromycin and penicillin (except Isolate #117). Most of the organisms were also insensitive to Novobiocin.

Sensitivity to antibiotics may be important to the taxonomy of bacteria. Members of *Pseudomonas* spp. are commonly insensitive to antibiotics especially the penicillins (Shewan *et al.*, 1954, 1960). However, antibiotic sensitivity is known in many other bacterial groups especially among the Enterobacteriaceae and the spectrum of resistance may depend on previous exposure to the antibiotics (Battacharya and Taylor, 1975; Anderson, 1968).

TABLE 10. Characteristics of bacterial isolates from Pembina crude oil.

Characteristic	2	103	117	120	200	213	218	224	230	P. fluorescens	P. putida	P. aeruginosa
*Growth at 4 C	+	-	+	+	+	+	-	-	+	+	-	-
37 C	+	+	+	+	+	+	+	+	+	+	+	+
41 C	+	+	+	-	+	-	+	+	-	-	-	+
Colonial morphology												
Gram reaction	-	-	-	-	-	-	-	-	-	-	-	-
Spore formation	rod	rod	rod	rod	rod	rod	rod	rod (short)	rod	rod	rod	rod
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Flagellation	polar	polar	polar	polar	polar	polar	polar	polar	polar	polar	polar	polar
Catalase	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+
Hugh-Leifson's												
(a) oxidative	acid	basic	acid	acid	alkaline	basic	acid	acid	basic	acid	acid	acid
(b) fermentative	-	-	-	-	-	-	-	-	-	-	-	-
α-ketogluconate formation												
Gelatin hydrolysis		+	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis	-	+	+	-	+	+	-	-	+	+	+	+
**Starch hydrolysis	n.d.	n.d.	n.d.	n.d.	+	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Urease	+	+	+	+	-	+	-	-	-	-	-	-
***Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-
(i) denitrification (gas)	-	-	+	+	+	-	±	±	+	+	+	+
(ii) nitrite formation	+	+	+	+	+	+	+	+	+	+	+	+

\*After 3 days of incubation except at 4 C which was incubated for about 10 days.

\*\*Very weak.

\*\*\*After 1 week incubation.

TABLE 11. Characteristics of bacterial isolates from Pembina crude oil.

Organism	Tripple sulpha	Streptomycin	Novobiocin	Tetracycline	Penicillin	Chloram- phenicol	Erythro- mycin
2	+	-	+	+	-	+	n.d.
103	-	+	-	+	-	+	-
117	+	+	+	+	+	+	-
120	+	+	-	+	-	+	-
200	+	-	+	+	-	+	-
213	-	+	-	+	-	+	-
218	-	+	-	+	-	+	-
224	+	+	-	+	-	+	-
230	-	+	-	+	-	+	-
<i>P. fluores-</i>							
<i>cens</i>	+	+	-	+	-	+	-
<i>P. putida</i>	+	+	-	-	-	+	-
<i>P. aerugin-</i>							
<i>osa</i>	+	-	+	-	-	-	-

(+) sensitive  
(-) insensitive

### Microscopy

Electron and epifluorescence microscope studies were undertaken to investigate the changes occurring on the surfaces of coupons immersed in cultures of bacteria isolated from Pembina oil. These changes arise from the loss of or deposition of corrosion products and from the possible attachment of the organisms on the surfaces of the coupons.

Plate 1 shows the typical scanning electron microscopy (SEM) of fine glass-blasted corrosion coupon before immersion in bacterial cultures. As is evident from the micrograph, the metal is free of any surface deposit and the clean metal, albeit rough, is exposed. When immersed in either B<sub>10</sub> or Butlin's medium used in these studies, however, the coupons were covered by a crystalline coating or deposit. Such crystalline deposit appeared to be dense (closely packed) as shown in Plate 2. The thick surface coating would probably create a barrier between the metal and its environment.

In the uninoculated B<sub>10</sub> medium (control), the coupons immersed for 6 days were almost completely (if not completely) obliterated by the surface deposition (Plate 3). On the other hand, when the coupons were immersed in B<sub>10</sub> medium inoculated with isolate #200 [Fe(III)-reducing] the surface coating was extensively removed, exposing the bare metal (Plate 4). The result was that the surface deposit occurred only as isolated patches. A similar observation was made when the coupons were similarly immersed in Butlin's medium, as can be seen in Plate 5 (control) and Plate 6 (inoculated with isolate #200). Thus, it appeared that isolate #200 could engender corrosion of the coupons by preventing the formation of possibly protective surface coating. Since these studies were carried out under microaerobic conditions,

Plate 1. Scanning electron microscopy of surface of fine glass-blasted  
unimmersed, mild steel coupon. X2485



K

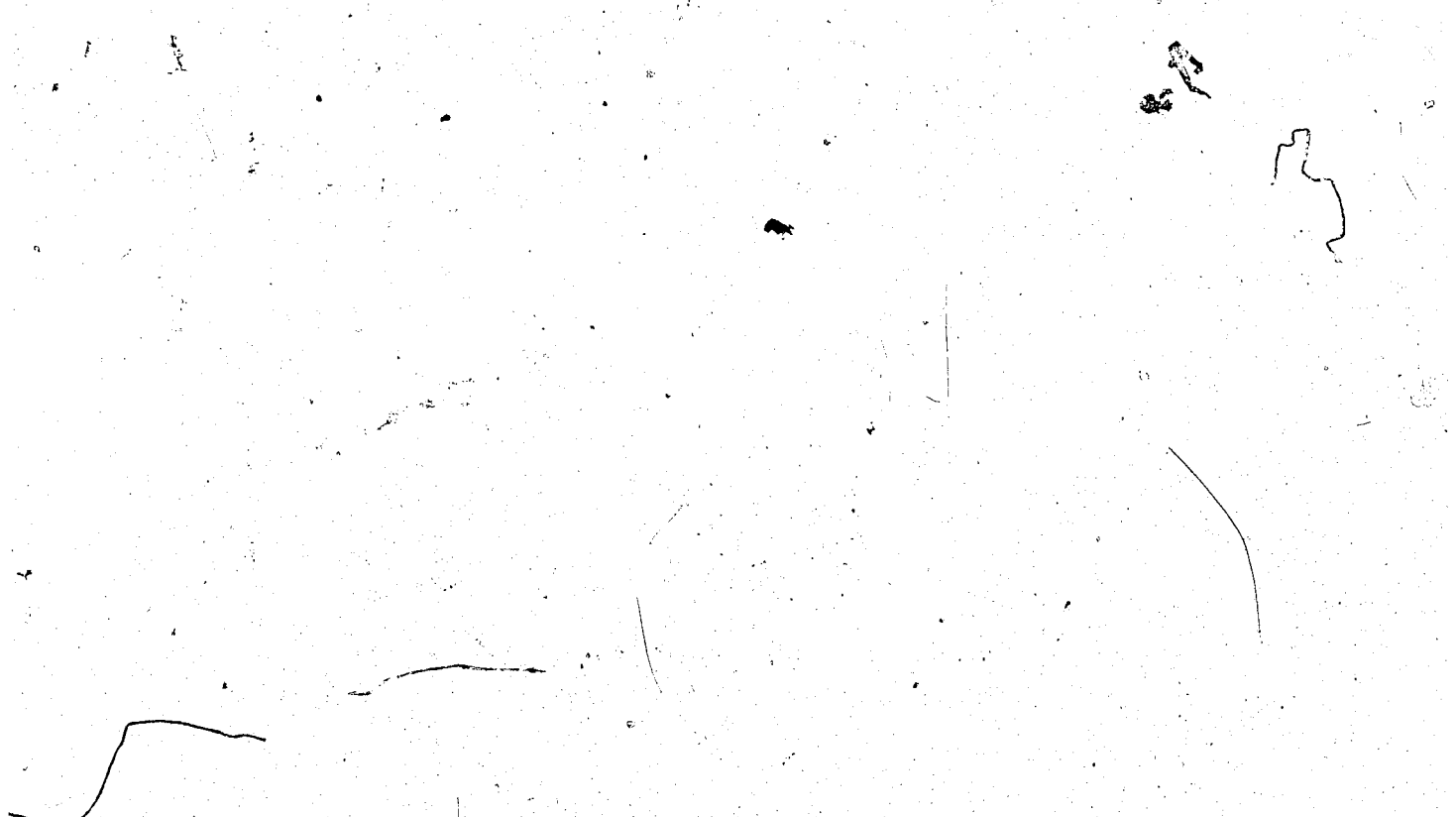
The image is a scanning electron micrograph (SEM) showing a highly textured, crystalline surface. The surface is covered with numerous small, irregular, and densely packed crystalline structures. The overall appearance is granular and uneven, with varying heights and depths of the crystalline growth. The background is a lighter, more uniform surface, likely the mild steel coupon mentioned in the caption. The image is labeled as Plate 2, X2275.

Plate 2. Scanning electron microscopy of densely packed crystalline  
surface covering of mild steel coupon incubated in B<sub>10</sub> medium  
for 6 days. X2275





Plate 3. Scanning electron microscopy of mild steel coupon incubated  
for 6 days in uninoculated (control) B<sub>10</sub> medium. X455



Plate 4. Scanning electron microscopy of mild steel coupon incubated  
for 6 days in B<sub>10</sub> culture of Isolate #200. X455



Plate 5. Scanning electron microscopy of mild steel coupon incubated  
for 6 days in uninoculated (control) Butlin's medium. X4550



Plate 6. Scanning electron microscopy of mild steel coupon incubated for  
6 days in Butlin's medium culture of Isolate #200. X4550





any product of coupon corrosion (*i.e.*  $\text{Fe}(0) \rightarrow \text{Fe}(\text{II})$ ) could be further oxidized by dissolved  $\text{O}_2$  to  $\text{Fe}(\text{III})$  state. Since the observed surface coating was minimal in cultures of isolate #200 (which can reduce  $\text{Fe}(\text{III})$  to  $\text{Fe}(\text{II})$ ), it was inferred that these surface deposits were  $\text{Fe}(\text{III})$  compounds which were solubilized in the presence of  $\text{Fe}(\text{III})$ -reducing bacteria. Presumably, the formation of the dense crystalline surface coating would reduce the contact between the metal and the environment and in that manner prevent or reduce corrosion. In contrast, the reduction of the generally insoluble  $\text{Fe}(\text{III})$  compounds to soluble  $\text{Fe}(\text{II})$  forms by isolate #200 (or any  $\text{Fe}(\text{III})$ -reducing organism) would expose the base metal allowing for continued interaction with its environment which might lead to greater corrosion.

The demonstration of the attachment of isolate #200 to coupons after short-term immersion (1-2 weeks) was not unequivocal, although bacteria-like particles were observed on the surface of the coupons. This problem arose because it was not possible to differentiate amorphous inorganic deposit from possible bacteria. The use of epifluorescence microscopy to study the surface of the previously immersed coupons was an attempt at solving the problem of bacterial identification. With this technique, stained cells would fluoresce and the bacterial shape would be evident (rod-like), thus differentiating animate from inanimate particles.

Epifluorescence microscopy revealed bacterial attachment to or close-association with the coupon surface. Rinsing of the coupon in running distilled water (4 litre/min) did not dissociate the bacteria from the coupon. It was, therefore, concluded that this association between bacterial cells and metal was strong enough to be considered




Plate 7. Scanning electron microscopy of mild steel coupon incubated  
for 9 weeks in B<sub>10</sub> culture of Isolate #200. X9200

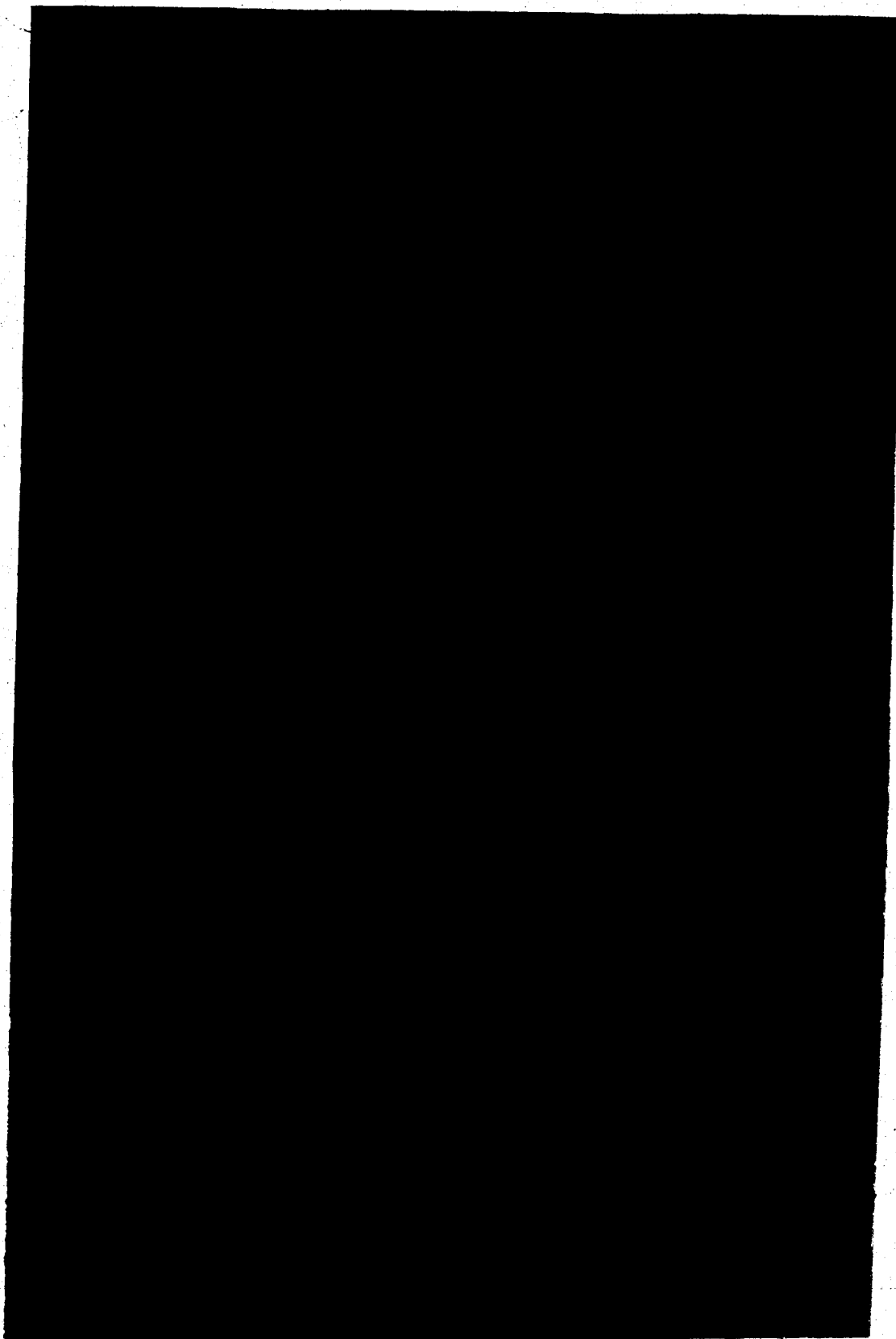


Plate 8. Transmission micrograph of sectioned cells of Isolate #200  
stained with ruthenium red. The cells were obtained from  
9 week B<sub>10</sub> culture of the organism to which mild steel  
coupons were exposed. (EP - Exopolysaccharide fibres)  
X40000



Plate 9. Electron micrograph of sectioned 18 hr cells of Isolate #2  
stained with ruthenium red. (EP - Exopolysaccharidic fibres)

X 37500

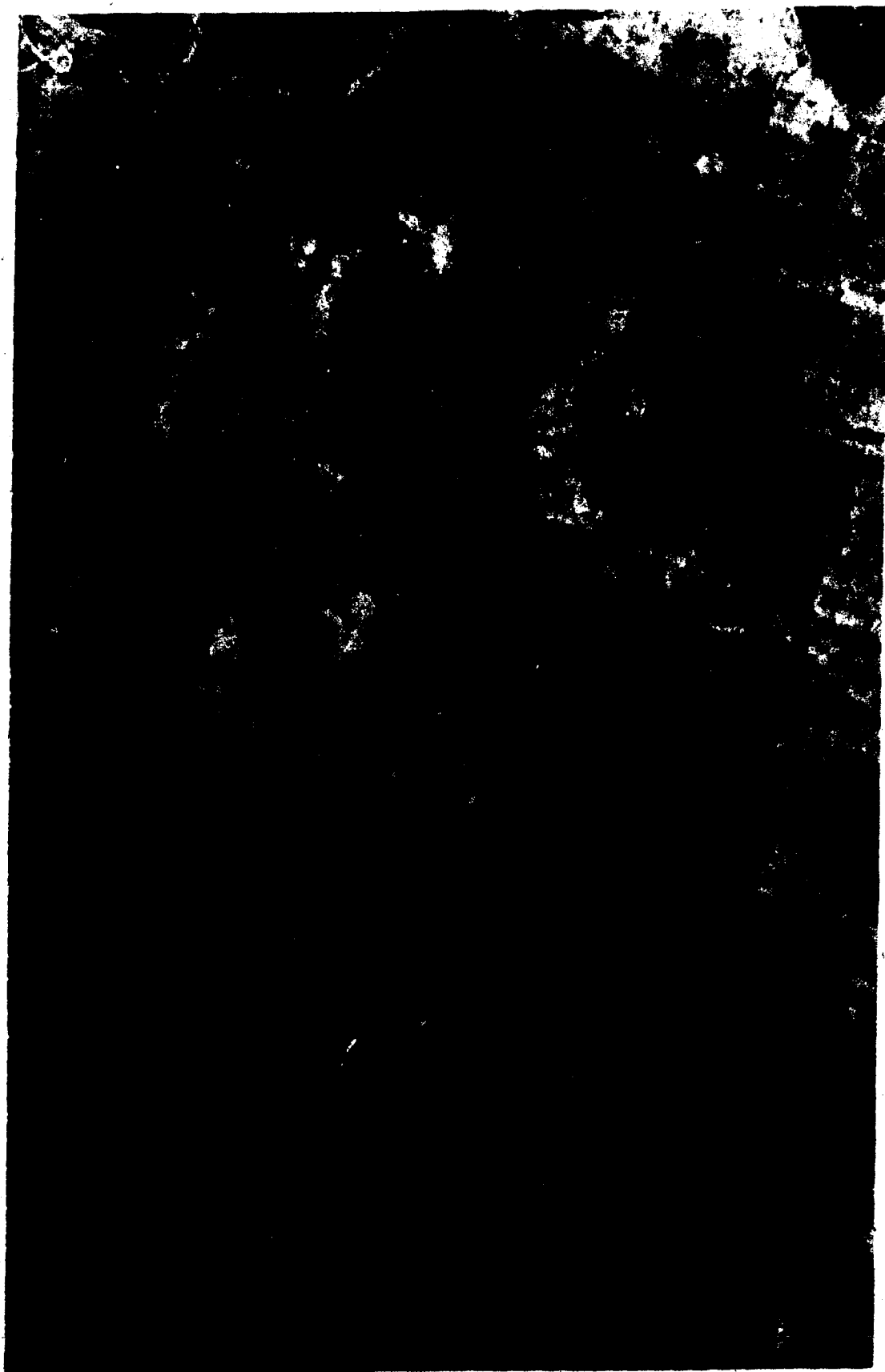


Plate 10. Scanning electron microscopy showing the attachment of cells  
of Isolate #66 to mild steel coupons after 2 weeks'  
incubation. X1416





Plate 11. Scanning electron microscopy showing attachment of cells of  
Isolate #42 to mild steel coupon after 2 weeks' incubation.

X1416



an attachment. Isolate #200 attachment to the coupon could not be quantitated (cell/unit area) because many of the cells were actually embedded in the uneven surface deposits and could be seen only at varied planes of focus.

During long immersion periods, isolate #200 produced thick fibrous exopolysaccharide material in which the organisms were entrapped and attached to the surface of the coupon (Plate 7). Ultrastructural studies using portions of cultures of isolate #200 into which coupons were immersed showed that this organism produced exopolysaccharide also in the liquid medium. The exopolysaccharidic material produced by, surrounding and connecting the cells of isolate #200 in culture is clearly shown in Plate 8. The culture material was stained with ruthenium red which selectively stains polysaccharides. Therefore, it is certain that isolate #200 has the capability to bind itself to metal coupon (by virtue of sticky exopolysaccharide produced) and did indeed attach itself to metal surface as was observed by epifluorescence microscopy. Other isolates from Pembina oil also produced sticky exopolysaccharide (Plate 9 for #2 isolate).

Under natural conditions, isolate #200 would exist together with other bacteria in Pembina crude oil. Amongst the isolates obtained from Pembina oil were several bacteria that produced extremely gummy colonies. These organisms readily attached to the surface of coupons suspended in their cultures. Such attachments after about 2 weeks of incubation in batch cultures are shown in Plates 10 and 11. Evidently, organisms which do not readily produce the slimy, adhesive materials would be easily entangled in the masses of the slime-producing bacteria and thus help in their attachment to metallic structures.

The importance of exopolysaccharide in the establishment of micro-organisms on different surfaces has been discussed by Costerton *et al.* (1978).

The relationship between bacterial binding or attachment to metal surfaces and the corrosion of such metallic structures is not known. However, formation of a mass of attached cells may cause development of concentration cells due to differential aeration. Presumably, for an organism to play a significant role in the corrosion process it should be closely associated with or attached to the corroding material. In the related phenomenon - ore leaching by bacteria - attachment of the corroding bacteria to the ore particles has been the rule (Berry and Murr, 1978). However, these workers maintained that attachment to the ore particles might not be indispensable for bacterial leaching of ore materials to occur.

### The Identity of Isolate #200

The bacterial organism isolated, and purified by restreaking on PCA medium, from Pembina crude oil and designated Isolate #200 is a Gram-negative, aerobic, oxidase-positive, non-sporing, motile rod possessing 2-4 polar flagella (Plate 12). When grown at 20°C in an unagitated Butlin's medium the organism produced predominantly 4 flagella per cell, but formed 2 flagella per cell when grown at higher temperature (30°C). Other characteristics that may help in the identification of Isolate #200 are shown in Tables 9, 10 and 11.

The positive oxidase reaction, aerobic character and the presence of polarly placed flagella are characteristics that may place this organism as a member of the genus *Pseudomonas*. However, in Hugh-Leifson's medium, 0.5 glucose and lactose broths, Isolate #200 showed alkaline reaction, instead of the commonly observed acidity. Alkaline reaction in Hugh-Leifson's medium is commonly associated with members of the genus *Alcaligenes*. *Alcaligenes* spp., in addition to the alkaline reaction in Hugh-Leifson's medium, are aerobic, Gram-negative, oxidase-positive, motile rods; characteristics shared with members of *Pseudomonas*. Although common with *Alcaligenes* spp. the alkaline reaction is by no means unique to these organisms. Shewan *et al.* (1960) described a group of aerobic *Pseudomonas* which showed alkaline reaction in Hugh-Leifson's medium. These organisms were classified by Shewan and co-workers (1960) as *Pseudomonas* group III. Although members of the genus *Alcaligenes* are described as having peritrichous flagellation, some are 'degenerately peritrichous' (Holding and Shewan, 1974).

It is often very difficult, however, to differentiate cases of

Plate 12. Electron micrograph of Isolate #200 (negatively stained)  
showing polar flagellation. X29700





degenerate peritrichous flagellation from polar or subpolar cases. It appears, therefore, that Isolate #200 could belong to *Pseudomonas* group of Shewan *et al.* (1960) or to the genus *Alcaligenes*.

Described species of the members of the genus *Alcaligenes* are lacking in their ability to utilize carbohydrates and sugar derivatives (except fructose) (Stanier *et al.*, 1966). Isolate #200, however, does grow on many carbohydrate substrates as the sole carbon and energy source. Moreover, *Alcaligenes* spp. have not been reported to liquify gelatin, neither do they commonly hydrolyse casein (*i.e.* proteolytic activity). In contrast, Isolate #200 vigorously hydrolyses gelatin and casein (in litmus milk). This proteolytic capability in Isolate #200 may well explain the predominant alkaline reaction observed in Hugh-Leifson's 0.5% glucose and lactose broths since these media contain peptone base. It is evident from the presence of proteolytic capability in Isolate #200 that this organism cannot be an *Alcaligenes* sp. All the evidence shown (Tables 9, 10 and 11) indicate that Isolate #200 has more than the physiological capabilities generally associated with members of the genus *Alcaligenes* and conform to the general attributes of the members of the genus *Pseudomonas*. Although the determined % G+C content (44.86) of Isolate #200 is lower than normally cited for members of *Pseudomonas*, Mandel (1966) reported the case of *Pseudomonas atlantica* NCMB 301 having a percent G+C content as low as 43.5.

In the most extensive taxonomic study so far undertaken on aerobic *Pseudomonads*, Stanier *et al.* (1966) reported that in all species of *Pseudomonas* examined that contained C-type cytochromes, the  $\alpha$ -band of the b-type cytochrome lies on the shoulder of C-peak (522 nm). In the study of the cytochrome content of Isolate #200, absorbance shoulders

were also observed around 522 nm. This shows that Isolate #200 has a common *Pseudomonas* characteristic. In addition, Isolate #200 utilized a variety of carbon compounds (hydroxy acids, and other carboxylic acids) considered the 'core substrates' for aerobic *Pseudomonas* by Stanier and his co-workers (1966). Moreover, Isolate #200 grew on some aromatic amino acids (Table 9) utilizing them as the sole carbon/energy source. The fair extent of nutritional versatility exhibited by Isolate #200 is reminiscent of the members of the genus *Pseudomonas*.

In a modification of Hugh-Leifson's test, in synthetic medium devoid of any proteinaceous material but containing only glucose, Isolate #200 indeed produced acid from glucose. Therefore, the strong alkaline reaction observed in the regular Hugh-Leifson's medium must be due to the ammonification of the peptone content. On the basis of these characteristics shown by this organism it is concluded that Isolate #200 is a *Pseudomonas* sp.

### Ferric Iron Reduction in Pembina Oil System

The occurrence of ferric iron-reducing bacteria may be regarded as an intrinsic characteristic of oilfield operations in North Central Alberta. Their presence has been detected at all times in produced water, oil-water emulsion as the emulsion comes out of the wells and in the crude oil samples transported through the Interprovincial-Lakehead Pipeline from Edmonton to Montreal, a distance of over 2000 miles (Dr. F.D. Cook, personal communication).

These organisms could be easily recognized by their characteristic concave colonial morphology on B<sub>10</sub> agar plates. When grown on complex media, they show light orange coloration but produced no diffusible pigment. These iron-reducers are Gram-negative, non-spore-forming, motile rods. Some Gram-positive spore formers are also known to occur and reduce Fe(III), too (F.D. Cook, unpublished data). Their constant occurrence and their presence as an appreciable proportion of the total bacterial flora in crude oil samples and their propensity to reduce ferric iron have made these organisms very attractive candidates for the studies of the possible role of iron-reducing bacteria in the corrosion process.

Of 235 organisms aerobically isolated from Pembina crude oil, 19 have shown the ability to reduce Fe(III) to Fe(II). The data in Table 12 shows the result of screening of the isolates for the ability to reduce ferric iron. The best iron-reducing isolate reported by Ottow (1968) was able to produce only 25 mg/L of Fe(II) after 5 days' incubation. All the isolates reported here have shown ferric iron-reducing ability several orders of magnitude greater than the bacteria

TABLE 12. Summary of Fe(II) produced in B<sub>10</sub> medium by Fe(III)-reducing isolates

Isolate	Mg Fe(II)/litre/mg dry wt of cells	
	Incubation period (hr)	
	2	4
77	224.7	319.7
78	418.3	651.7
134	462.0	628.3
167	440.6	573.9
169	397.9	448.1
180	369.0	480.3
181	472.1	560.8
198	308.7	357.5
200	538.6	693.9
201	484.8	582.6
202	381.7	519.6
213	280.7	513.3
216	279.1	412.9
217	246.8	363.3
230	389.1	675.0
232	287.5	557.3
233	69.1	95.5
234	88.7	129.6
235	237.3	312.5

isolated by Ottow from soils. It would then be apparent that the ability to reduce ferric iron by these organisms is an innate characteristic (purposeful) and not due to reduction of Fe(III) by the metabolic products (incidental reduction). Isolate #200 showed the greatest capacity to reduce Fe(III) and was, therefore, chosen for subsequent investigations.

Although the role of iron-reducing bacteria in soil nutrient cycle has been recognized and studied (Kamura *et al.*, 1963; Gotoh and Yamashita, 1966) only very little information is available on the reduction of iron by pure cultures or membrane preparations (Lascelles and Burke, 1978). Moreover, the mechanism of ferric iron reduction is not well understood. Because Isolate #200 reduces Fe(III) fast, it would serve as a very convenient organism with which to investigate the characteristics of microbial reduction of Fe(III).

#### Ferric Iron Reduction by Resting Cells of Isolate #200

##### Establishment of assay conditions

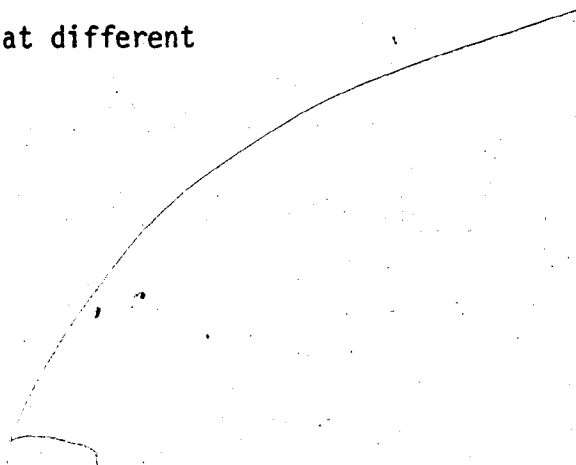
Initial experiments were designed to establish the conditions that were favourable for Fe(III) reduction by Isolate #200. The influence of temperature, the nature of electron acceptor, and the scope of electron donors were investigated. To ascertain that the cells were actually utilizing the added energy sources, two controls were employed: uninoculated and endogenous controls. Although the possibility of substrate ( $e^-$  source) carry-over was obviated by multiple washing of cells in buffer solutions, it was still possible that endogenous activity might contribute to observed Fe(III) reduction. Consequently, the cells were incubated for 30 minutes,

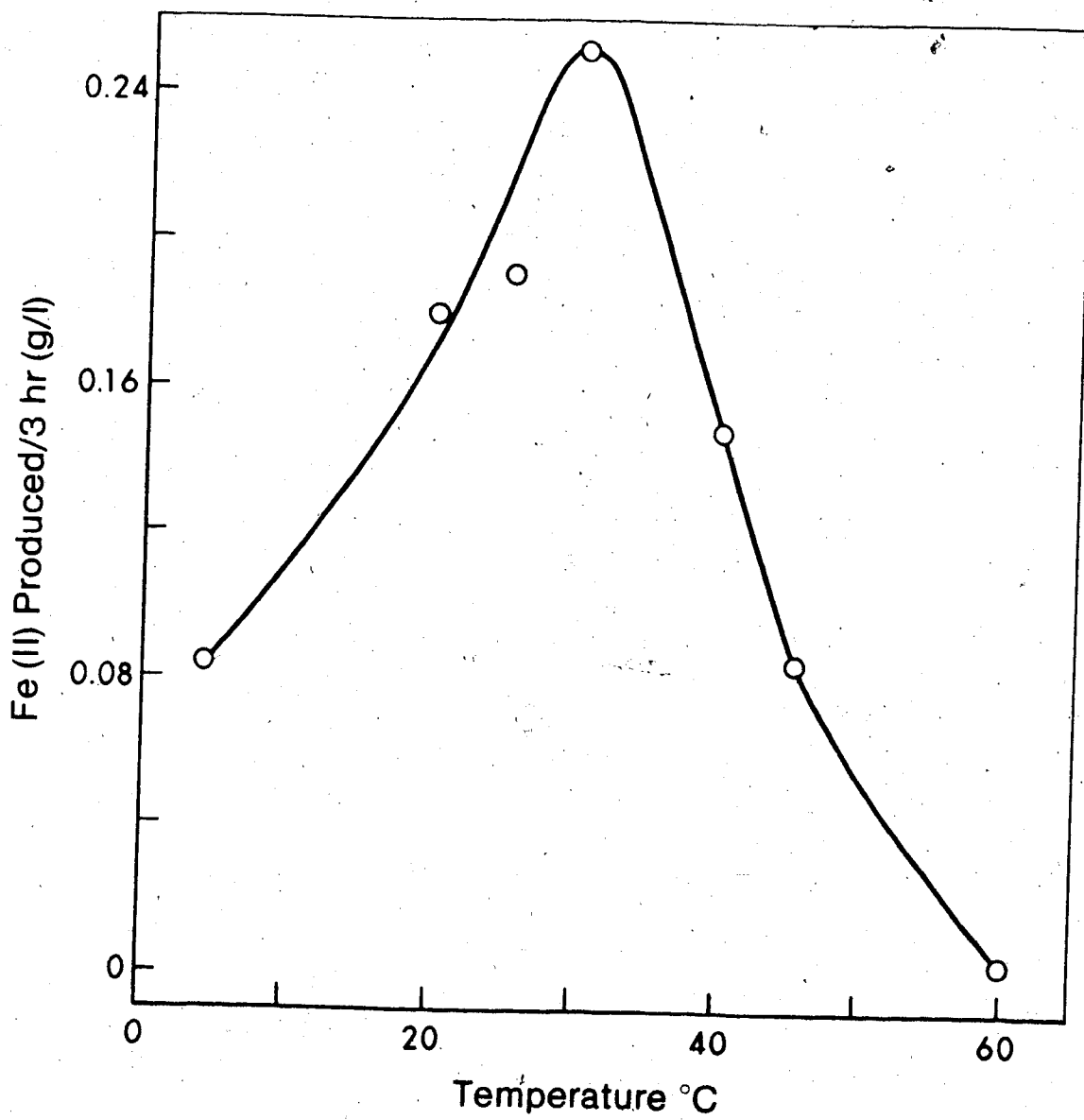
before use, to deplete any stored energy sources. In most cases, however, cell preparations were used after several days of storage during which any energy reserve would have been depleted. No Fe(III) reduction was observed in the absence of added energy source (endogenous control). It was concluded, therefore, that endogenous contributions to overall Fe(III) reduction did not exist under the experimental conditions employed. Subsequently, all experiments employed only uninoculated controls.

*Electron acceptors:* Of the soluble ferric compounds, ferric phosphate ( $\text{FePO}_4$ ) and ferric ammonium citrate were examined as possible electron acceptors for Isolate #200. The organism reduced both compounds but reduced  $\text{FePO}_4$  faster. It was observed with ferric ammonium citrate however, that the organism could reduce Fe(III) in the absence of any added energy source. This would mean that the citrate of ferric ammonium citrate also served as an energy source. This was not surprising since it was found that Isolate #200 could grow on citrate as the sole energy source. The ammonium ion present in this compound would serve as a nitrogen source and there would be an accompanying increase in population; a condition not desirable in resting cell experiments. Therefore,  $\text{FePO}_4$ , at 0.4% final concentration, was the preferred electron acceptor and used through the work. Higher concentration of  $\text{FePO}_4$  was not used as a precipitate developed in course of the reduction process.

*Temperature:* The rates of  $\text{FePO}_4$  reduction by Isolate #200 at temperatures ranging from 4° to 60°C with lactate as electron donor are shown in Fig. 9. The ability of the organism to reduce Fe(III) increased with increase in temperature up to 30°. Above 30°, there was a sharp

Fig. 9. Fe(III) reduction by cells of Isolate #200 at different temperatures.





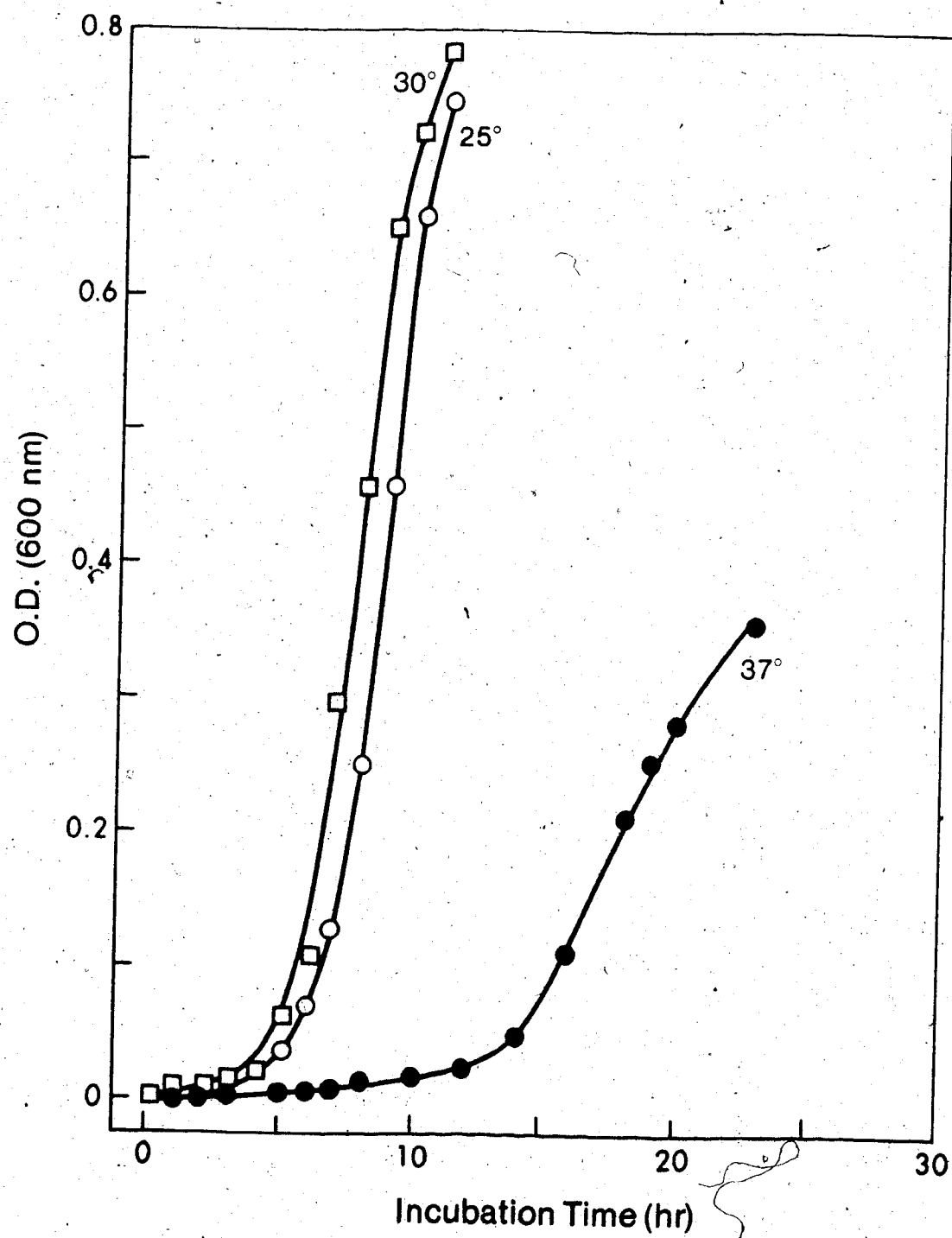


decline in its Fe(III)-reducing ability, and was almost completely inhibited at 60°C. The optimum temperature for Fe(III) reduction corresponded to that of growth (Fig. 10), although under the experimental conditions employed for Fe(III) reduction reactions there was no accompanying increase in cell population. Therefore, the observed activity at 30° cannot be attributed to increased cell number.

The temperature of the crude oil-water system in the pipeline ranges from 7°, in winter, to about 20°C, in summer. Therefore, under field conditions, the iron-reducing capability of the organism would be reduced but not abolished by temperature changes. Consequently, iron-reduction reaction must be considered an ever-going reaction in the pipeline system which carry these organisms.

*Electron donors:* This study was undertaken to determine the scope of potential electron donors for Fe(III) reduction. Initially, it was observed that ample Fe(III) reduction could occur in inoculated produced water without any extraneous supply of energy source. This would indicate that the produced water contained some available energy source(s) which served as electron donor for the reduction reaction. Since the crude oil-water system in which #200 occurred contained a mixed population with varied metabolic activities, it was possible that end products of their metabolism could serve as the available energy sources. Similar observation was made with respect to the ability of sulphate reducers to utilize crude oil. Jobson *et al.* (1979) reported that although an isolate of *Desulfovibrio* sp. from crude oil sample could not utilize directly crude oil as energy source for sulphate reduction, intermediate products of crude oil degradation by oil-degraders readily served as electron donors for sulphate reduction.

Fig. 10. Growth of Isolate #200 in Butlin's medium at different temperatures.



Equimolar concentrations (30  $\mu$ moles/ml) of a variety of organic compounds were tested to determine the preferred electron donors for Fe(III) reduction. All the compounds tested were able to serve, to different degrees, as  $e^-$  donors for Fe(III) reduction by Isolate #200 (Table 13). In general, although the three- and four-carbon compounds could be utilized, the hexoses and disaccharides were most favoured. Of the two best utilized 3-carbon compounds, lactate was preferred over pyruvate. The utilization of the hexoses and disaccharides was slow initially but later increased with prolonged incubation. Although all the substrates were supplied at equimolar concentrations, the total energy available in glucose would be at least twice that of lactate since two molecules of lactate could be produced from one of glucose. Thus, the greater amount of Fe(II) produced with the higher molecular substrates (*e.g.* hexoses) as the electron donors can be attributed to a greater overall available energy.

Although lactate was the preferred energy source, the concentration at which it was supplied affected Fe(III) reduction (Fig. 11). At low concentrations, increase in Fe(III) reduction was observed with the rise in the level of lactate (sodium lactate) supplied, up to a maximum of 30  $\mu$ moles/ml of reaction mixture. At concentrations above this, lactate was inhibitory to Fe(III) reduction. In all subsequent experiments, lactate was the energy source employed at the optimum concentration for Fe(III) reduction.

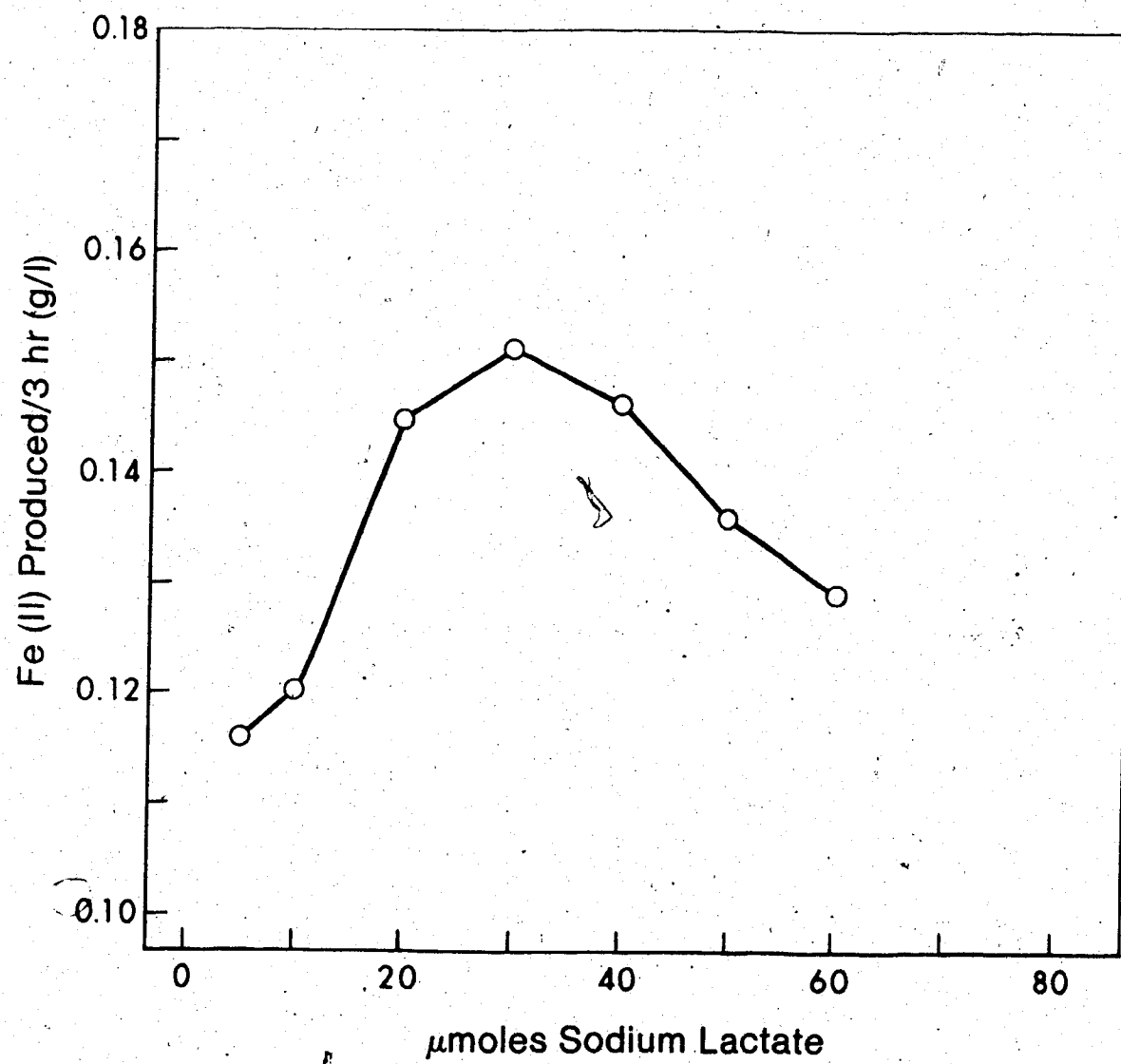
The pH of the incubation medium was adjusted to 7.2. This was chosen because it had earlier been found that pH range of 7.2-7.4 was optimum for Fe(III) reduction by a similar organism (Halasa, personal communication).

TABLE 13. Summary of Fe(III) reduction by isolate #200 using different potential electron donors<sup>1</sup>

Electron donor	Amount of Fe(II) produced (g/L)	
	Incubation period (hr)	
	3	72
Na-acetate	0.03	0.15
Na-succinate	0.03	0.23
Na-citrate	0.01	0.14
Na-tartarate	0.01	0.01
K-fumarate	0.08	0.11
Na-malate	0.01	0.06
Na-lactate	0.27	0.51
Na-pyruvate	0.11	0.43
Glucose	0.01	0.42
Sucrose	0.00	0.33
Maltose	0.04	0.41
Galactose	0.02	0.38
Mannitol	0.24	0.39

<sup>1</sup>Potential electron donors were supplied at the rate of 30  $\mu$ moles/ml.

Fig. 11. Effect of concentration of electron donor (lactate) on the reduction of Fe(III) by Isolate #200.



### Effect of cell number on Fe(III) reduction

Aliquots containing 0.1, 0.2, 0.3, 0.5, 1.0 and 2.0 ml of the standard washed cell suspension (1.0 g wet wt/80 ml buffer) were used to reduce Fe(III) (0.4%  $\text{FePO}_4$ ), with lactate (30  $\mu\text{moles/ml}$ ) as the electron donor. One millilitre of the standard cell suspension contained  $30 \times 10^7$  cells.

Data shown in Fig. 12 show the effect of Isolate #200 cell number on Fe(III) reduction. The total Fe(II) produced increased linearly (*i.e.* constant rate of Fe(III) reduction) with increase in cell number up to  $30 \times 10^7$  cells (equivalent to 1 ml of the standard cell suspension). Above this number of cells, the linear relationship was lost and the rate decreased. The decrease in rate at the high cell number was probably due to the Fe(III) or the electron donor (lactate) limitation. Therefore, for all subsequent Fe(III) reduction experiments involving resting cells (washed cells) 1 ml of the standard cell suspension was employed. This is because this number of cells gave the greatest total Fe(II) production and also fell within the constant reduction rate region.

### Effect of storage of washed cells on ferric iron reduction

It was not convenient to prepare fresh cell suspension every time resting cells were needed. It was considered necessary to investigate the effect of storage at 4°C and storage medium on ferric iron-reducing cell suspensions.

Two buffers - phosphate and Tris-HCl - were employed.

Data shown in Fig. 13 show Fe(III) reduction by cell suspensions stored






Fig. 12. Effect of cell number on the reduction of Fe(III) by Isolate #200.

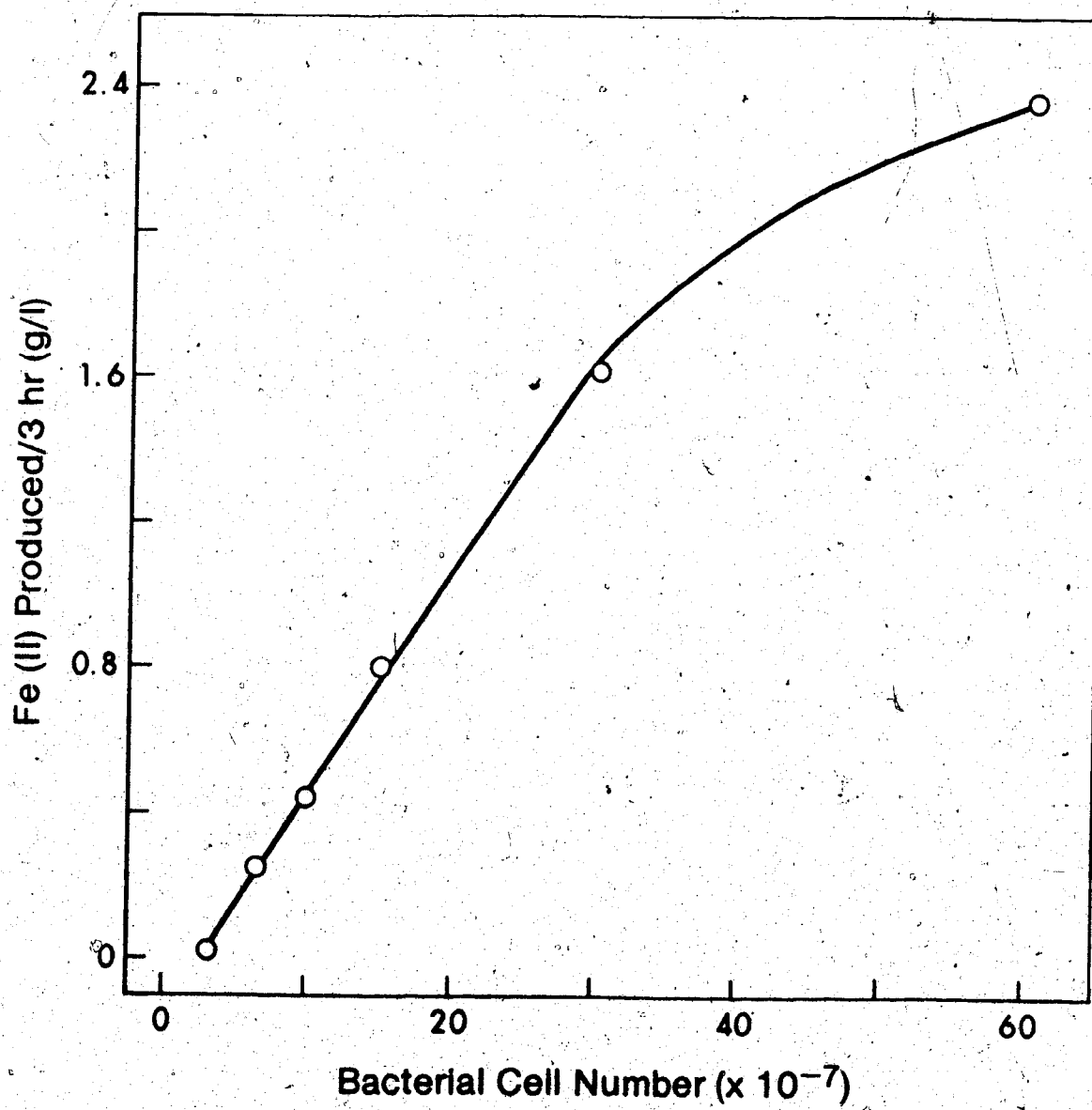
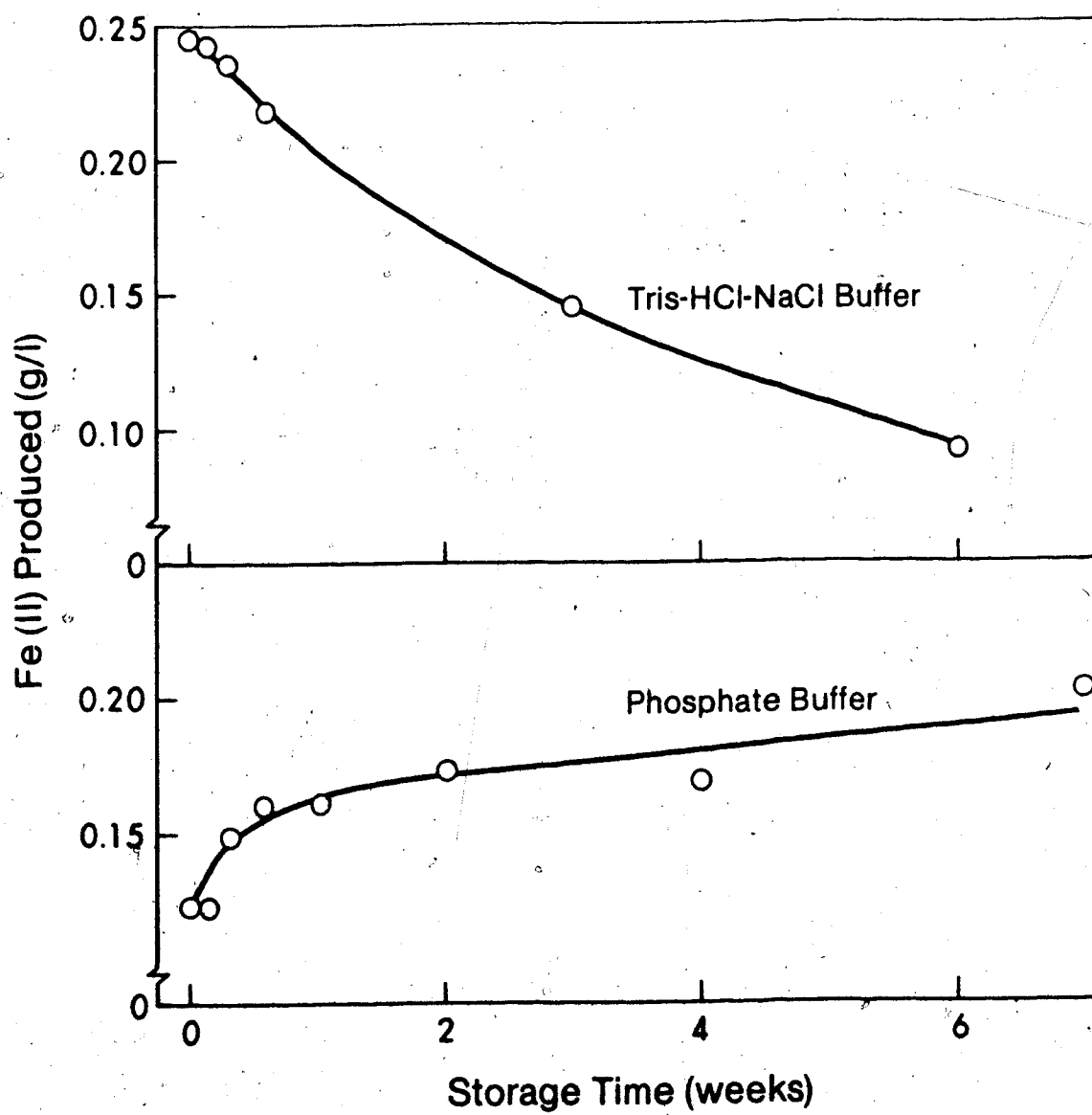


Fig. 13. Fe(III) reduction by Isolate #200 after storage in 0.1 M phosphate and 0.1 M Tris-HCl-NaCl (pH 7.2). Assays were done with different cell preparations from portions of the same culture, washed with and resuspended in the requisite buffer.



in 0.1 M Tris-HCl-NaCl and 0.1 M phosphate (pH 7.2) respectively. In the phosphate buffer, the cells maintained an unimpaired ability to reduce Fe(III) during long storage. A slight increase in activity was observed after 1 day storage. Subsequently, the cells maintained an unchanged ferric iron reducing capability up to 4 weeks of storage. With longer storage, a slight increase in reductive activity was observed which was ascribed to a probable increase in cell concentration due to loss of water by evaporation, although the cells were stored in capped 250 ml nalgene bottles.

On the other hand, the cells suspended in Tris-HCl buffer soon began to lose their ferric iron reducing ability. By the end of 6 weeks of storage a loss of about 65% of the initial activity was observed. Therefore, Tris-HCl buffer impaired the cells capability to reduce ferric phosphate on storage. The change in viable cell population with storage was not followed, so it was not possible to ascribe the loss of activity to cell death in Tris-HCl buffer. On the basis of these results, all resting cells were prepared by washing and resuspending for storage in phosphate buffer.

#### The Physiology of Iron Reduction by Isolate #200

The mechanism of Fe(III) reduction in soils was proposed by Kamura *et al.* (1963) as being partly due to direct bacterial effect and indirectly by the reductive property of exogenous bacterial products. In determining the existence of such dual effects on Fe(III) reduction by Isolate #200 it would be necessary to employ culture of the organism in a suitable growth medium. Presumably, if Isolate #200 reduced Fe(III) because of the formation and secretion of reductive products, such

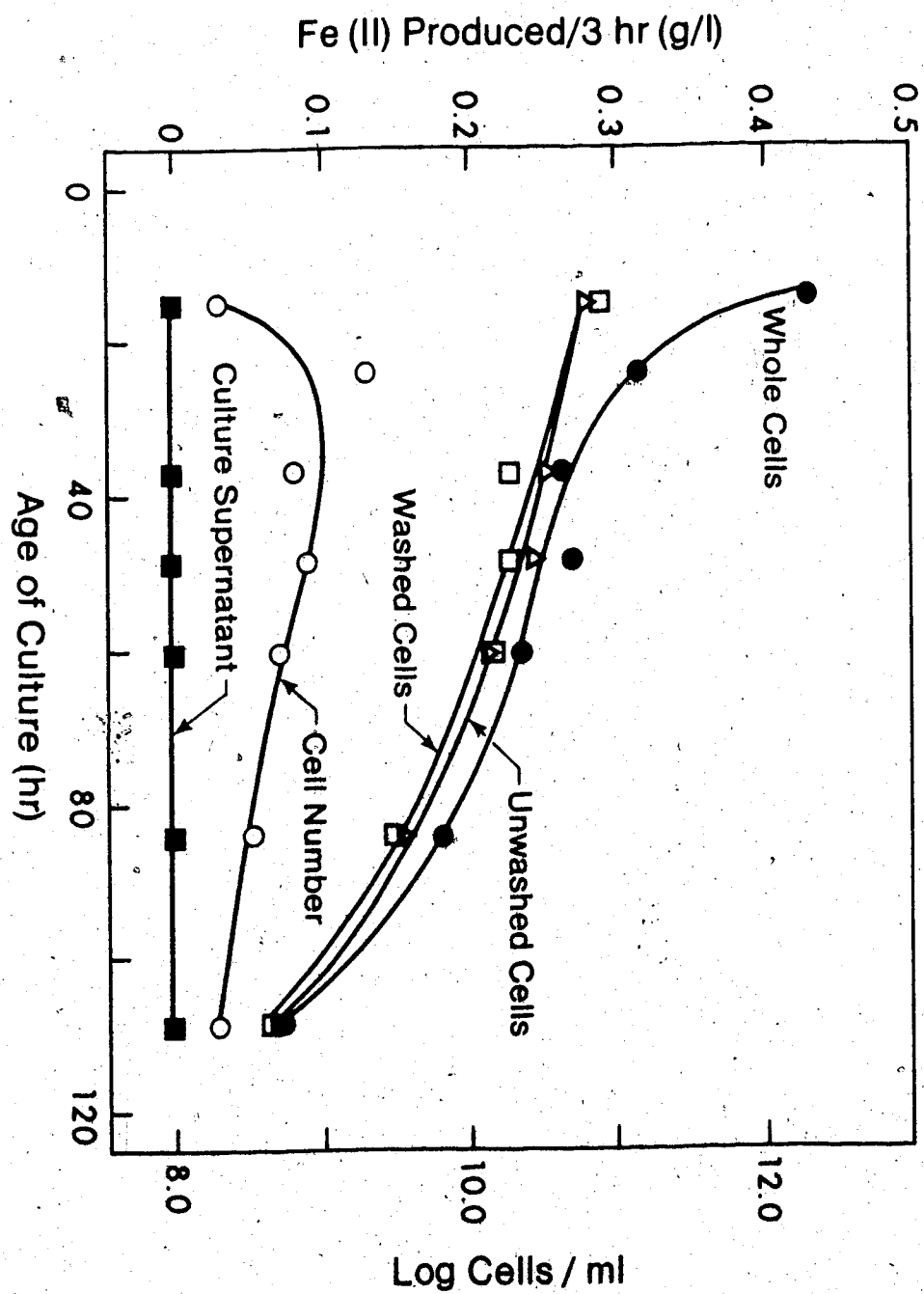
products would be expected to accumulate in the culture supernatant with incubation period.

#### Effect of Age of Cell Culture on Fe(III) Reduction

The data in Fig. 14 show the time course of ferric phosphate reduction by whole culture of different ages and washed cells of Isolate #200 grown in Butlin's medium and by the culture supernatants. Ferric iron reduction by the organism was highest after 14-hour incubation and decreased with age. No reductive activity was observed with the supernatant fluids. Whole culture inoculum (unwashed) showed a slightly better reductive activity than washed cells. This was probably due to incomplete recovery of cells after centrifugation at 5910 *g* for 5 min. Since washed cell samples were prepared by resuspending pelleted cells to their original volume, unsedimented cells were lost with the supernatant.

Although the activity of cell suspensions or cultures decreased with culture age, there was no accompanying activity in the culture supernatant. Therefore, the decrease in cell activity could not be due to secretion of the active agent (enzyme) into the surrounding medium. Furthermore, there was no difference in the Fe(III)-reducing activities of washed and unwashed cells. No Fe(III)-reducing activity was present in the recovered buffer used in washing cells. It is, therefore, evident that if iron-reducing activity of the cells was associated with the cell wall, it must be firmly attached. The decline with age in ferric-reducing activity of the culture may be explained by a general decline in physiology with aging.

Fig. 14. Fe(III) reduction by culture supernatants, cells and whole cultures of Isolate #200 of different ages.





Effect of Growth Medium on Fe(III)-reducing Capability  
of Cells of Isolate #200

This investigation was carried out with a view to understand some environmental factors that may influence ferric iron reduction by the organism. In nature, the mottled appearance of gley soil would indicate that iron-reducing activity of soil organisms is discontinuous. Factors which might be responsible for favoured activities in different soil patches include availability of utilizable energy sources (Takai *et al.*, 1963) and, of course, the availability of reducible ferric compounds (*ibid*). Since soil contents of iron and energy sources vary, it was considered necessary to investigate the effects of such variations on the capability of potential ferric iron-reducing organisms.

To carry out this investigation, it was necessary to grow the organism in synthetic medium where the iron content could be closely controlled, and in rich medium. In order to ensure the depletion of possible iron reserve, where necessary, the culture was passed through the synthetic medium (Appendix 1e) lacking added iron for two successive 14-hour incubation periods. To produce inoculum in more complex media, Butlin's medium, with or without added iron, and C<sub>4</sub>F<sub>1</sub> medium (mineral salts + yeast extract and nutrient broth; Appendix 1c) were employed.

Quantitative as well as qualitative differences were observed in the biomass produced in the different media. Cells grown in iron-free minimal medium lacked any visible pigmentation. On the other hand, cells grown in Butlin's or B<sub>10</sub> media showed orange pigmentation. However, the pigmentation dissociated with cells grown in C<sub>4</sub>F<sub>1</sub> medium was

more intense than those observed in Butlin's or iron-containing minimal medium, in that order. Biomass production was highest in  $C_4F_1$  and least in iron-free minimal medium. In general, complex media favoured biomass production and the absence of added iron was inimical to cell growth. Understandably, the addition of nutrient broth (Difco) and/or yeast extract (Difco) with their content of free amino acids and growth promoters (including Fe) would facilitate cell growth in the complex media.

The iron-reducing activities of Isolate #200 grown in different media are present in Table 14. As can be seen, cells grown in more complex media reduced ferric iron faster than those grown in minimal medium. The iron-reducing capability decreased with decrease in medium complexity. In addition, growth of the organism in iron-containing medium enhanced the ferric iron-reducing activity of the organism. Supposedly, cells grown in complex medium were in better physiological state than those grown in minimal medium. Since iron is a component of several proteins (e.g. cytochrome) its absence in a growth medium would be expected to limit the synthesis of such metaloproteins. As a result, there would be a decrease in the general activity of the cells. Also, associated with ferric-reducing ability was the intensity of pigmentation. Cells which showed the highest intensity of pigmentation also showed the greatest ability to reduce ferric phosphate. Since many iron-containing proteins are pigmented, the absence of such iron-protein molecules might be responsible for lack of pigmentation observed in iron-free growth.

A much closer look at the effect of iron-limitation on the ability of Isolate #200 to reduce  $Fe(III)$  to  $Fe(II)$  showed a marked kinetic

TABLE 14. Fe(III) reduction by cells of isolate #200 grown in different media.

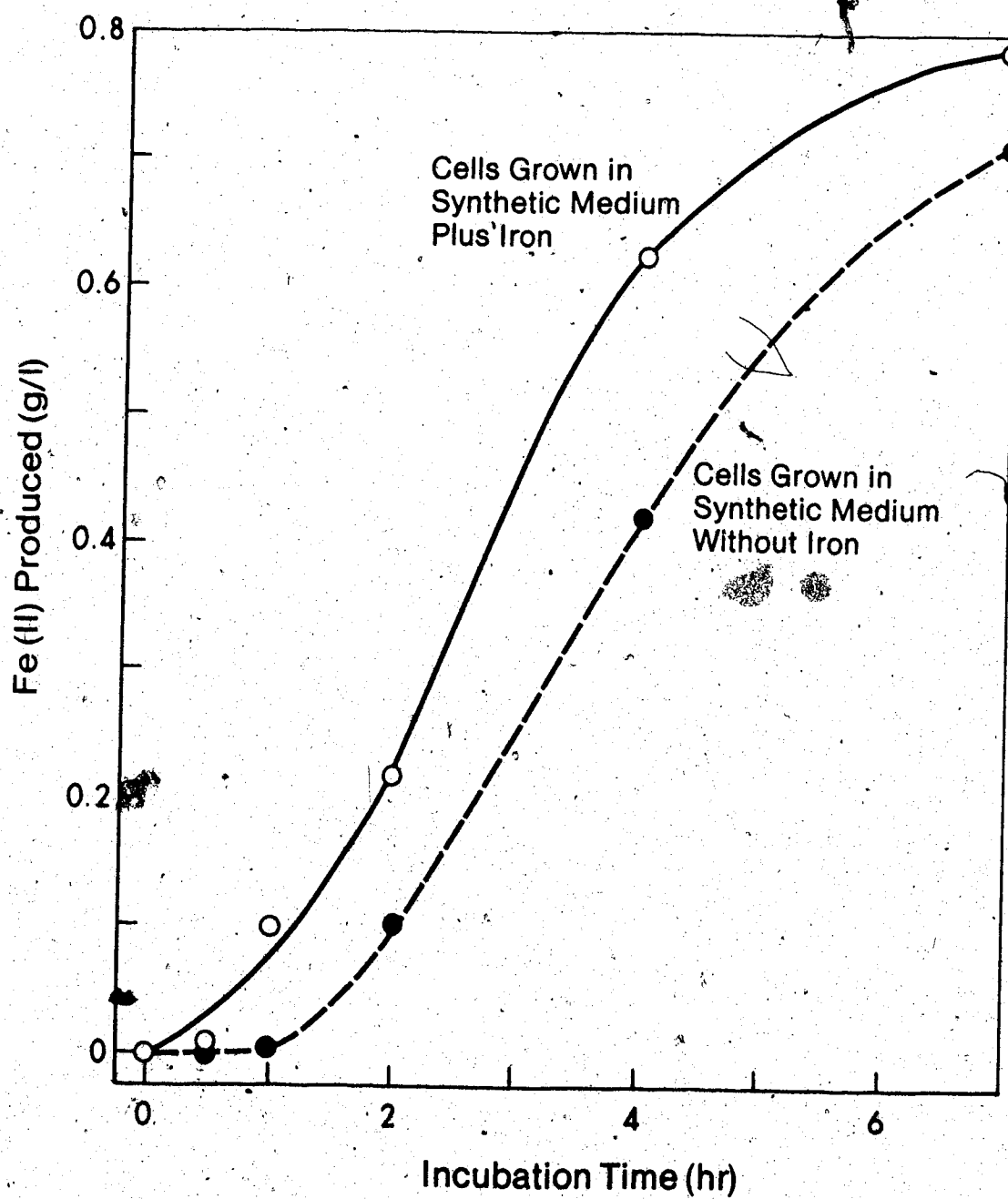
Medium	Fe(II) produced (mg/l)/3 hr
Iron-free minimal medium	7.5
Iron-positive minimal medium	21.3
Butlin	102.5
C <sub>4</sub> F <sub>1</sub>	161.3

All cells were washed three times in 0.1 M phosphate buffer and suspended in same buffer to a final concentration of 1 g wt wet cells per 80 ml of buffer.

difference in the reduction by iron-starved and non-limited inocula. The data in Fig. 15 show ferric iron reduction by cells of #200 grown under iron-starved/iron-rich conditions. Cells grown in iron-containing synthetic medium reduced altogether more Fe(III) than those grown in synthetic medium without added iron. An initial lag was observed with cells grown in the iron-free medium during the first hour of incubation after which Fe(III) reduction proceeded at a comparable rate as with the iron-positive cells. However, when the inocula were grown in Butlin's medium, with or without added iron, ferric iron was reduced with equal facility (Fig. 16) and no lag phase was observed in either of the inocula. Since only trace amounts of iron occur in yeast extract and other chemical components of Butlin's medium, it could be inferred that only trace amounts of iron were necessary to abolish the initial lag period observed with iron-starved cells (Fig. 15).

The occurrence of the early lag phase in Fe(III) reduction observed in iron-starved cells could be the result of several factors (Mandelstam and McQuillen, 1973). First, the lag could be indicative of the induction of the enzyme(s) necessary for Fe(III) reduction. Secondly, it was also possible that the lag defined a time requirement for the transport of Fe(III) to the appropriate site for the reduction process. Transport of Fe(III) to a reduction site would necessarily mean a need for transport protein-permease. Therefore, it was possible that the lag period represented an induction period for the synthesis of the appropriate permease, as different from a protein that reduces Fe(III) as mentioned earlier. Furthermore, another possibility that the lag might be a result of an adjustment period due to change in the physical environment (like transfer from Fe-free to Fe-containing medium), that

Fig. 15. Fe(III) reduction by cells of Isolate #200 grown in synthetic medium, with or without added iron.




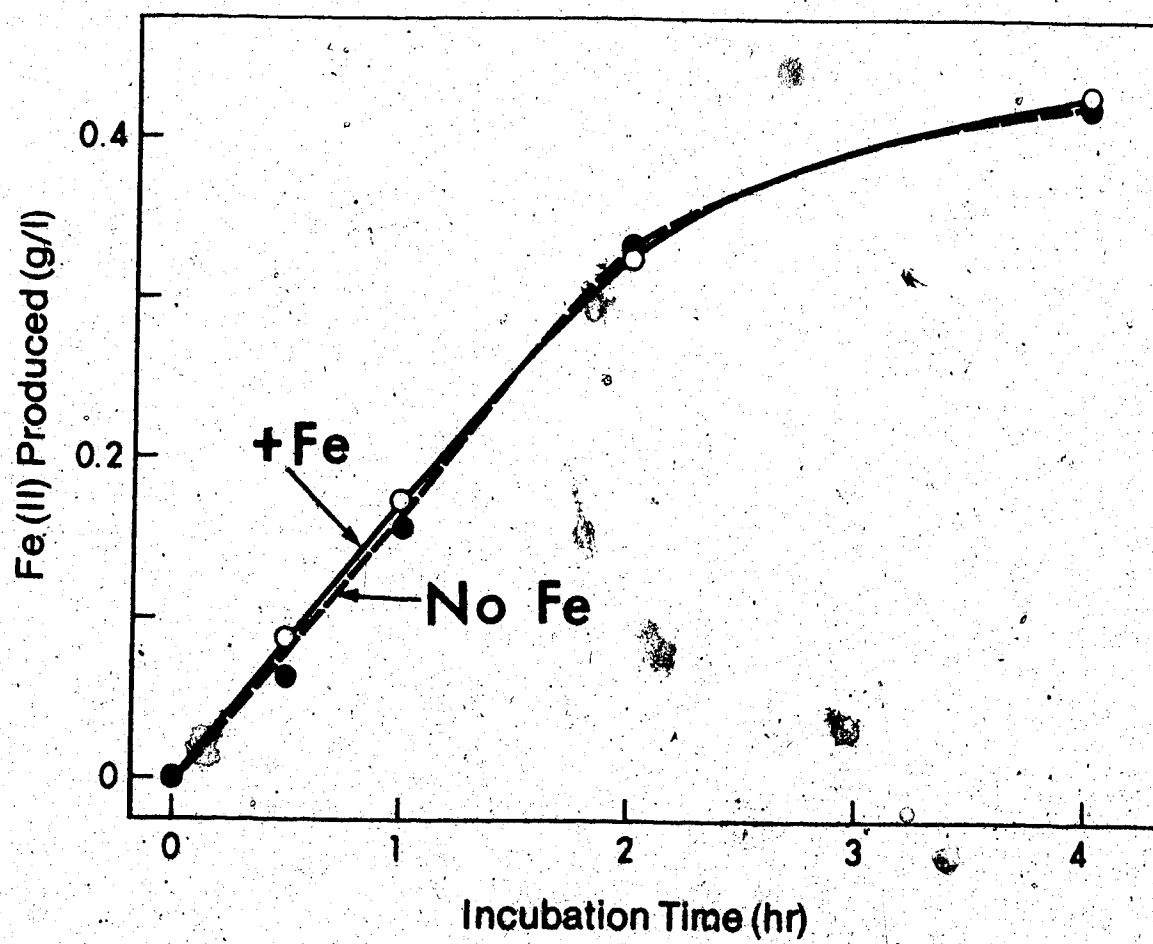


Fig. 16. Fe(III) reduction by cells of Isolate #200 grown in Butlin's medium, with or without added iron.





does not involve changes in biochemical activities, should not be discountenanced.

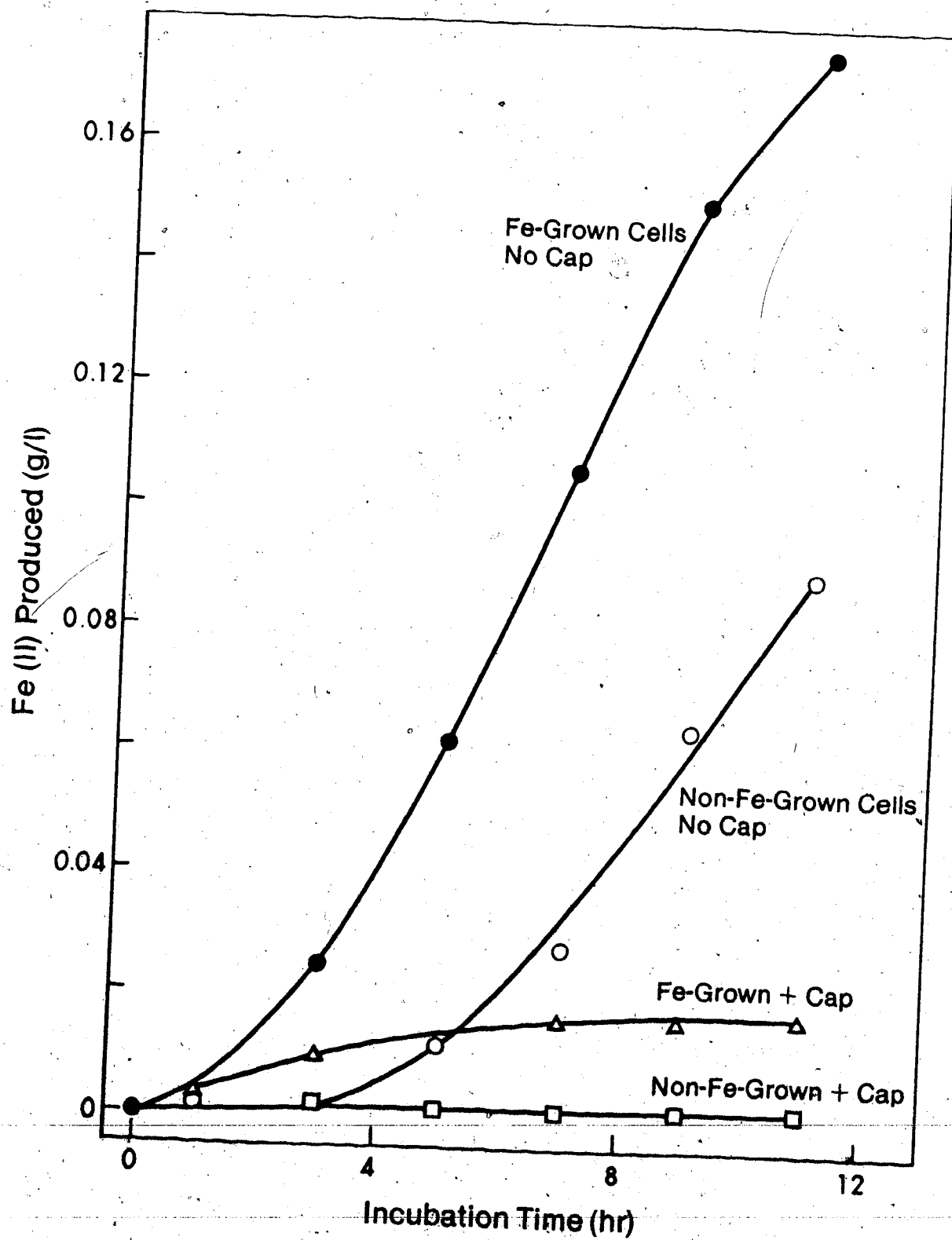
Ferric Iron Reduction by Iron-starved and Non-starved  
Cells in the Presence of Chloramphenicol

Several factors have been suggested as the probable cause of a lag period during Fe(III) reduction by iron-starved cells of Isolate #200. If, however, the observed kinetics of Fe(III) reduction by iron-starved cells did not involve biochemical changes, this kinetics would not be expected to change in the presence of a metabolic inhibitor like a protein synthesis inhibitor. Contrarily, the presence of a potent inhibitor of protein synthesis would be expected to markedly change the kinetics of ferric iron reduction, if it was preceded by a *de novo* synthesis of protein. To investigate these possibilities, chloramphenicol was added to the reaction medium at a final concentration of 0.3 mg/ml. Chloramphenicol inhibits protein synthesis by preventing the initiation of protein synthesis.

The data in Fig. 17 show the effect of chloramphenicol on ferric iron reduction of iron-starved and non-starved cells. The presence of chloramphenicol inhibited Fe(III) reduction by Isolate #200 whether the organism had been previously starved of iron or not. With cells grown in iron-containing medium, about 90% inhibition of Fe(III) reduction was observed at the end of 11 hours of incubation. Again, no lag period occurred. When the cells had been previously starved of iron, chloramphenicol perpetuated the lag phase. The meagre activity observed after long incubation could be ascribed to basal activity present originally in the cells.

Fig. 17. Fe(III) reduction by cells of Isolate #200 in the presence and absence of chloramphenicol (0.3 Mg/ml).

Cells were previously grown in synthetic medium, with or without added Fe.



The possibility that the decrease in Fe(III) reduction was the result of a decline in the original cell population due to possible bacteriocidal effect of chloramphenicol at such a high concentration employed was not supported by data obtained. Table 15 shows the number of cells in the reaction mixture during the first 6 hours of reaction in the presence of chloramphenicol. No change in cell number occurred. However, colonies of the organism incubated for 11 hours in chloramphenicol had a smaller colony diameter.

Since the role of chloramphenicol was to inhibit *de novo* synthesis of proteins by preventing the initiation of synthesis of fresh molecules, it can be concluded that Fe(III) reduction involved the induction of a protein factor which was necessary for the reduction process. The synthesis of this protein was either inducible by iron or that iron was a necessary component of the functional protein. However, the exact nature or role of the protein factor cannot be inferred at this stage. It could be a transport protein (permease), necessary for the transport of Fe(III) to the appropriate reduction site. Also, the protein factor could be the enzyme whose specific function is to reduce Fe(III) to Fe(II) (iron reductase). A third possibility is that of an iron-protein that participates in electron transport to Fe(III) where Fe(III) acts as terminal electron acceptor. The associated loss of pigmentation with iron starvation might support the possibility that this protein factor is an iron-protein conjugate. Many iron-protein compounds are coloured and if this iron-protein molecule is a cytochrome, its absence would be expected to impair electron transport to Fe(III) and a general decline in physiological activities. Peters and Warren (1968) suggested the inducibility of Fe(III) transport protein in *Bacillus subtilis*.

TABLE 15. Counts of number of cells of isolate #200 during Fe(III) reduction in the presence of chloramphenicol<sup>1</sup>

Incubation time (hr)	Cell density ( $\times 10^{-6}/\text{ml}$ )
0	113.7
3	110.0
6	112.0

<sup>1</sup>Incubation was at 30°C. Counts were made on Trypticase Soy Agar plates after 48-hour incubation.

Suspicion that Fe(III) was an inducer was heightened by the observation that the need for induction was noticed in iron-starved cells only.

### Reduction of Fe(III) by Isolated Cell Fractions

#### Enzymatic activities and ferric iron reduction by shocked cells and shock fluids

##### Alkaline phosphatase activity of shock fluid

It has been demonstrated earlier that ferric iron-reducing enzyme(s) was not secreted into the surrounding medium by the Gram-negative bacterium, Isolate #200. However, many enzymatic activities have been associated with the periplasmic fluid of many Gram-negative bacteria (Heppel, 1967; Nossal and Heppel, 1966; Bhatti *et al.*, 1976; Malamy and Horecker, 1960). It was in recognition of this fact that the presence of ferric iron-reducing activity was sought in the periplasmic fluid. *Escherichia coli* C<sub>4</sub>F<sub>1</sub> is constitutive for alkaline phosphatase which is known to be released by osmotic shock (Bhatti *et al.*, 1976). Therefore, alkaline phosphatase activities in *E. coli* C<sub>4</sub>F<sub>1</sub> and Isolate #200 were assayed as marker for the release of periplasmic proteins by the organisms.

Table 16 shows the phosphatase activities of the shock fluid from the marker organism (*E. coli*) and the experimental organism (#200). The level of phosphatase activity in *E. coli* was very high and amounted to about 27 times that in Isolate #200. The low activity obtained in #200 shock fluid would be due to the repression of the enzyme synthesis by the presence of sufficient inorganic phosphate (Torriani and Rothmans, 1960). The *E. coli* mutant was able to produce large amounts of the

TABLE 16. Alkaline phosphatase activities of shock fluids.

Organism	Enzyme activity (enzyme units/ml <sup>1</sup> shock fluid)
<i>Escherichia coli</i> C <sub>4</sub> F <sub>1</sub>	19.6
#200 (C <sub>4</sub> F <sub>1</sub> medium-grown)	0.72
#200 (Butlin's medium-grown)	0.68

<sup>1</sup> One enzyme unit = 1  $\mu$ mole p-nitrophenol produced per hr .

enzyme, the presence of inorganic phosphate notwithstanding. However, the demonstration of alkaline phosphatase activity (albeit low) in the shock fluids clearly indicated that periplasmic protein was released by Isolate #200 in the procedures employed.

#### Ferric iron reduction by shocked cells

The iron-reducing activities of the osmotically shocked cells and shock fluid of Isolate #200 are shown in Fig. 18. This figure depicts the activities obtained with cells grown in Butlin's medium, and those obtained with cells grown in  $C_4F_1$  medium. Whole, untreated cells reduced Fe(III) much better than shocked cells. The osmotic shock resulted in the loss of 30-40% of the iron-reducing capability of the organism. Washed, shocked cells reduced slightly less as much Fe(III) as the reconstituted shocked cells (shocked cells plus shock fluid). The slight difference obtained in their activities might be due to incomplete recovery of cells during washing or that the shocked fluid contained a complementary component.

The decline in the Fe(III)-reducing activity of shocked cells might indicate a possible loss of activity to the shock fluid. However, the results obtained did not support this view; no activity was found in the shock fluid. It could be surmised from this observation that either there was no Fe(III)-reducing activity (enzymatic) in the periplasmic space, or that the enzyme, if present, had to maintain a specific conformation which was disrupted by osmotic shock. Another possibility was that the decline in ferric iron reduction by shocked cells was the result of a partial loss of transport protein. Such a partial loss of a transport protein would necessarily cause a decline



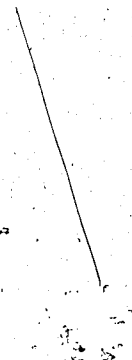
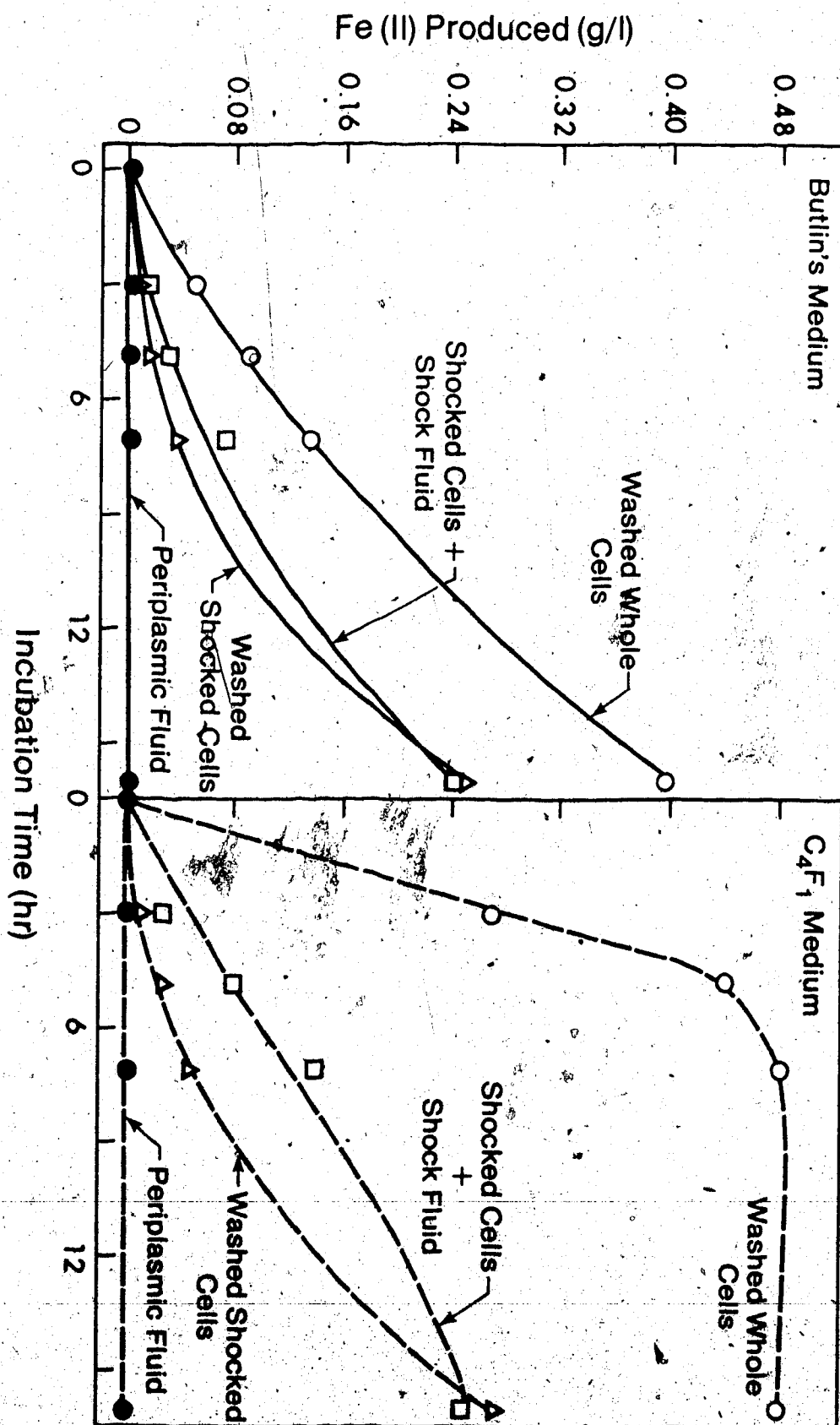


Fig. 18. Fe(III) reduction by cells, osmotically shocked cells and periplasmic fluid (shock fluid) of Isolate #200.



in the amount of Fe(III) available to the organism to reduce. Since the reconstitution of the shocked cells with the periplasmic (shock) fluid did improve only slightly the cells' activity, it suggested that a stringent conformational relationship between the cells and the lost material (*i.e.* material in the shocked fluid) was necessary for transport or enzymatic reduction to occur. Partial loss of transport protein by osmotic shock have been observed in bacteria. Kundig *et al.* (1966) reported a reduction in the transport of glycosides in *E. coli* by osmotic shock. However, the transport activity was subsequently regained by incubation with the cell extract. Also, osmotic shock led to a partial loss of an *E. coli* strain to take up several acids from solution (Piperno and Oxender, 1966). In *Salmonella typhimurium*, osmotic shock caused the release of sulphate iron transport factor (Pardee *et al.*, 1966). On the other hand, Heppel (1967) noted that a general increase of cell sensitivity to adverse environment and decline in overall cell activity often accompany osmotic shock. This decline, rather than the loss of specific factor, might well account for the partial loss of Fe(III)-reducing ability with osmotic shocking. However, the slight increase in activity of the reconstituted shocked cells has made the loss of a factor the more likely reason.

#### Ferric iron reduction by spheroplast preparations

Spheroplasts are cell fractions remaining after the cell envelope has been partially removed by the action of lysozyme. It was hoped that by gradual dissolution of the cell, the loss of activity with each fractionation procedure will help identify the cell component.

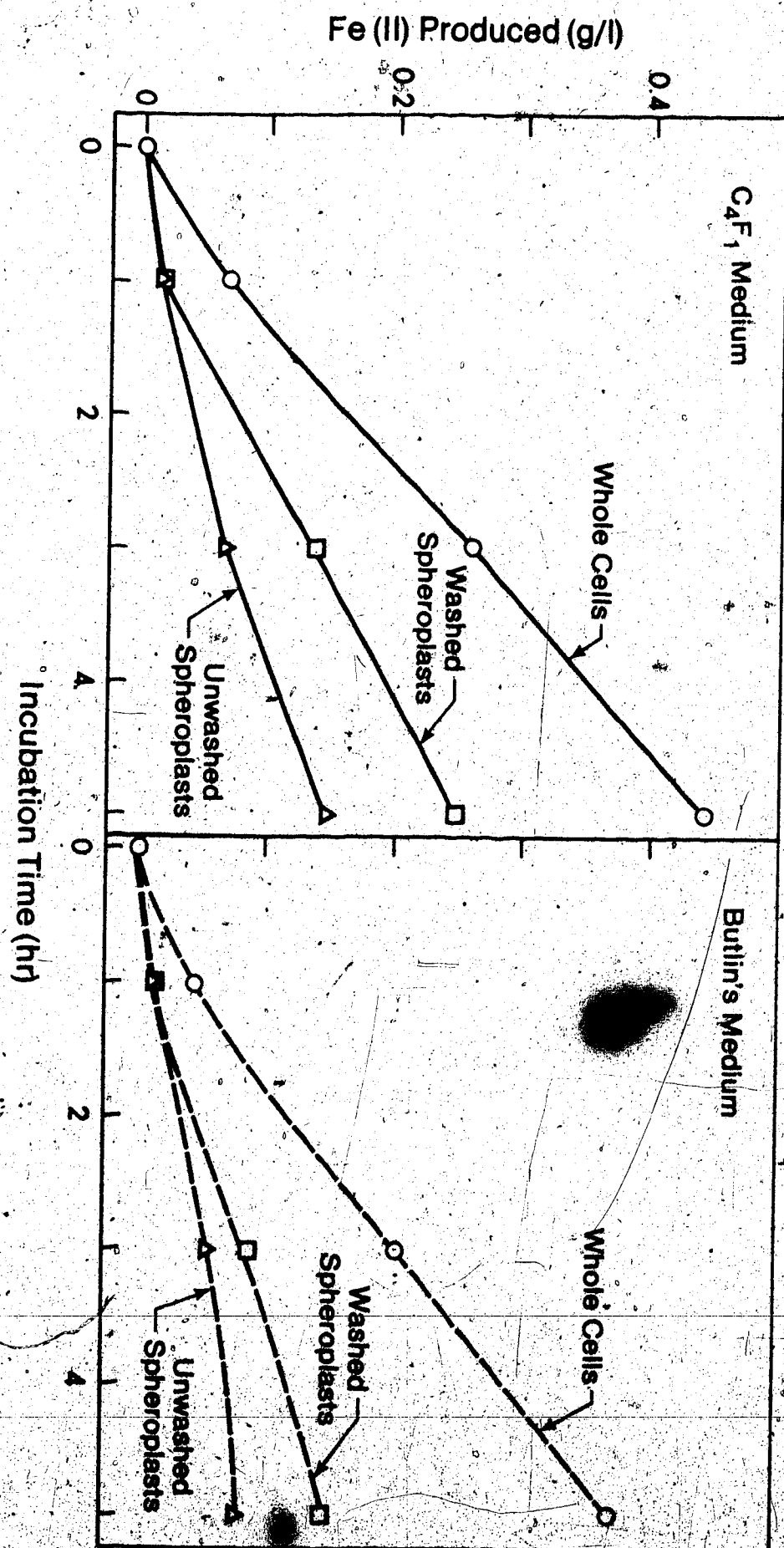
Initial attempts at spheroplast preparations were carried out

using cells grown in  $C_4F_1$  medium (mineral salts + yeast extract and nutrient broth; Appendix 1c) and the Tris-HCl buffer system as washing and stabilizing medium. No ferric iron reduction occurred with such spheroplast preparation. As a result, a fresh attempt was made using cell samples grown in  $C_4F_1$  and Butlin's media, although previous work had shown that greater activity was present in  $C_4F_1$ -grown cells. Also, because there was no activity in the equilibrated spheroplasts prepared previously, in subsequent preparation the iron-reducing activities at the various stages of spheroplast preparation were monitored. This was to check whether the absence of activity in the final product was due to a loss at a particular stage of specimen preparation. Again, no ferric iron-reducing activity was observed at all stages of spheroplast preparation except with the untreated whole cells. So, the treatment with lysozyme and EDTA-Tris buffer abolished the ferric iron-reducing ability of the cells.

In a previous experiment, it was reported that whole cells washed and suspended in Tris-HCl buffer soon partially lost their iron-reducing capability. It was thought, therefore, that Tris-HCl buffer impaired the cells' ability to reduce  $Fe(III)$  and was probably responsible for the loss of activity in spheroplast preparations. As a result, it was considered necessary to substitute the Tris-HCl buffer with 0.1 M phosphate as the washing and the stabilizing medium. The results of iron-reduction by spheroplasts prepared in the phosphate buffer system is compared to that of the whole cells in Fig. 19. Spheroplast preparations, whether grown in  $C_4F_1$  or Butlin's were able to reduce ferric to ferrous compounds. However, the spheroplast preparations reduced much less  $Fe(III)$  than whole cells. Losses of about 44 and 60% of the

Fig. 19. Fe(III) reduction by whole cells and spheroplast preparations from Isolate #200.

o, whole cells; x, spheroplast preparation washed and stabilized in 20% sucrose- $5 \times 10^{-4}$  M  $\text{MgCl}_2$ - $1 \times 10^{-4}$  M phosphate buffer; o, unwashed spheroplast preparation (*i.e.* suspended in 20% sucrose-EDTA-lysozyme mixture).



activities, compared to the whole cells, were observed in spheroplasts prepared from cells originally grown in  $C_4F_1$  and Butlin's media, respectively. When washed and resuspended in stabilizing sucrose- $MgCl_2$ -phosphate solution, the spheroplasts showed a much higher activity than that obtained with unwashed preparations. Either the presence of lysozyme and EDTA inhibited the activity or that  $MgCl_2$  enhanced the spheroplast activity. The latter suggested was a more likely situation. Nossal and Heppel (1966) reported an increase in viability of treated cells in the presence of  $Mg^{2+}$ .

Phase contract microscopy revealed that the spheroplast preparation contained cells which had assumed various shapes. There was no obvious spherical spheroplasts as would be expected in lysozyme treatment of sensitive Gram-positive bacteria (protoplasts). Some of the cells still maintained their regular shapes and no obvious change was noticeable. However, there was a tendency of the treated cells to clump. Obviously this clumping tendency indicated that the lysozyme treatment had wrought some change in the cell envelope. Dilution (1:10) of the lysozyme-treated cells in distilled water yielded only 14.3% decrease in optical density compared to untreated cells. Thus, about 14% of the cell population was modified enough to make them osmotically fragile. It was possible, therefore, that lysozyme treatment was effective only on a fraction of the original cell population. If this were so, then the ferric iron reduction observed would be due, in part or wholly, to the activity of the unmodified cells. Direct plating of the lysozyme-treated cells resulted in very profuse growth on PCA plates after 48-hour incubation at  $30^\circ C$ . However, the proportion of the viable cells present in the preparation before and after the enzyme

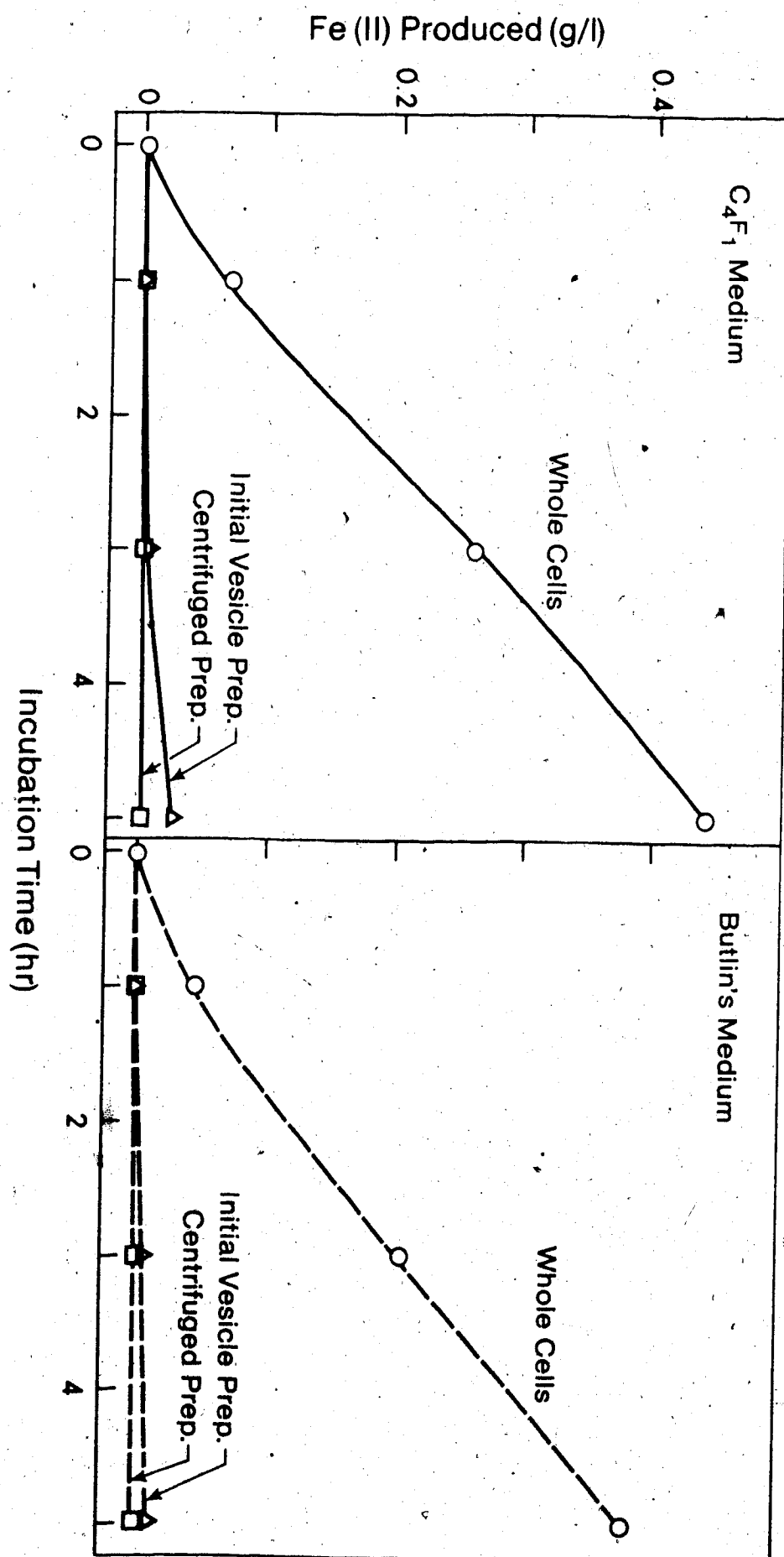
treatment was not determined. Since spheroplasts are capable of regenerating the lost cell wall and growing when transferred to lysozyme-free medium, it was not possible to ascribe the growth only to unaffected cells. The tendency to clump and the fact that spheroplasts maintain, at least, part of their cell wall made it difficult to separate affected and unaffected cells by centrifugation or delineate them morphologically. Since the exposure of lysozyme-EDTA-treated cells to Tris-HCl buffer led to a complete loss of iron-reducing activity, but only partially so with whole cells, it must be concluded that the lysozyme-EDTA treatment actually affected and modified all the cells. So the treated cells were susceptible to the deleterious Tris-HCl effect because the cell wall was impaired. Therefore, the reduction of ferric iron as observed in this experiment must be due to the spheroplasts and not just the unaffected cells.

#### Ferric iron reduction by membrane vesicle

The reduction of ferric iron by membrane vesicle prepared by lysis of spheroplasts in  $MgCl_2$  solution closely resembled that obtained with the spheroplasts. The vesicles prepared in Tris-HCl buffer system failed to reduce  $Fe(III)$ . When, however, preparations were made in phosphate buffer system, an apparent activity was observed (Fig. 20). This activity was lost when the final preparation was centrifuged at 3000  $g$  for 5 min. This treatment was enough to sediment contaminating whole or unlysed cells while leaving the vesicle still in suspension. Plating a loopful of the initial vesicle preparation (before centrifugation) on  $B_{10}$  medium gave rise to growth of colonial types characteristic of Isolate #200. Therefore, the membrane preparation lacked ferric



Fig. 20. Fe(III) reduction by whole cells and membrane (vesicle) preparations from Isolate #200. Centrifugation of the vesicle preparation was at 3000 *g* x 5 min.



iron-reducing activity and appeared to do so only because the initial preparation was contaminated with unlysed cells (spheroplasts). In an independent work, no ferric iron-reducing activity was associated with membrane preparations from a similar organism (Halasa, personal communication). This observation differs from that of Lascelles and Burke (1978) who reported Fe(III) reduction by membrane preparations from *Staphylococcus aureus*.

#### Ferric iron reduction by cytoplasmic content

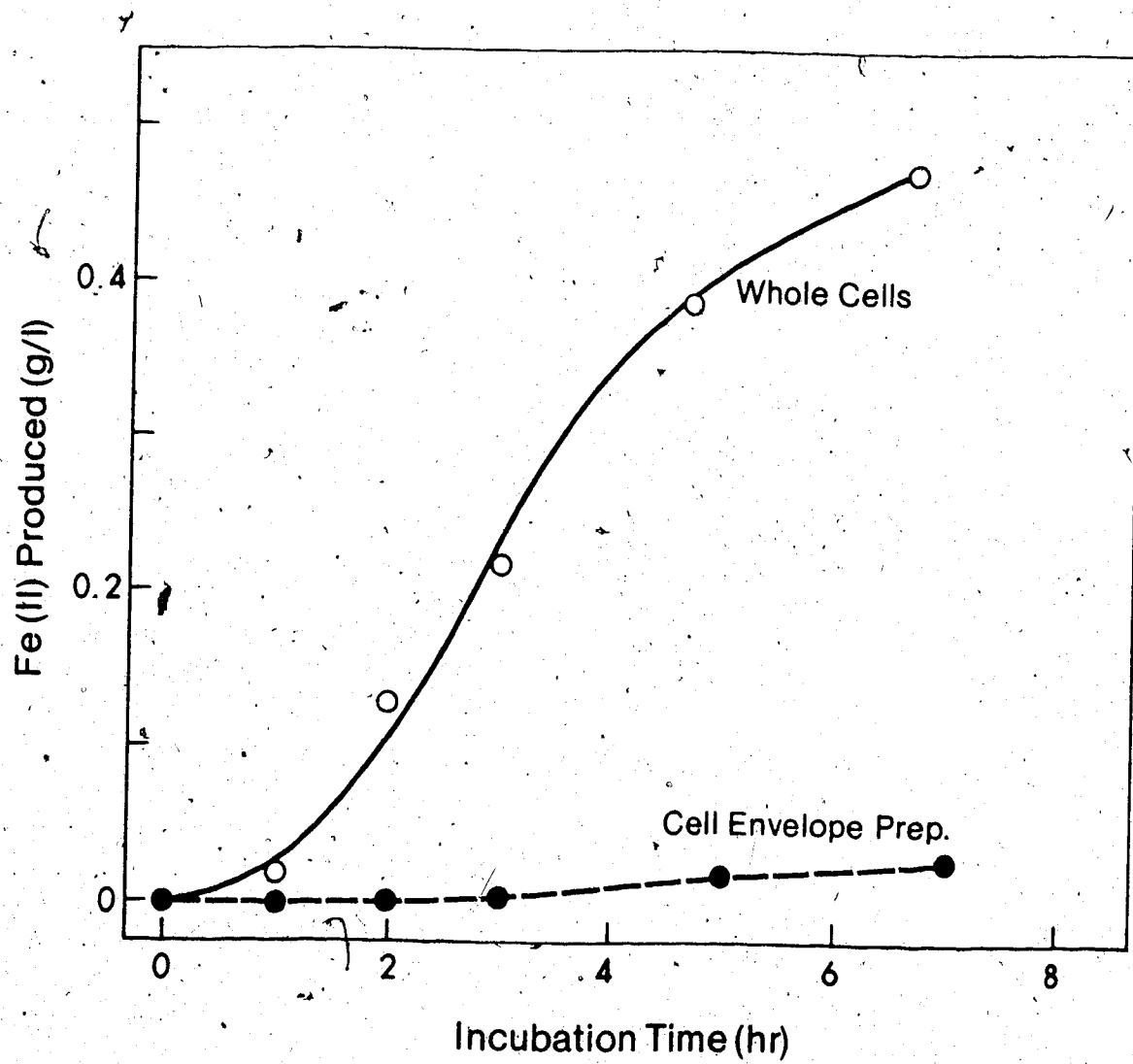
The cytoplasmic content of Isolate #200 prepared by lysis of the spheroplasts lacked any ferric iron-reducing activity.

#### Ferric iron reduction by cell envelope preparation

The reduction of Fe(III) by cell envelope preparations compared to untreated cells is shown in Fig. 21. Only about 5% of the original cell activity was obtained with cell envelope preparation. This activity, insignificant when compared to whole cell suspension, would indicate that cell envelope preparation of this organism did not contain the functional iron-reducing system. This view was supported by the observation that plating the cell envelope preparation revealed the existence of viable cells. Several attempts by low speed centrifugation of the resuspended preparation failed to yield a produce devoid of contaminating cells (viable cells). It was possible, therefore, that the low activity present in the cell envelope preparation was actually due to a small number of contaminating whole cells.

The above conclusion presupposed that all the starting cell material was wholly converted to the envelope and that there was no

Fig. 21. Fe(III) reduction by whole cells and cell envelope  
preparation from Isolate #200.



loss of material in course of the preparation process. This was not likely to be so. The initial differential centrifugation at 3020  $g$  for 5 min yielded a pellet which was definitely unbroken cells. So, only a fraction of the starting cell suspension yields the cell envelope preparation used in the Fe(III) reduction reaction. Thus, the comparatively low activity associated with the envelope preparation could be due to the very small portion of the original starting material that was actually present in the final preparation. The problem of the iron-reducing ability of cell envelope preparations was compounded by the presence of the contaminating cells which could not be gotten rid of despite repeated low speed centrifugations. It was not possible, therefore, to ascribe exclusively and unequivocally the observed activity to either the contaminating cells or to the cell envelope which represented only a fraction of material present in the starting material.

In the case of the isolated membranes, it was possible to conclude that no activity resided in the product. Although the loss of cell material in the course of material preparation could account for a case of low activity when compared to whole cells, it could not explain the absence of any activity when the membrane preparation was free of the contaminating cells. If the membrane preparations from Isolate #200 were actually effective in Fe(III) reduction, a small activity would necessarily be present in the preparations, the loss of material during the preparative steps notwithstanding. The problem of arriving at any conclusion from this work regarding the ability of cell envelope (membrane plus cell wall) to reduce ferric iron has been mentioned earlier. The work of Tano and Lundgren (1978) showed that considerable

enzymatic activity might be present in the bacterial cell envelope.

Relationship Between Cytochrome Content and Fe(III)-reducing  
Ability of the Cells

It was observed that cells which showed deeper orange coloration (probably due to pigment formations) reduced more Fe(III) than the cells which lacked or showed little of such coloration. The possibility that the coloration was due to the cytochrome content was also suggested. However, any such relationship between cytochrome content and the ability of the cells to reduce Fe(III) may be indicated by actually demonstrating that the cells which showed much deeper coloration (and reduced greater amount of Fe(III)) did indeed contain more cytochromes (qualitatively and/or quantitatively).

Although the spectral characteristics of different cytochromes may overlap and make them difficult to differentiate, the different components may have different absorption maxima at different wavelengths depending on whether the cytochromes are in the oxidized or reduced state. Moreover, in the reduced or oxidized condition differential reactivity with certain respiratory inhibitors, like carbon monoxide, could cause spectral shifts, or the abolition of certain absorption peaks while at the same time increasing the absorbance of other components. Such differential reactivity and absorption characteristics among the different cytochromes could be used to differentiate between the different cytochromes and identify their presence (Keilin and Hartree, 1939).

## Absorption Spectra of Washed Whole Cells of Isolate #200

### Absolute absorption spectra of the cells

The absolute absorption spectra of washed cells of Isolate #200 are shown in Figs. 22, 23 and 24. The data in Fig. 22 show the absorption spectra of untreated whole cells, while Figs. 23 and 24 show the absolute absorption of whole cells oxidized and reduced, respectively. As is evident from Figs. 22 and 23, the cells grown in the rich medium ( $C_4F_1$  medium) also showed the greatest total absorption especially in the Soret band. The Soret band is the characteristic absorption of tetrapyrrole in the region of 400-450 nm. Cells grown in the synthetic medium lacking added iron showed the least absorption. In the reduced state, the cells grown in  $C_4F_1$  medium ( $C_4F_1$  medium, spectrum 1) showed three prominent peaks at 552 nm, 522 nm and one at 420 nm at the Soret region. These absorption peaks are characteristic of cytochrome type C. No such absorption maxima were evident in the cells grown in the synthetic medium, with or without added iron (Fig. 24, spectra 2 and 3, respectively).

### Reduced-minus-oxidized spectra

The reduced-minus-oxidized difference spectra for Isolate #200 grown in different media are shown in Fig. 25. These spectra contained prominent peaks at 552, 510-530 and 420 nm. However, these peaks were observed only in cells grown in the rich  $C_4F_1$  medium (Fig. 25, spectrum 1) and in synthetic medium containing iron (Fig. 25, spectrum 3). Cells grown in the synthetic medium lacking iron (Fig. 25, spectrum 2) did not absorb within the range of wavelengths employed. It can be



Fig. 22. Absolute absorption spectra of untreated cells of Isolate #200.

Cell concentration = 0.25 g wet wt./80 ml buffer.

1,  $C_4E_1$  grown cells; 2, cells grown in synthetic medium + iron; 3, cells grown in synthetic medium without added iron.

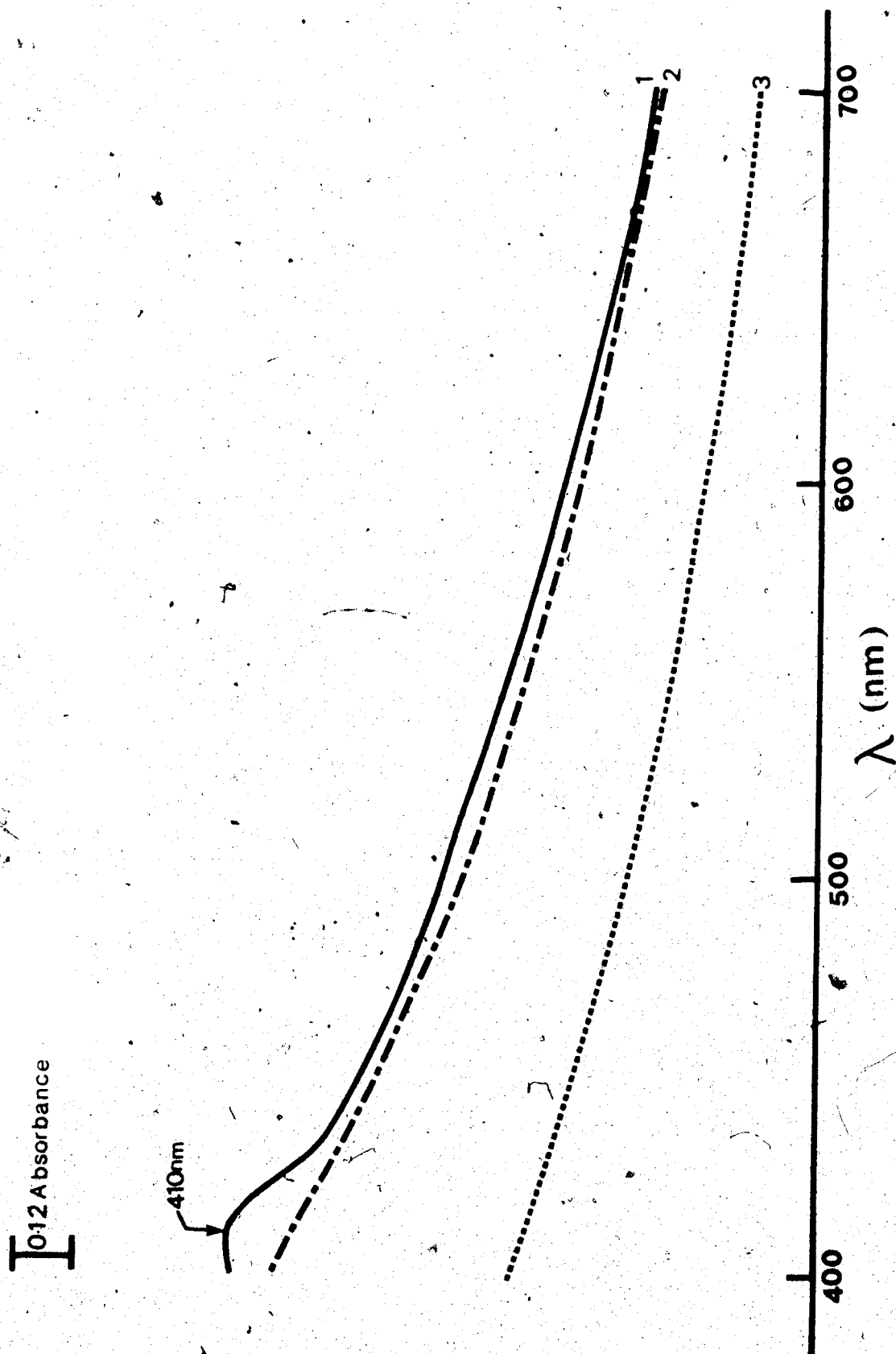


Fig. 23. Absolute absorption spectra of oxidized cells of Isolate #200.

1, cells grown in  $C_4F_1$  medium; 2, cells grown in synthetic medium + iron; 3, cells grown in synthetic medium without added iron.

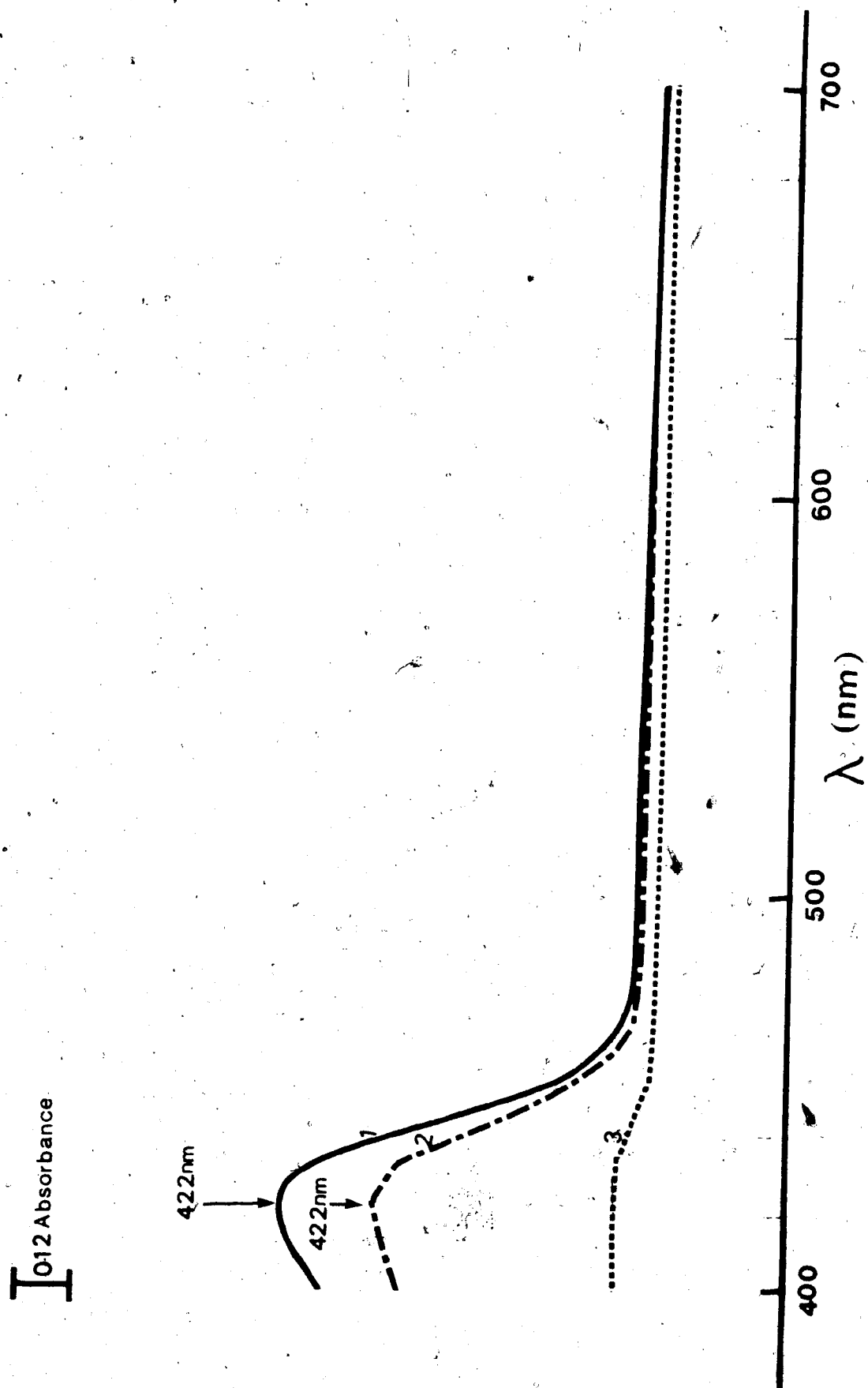


Fig. 24. Absolute absorption spectra of reduced whole cells of Isolate #200.

1, C<sub>4</sub>F<sub>1</sub> grown cells; 2, cells grown in synthetic medium + iron; 3, cells grown in synthetic medium without added iron.

0.12 Absorbance

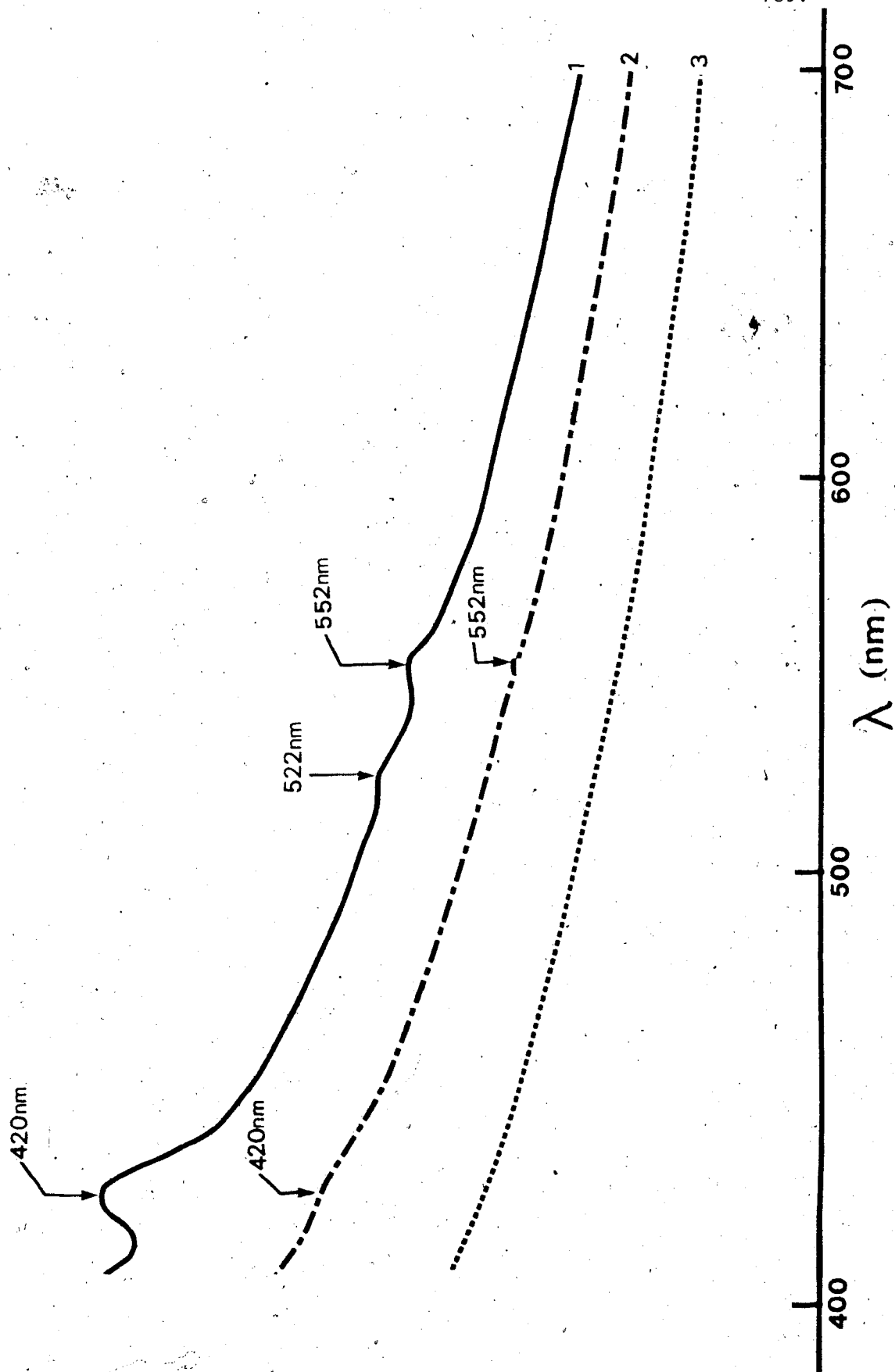
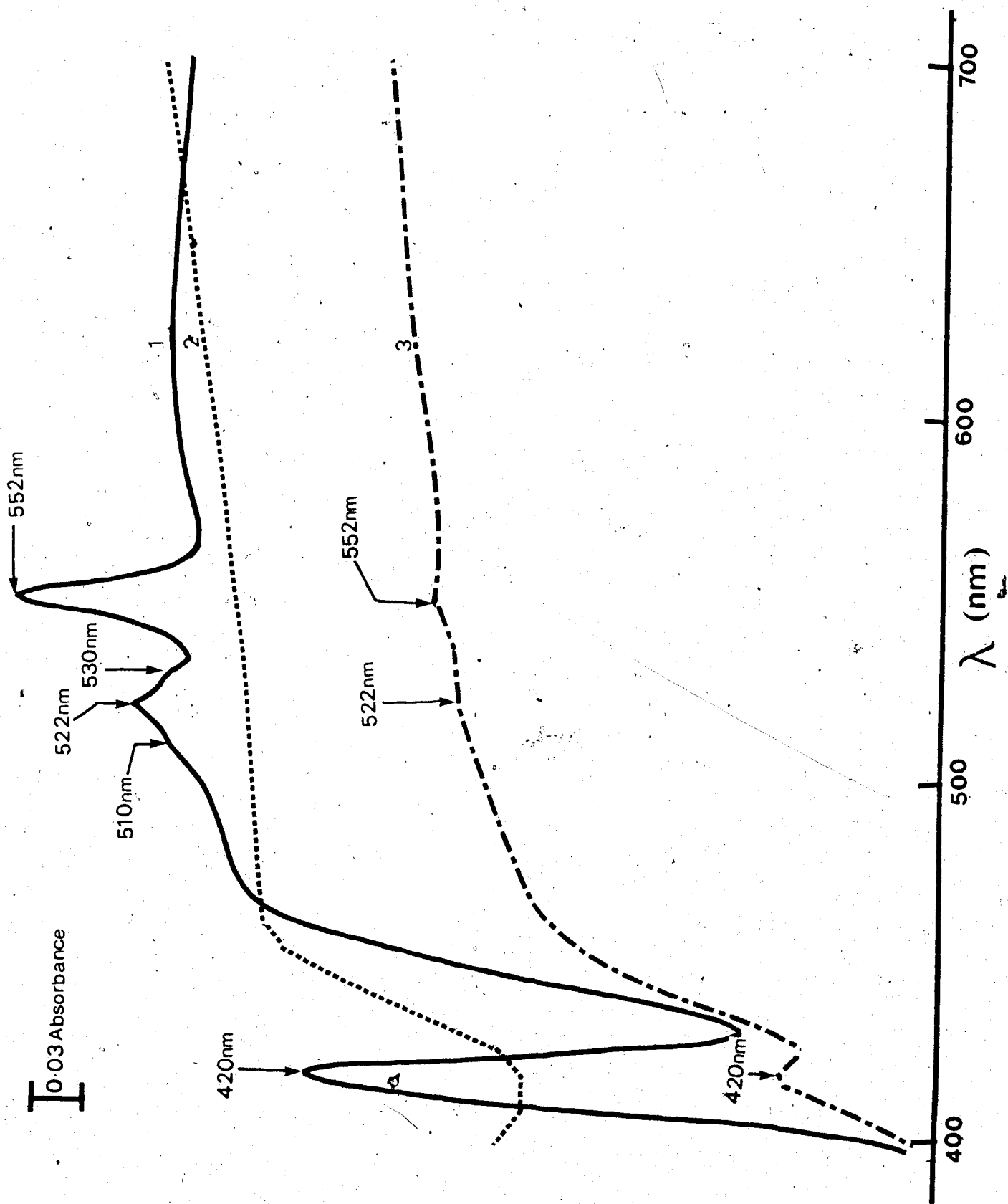


Fig. 25. Reduced minus oxidized cytochrome difference spectra of cells of Isolate #200.

1, cells grown in  $C_4F_1$  medium; 2, cells grown in synthetic medium without added iron; 3, cells grown in synthetic medium + iron.





inferred, therefore, that iron starvation prevented the synthesis of the absorbing substance(s) in Isolate #200 and would indicate that iron was necessary for the synthesis of such component(s).

Although the absorption peaks for  $C_4F_1$ -grown and the synthetic medium-plus-iron-grown cells were generally similar, some qualitative as well as quantitative differences occurred. The cells grown in rich medium ( $C_4F_1$ ) contained absorption peaks 10 times higher than those grown in the synthetic media. Thus, there was a greater synthesis of the colour-imparting substance(s) in rich than in the synthetic media. This result was in accordance with what was observed visually and further supported by the absolute absorption spectra obtained with untreated cells (Fig. 22), oxidized cells (Fig. 23) and reduced cells (Fig. 24). Two shoulders of absorption (Fig. 25, spectrum 1) occurred in 510-530 nm range in the  $C_4F_1$ -grown cells but were absent in cells grown in the synthetic media. Thus, while cells grown in synthetic-plus-iron medium showed a single peak at 522 nm, three peaks at 510, 522 and 530 nm were observed in  $C_4F_1$ -grown cells.

The predominance of absorption peaks of reduced-minus-oxidized spectra (Fig. 25, spectrum 1) in the region of 522, 552 and 420 nm would indicate the presence of C-type cytochrome. A similar absorption pattern in *Desulfovibrio* sp. was considered characteristic of C-type cytochrome by Postgate and Campbell (1966) and Jones (1972). The presence of absorption shoulders around 522 nm in  $C_4F_1$ -grown cells probably indicated that more than one form of cytochrome C was elaborated under the cultural condition or due to overlap of absorption by cytochrome b (Stanier *et al.*, 1966). On the other hand, the broad peak in the region of 570-670 nm, if real, might indicate the presence

of a-type cytochrome, too.

### Carbon Monoxide-reacted Difference Spectra

#### Reduced-plus-carbon monoxide minus reduced difference spectra

The difference spectra between carbon monoxide-treated reduced cells and reduced cells of Isolate #200 are shown in Fig. 26. The spectra obtained with C<sub>4</sub>F<sub>1</sub>-grown cells were similar to those obtained with cells grown in synthetic medium containing iron, except that the absorption peaks were higher in cells grown in the more complex medium. No absorption peaks or troughs were evident with cells grown in the synthetic medium that lacked iron. The observed absorption maxima at 410, 537 and 567 nm and minima at 425, 522 and 552 nm corresponded to those reported as carbon monoxide-reactable C-type cytochrome in *Desulfovibrio gigas* NCIB 9332 by Jones (1972), *Desulfovibrio africanus* (Jones, 1971) and Stanier *et al.* (1966) for *Pseudomonas* spp.

#### Reduced-plus-carbon monoxide minus oxidized difference spectra

No absorption peaks or troughs were observed with cells grown in iron-free synthetic medium (Fig. 27, spectrum 2). With cells grown in C<sub>4</sub>F<sub>1</sub> medium prominent peaks occurred at 522, 530 and 558 nm (Fig. 19, spectrum 2), while a broad absorption peak occurred in the region of 620-650 nm, with maximum at 638. Only a single peak at 558 nm was evident in cells grown in the iron-containing synthetic medium (Fig. 27, spectrum 3). Absorptions at 558 nm and 530 nm are considered characteristic of b-type cytochrome and have been specifically observed in *P. maltophilia* (Stanier *et al.*, 1966), while that at 638 nm

Fig. 26. Reduced plus CO minus reduced cytochrome difference spectra of cells of Isolate #200.

1, cells grown in synthetic medium without added iron; 2, cells grown in  $C_4F_1$  medium; 3, cells grown in synthetic medium + iron.

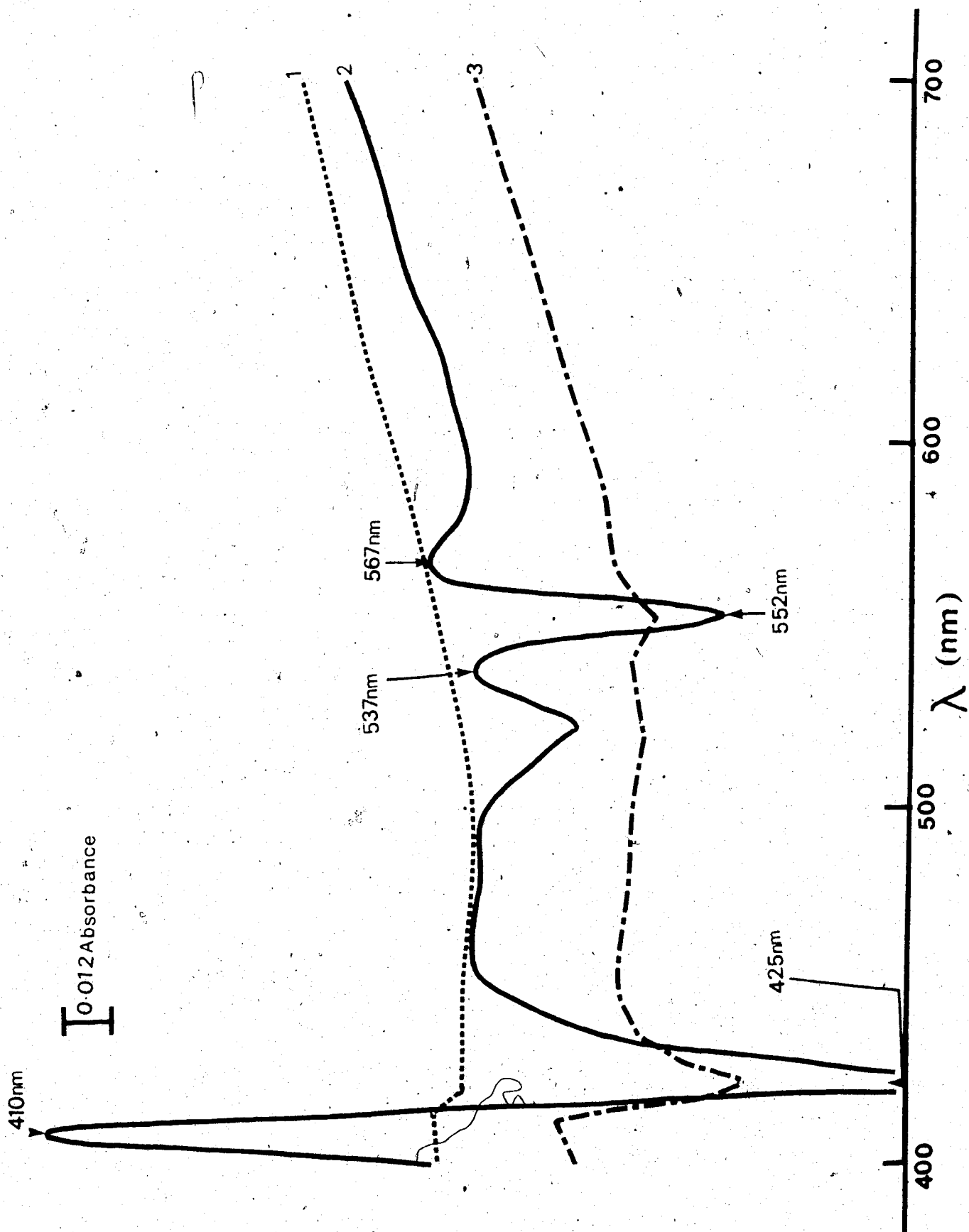
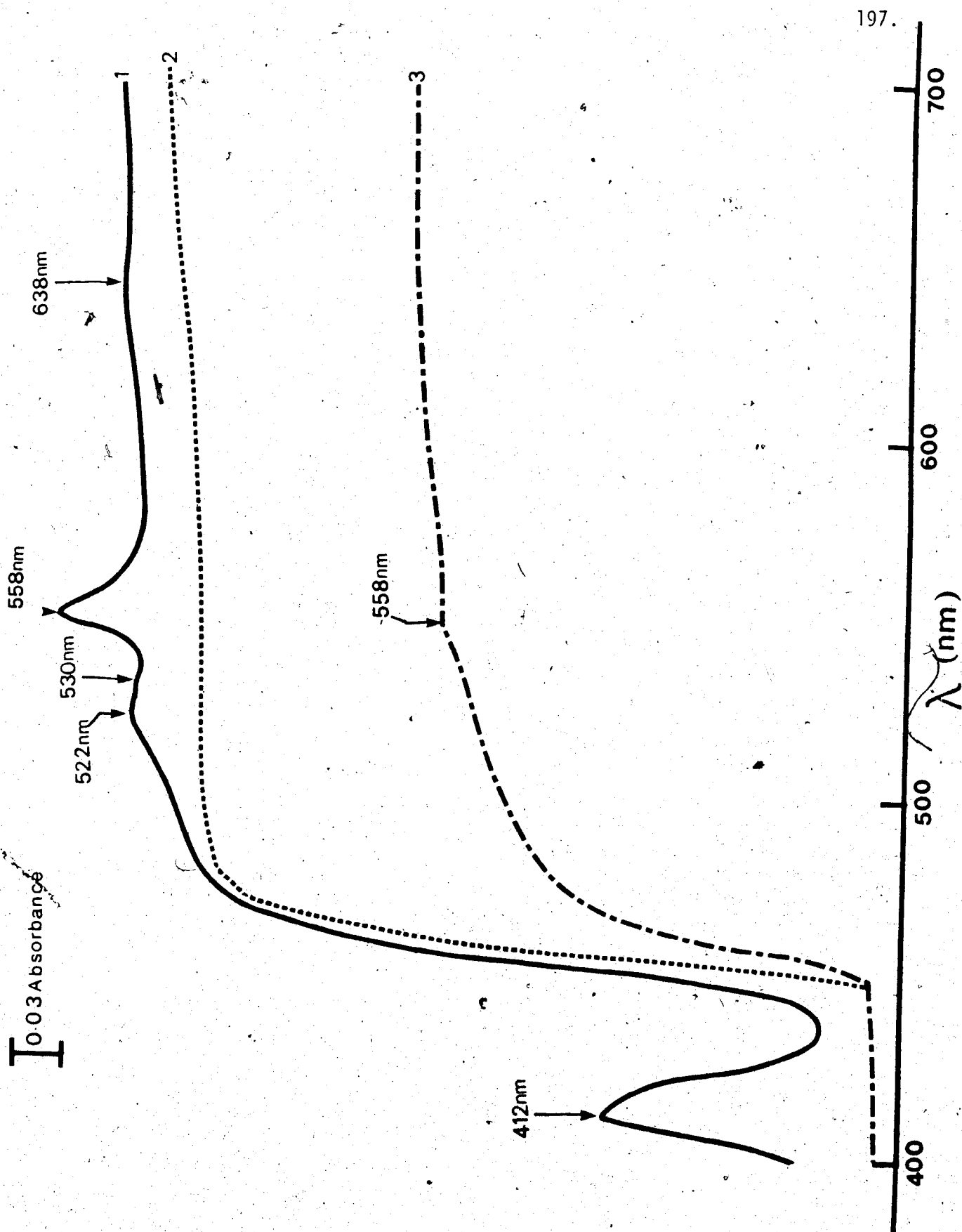


Fig. 27. Reduced plus CO minus oxidized cytochrome difference spectra of cells of Isolate #200.

1, C<sub>4</sub>F<sub>1</sub> medium-grown cells; 2, cells grown in synthetic medium without added iron; 3, cells grown in synthetic medium + iron.



has been associated with cytochrome  $a_2$  in *Azotobacter* sp. (White and Sinclair, 1971).

Evidence obtained from these studies on the cytochrome spectra of Isolate #200 indicated that this organism was capable of elaborating a variety of cytochromes. However, this capability was affected by medium composition and inhibited to non-detectable levels by the absence of iron, a component of the molecule. The wide variation in cytochrome composition in response to different growth conditions had been pointed out by White (1962).

An attempt has been made to identify the reported absorption peaks. The identity of Isolate #200 is not yet known with certainty, so the observed spectra could not be compared directly with any previously observed in the same organism. Because of this, the identity of the peaks were inferred from work published on other organisms, especially *Desulfovibrio* spp., *Azotobacter* spp., and *Pseudomonas* spp. Considerable overlap of spectra of components of bacterial cytochrome system is known to occur. For example, absorption maximum at 560 nm traditionally assigned to cytochrome b is known to be complicated by contribution by cytochrome o, and a 25% contribution has been reported by White and Sinclair (1971).

The significance of the result on cytochrome spectra studies is not on the identification of the various components but in the demonstration of the existence of such different components and their differences in cells grown under various conditions. Associated with the observed variations in cytochrome composition was the ability of the cells to reduce ferric iron. Thus, the  $C_4F_1$ -grown cells which had the greatest amounts and variety of components had the highest capacity

to reduce ferric iron. On the other hand, cells grown in iron-free medium, with their non-detectable cytochrome content, exhibited very low initial Fe(III)-reducing ability. It can, therefore, be inferred that a direct relationship exists between the cells' cytochrome content and the capacity to reduce ferric iron.

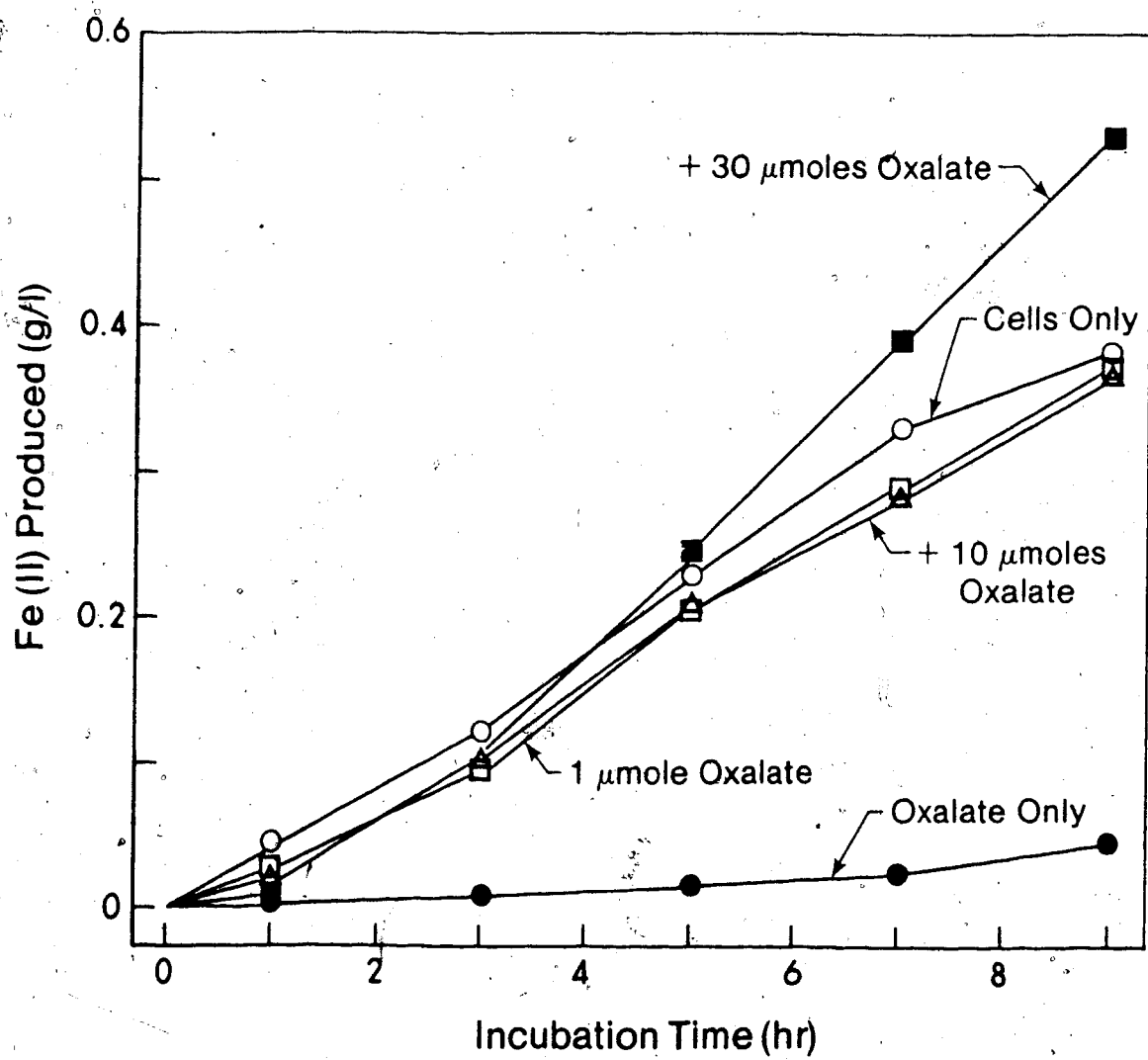
#### Reduction of Ferric Iron in the Presence of Electron Transport Inhibitors

A relationship between ferric iron reduction and cytochrome composition was suggested by results obtained in spectral studies. Since the cytochromes are known to be involved in electron transport, studies with specific electron inhibitors would confirm the involvement, or otherwise, of electron transfer by way of cytochromes to Fe(III). For these studies, 2-n-heptyl-4-hydroxyquinoline-N-oxide (HQNO), sodium amytal, sodium oxalate, carbon monoxide and sodium cyanide, whose specificities of electron transfer inhibition have been reported (Cox *et al.*, 1970; Konings, 1977; Lascelles and Burke, 1978) were tested as potential inhibitors of Fe(III) reduction.

The data shown in Fig. 28 indicate the effect of oxalate on Fe(III) reduction by Isolate #200. Oxalate did not inhibit ferric iron reduction and at higher concentration appeared to increase it. Sodium oxalate was reported to be a specific inhibitor of D-lactate dehydrogenase in *E. coli* (Konings, 1977) and was shown to inhibit ferric iron reduction in membrane preparations from *Staphylococcus aureus* (Lascelles and Burke, 1978). It is evident from the result obtained that the lactate dehydrogenase of Isolate #200 was not inhibited by oxalate. Indeed, oxalate alone could serve as an electron donor, albeit poorly,



Fig. 28. Effect of sodium oxalate on Fe(III) reduction by cells of Isolate #200.



for Fe(III) reduction.

Sodium amytal, HQNO and cyanide individually inhibited the reduction of Fe(III) (Fig. 29). The degree of inhibition, however, differed markedly with these inhibitors. After 9 hr of incubation, sodium amytal inhibited Fe(III) reduction by 9%; HQNO, 30% and sodium cyanide, 49%. HQNO inhibits electron transport from cytochrome b. Thus, the inhibitory effect of HQNO indicated the participation of cytochrome b in the transfer of electrons to Fe(III). Sodium amytal is reported to inhibit electron transfer to cytochrome b (Cox *et al.*, 1970), and in *E. coli* the location of amytal-sensitive site in the respiratory chain was in the flavin group between D-lactate dehydrogenase and cytochrome b (Konings, 1977). The poor inhibitory activity of sodium amytal and the absence of any inhibition with oxalate might indicate that electron transfer to Fe(III) is branched at the point of lactate dehydrogenase activity. It is also possible that the lactate dehydrogenase system of Isolate #200 is inately insensitive to these compounds. Lascelles and Burke (1978) reported that oxalate inhibited Fe(III) reduction by membrane preparation from *S. aureus*, but HQNO did not but actually appeared to increase iron reduction. This observation is contrary to what was observed in whole cells of Isolate #200. Assuming that membrane preparations respond in a similar manner as the whole cells from which they were derived, then *S. aureus*, as reported by Lascelles and Burke (1978) must have a (pattern of electron transfer for Fe(III) reduction) mechanism of Fe(III) reduction fundamentally different from what was obtained in Isolate #200. Such differences are consistent with Fig. 30 intended to explain the difference in the electron transfer pathways in the two organisms.

Fig. 29. Effect of electron transport inhibitors on Fe(III) reduction  
by Isolate #200.

S

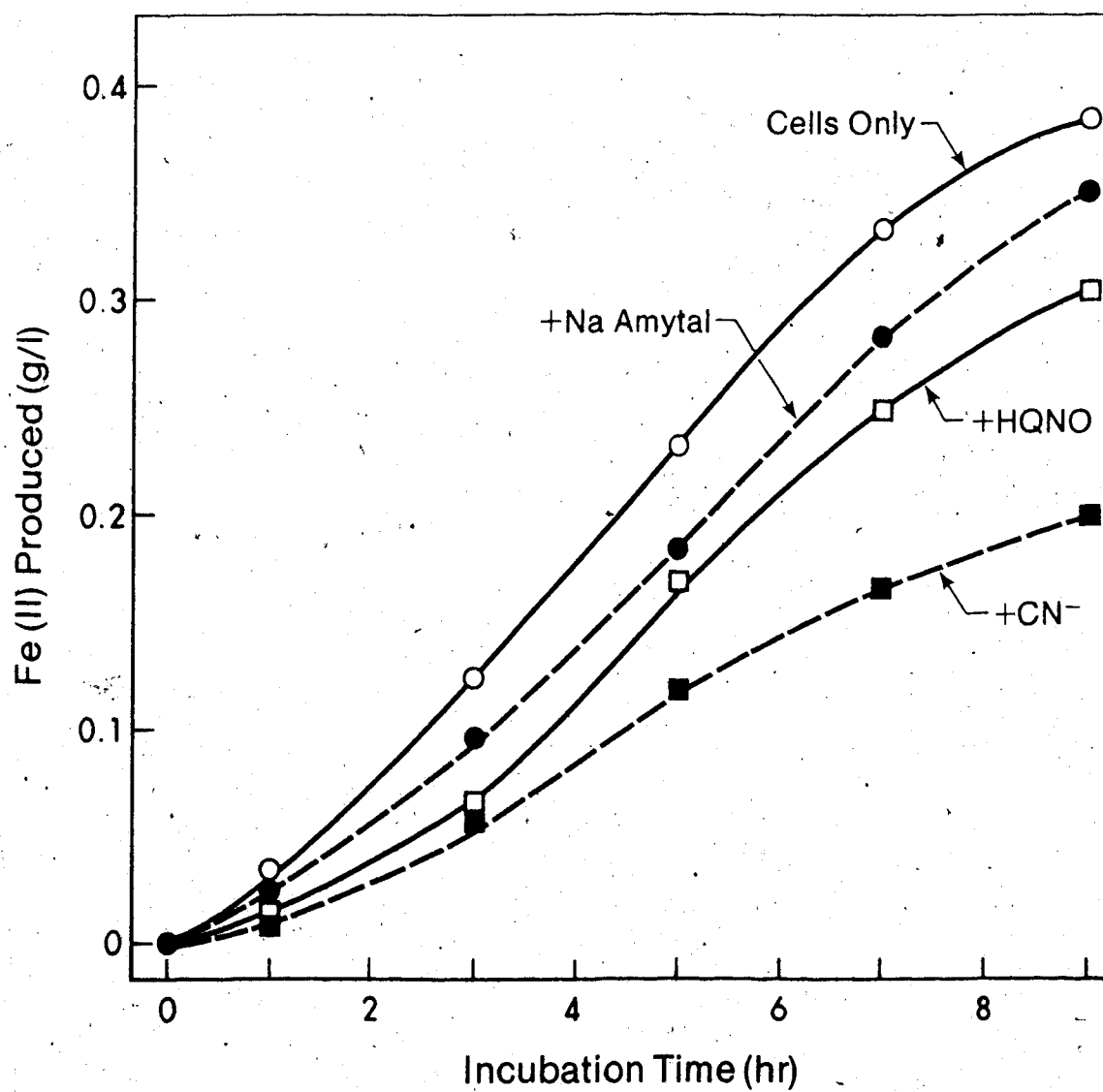
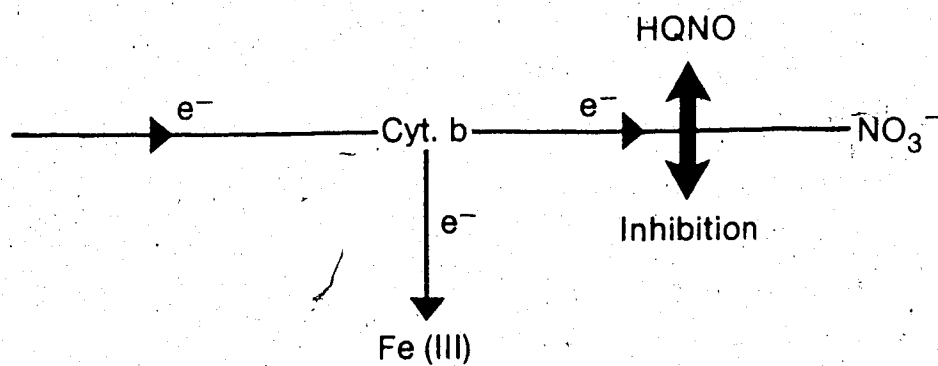
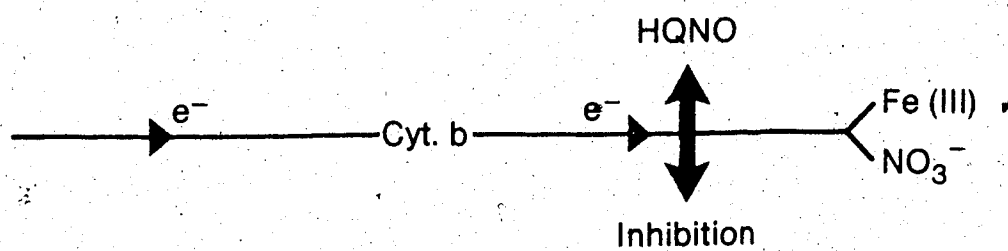


Fig. 30. Scheme explaining the difference in  $e^-$  transport and reduction of Fe(III) in *Staphylococcus aureus* (Lascelles and Burke, 1978) and in Isolate #200. This scheme is based on the result of effect of  $e^-$  transport inhibitors on Fe(III) reduction.



*Staphylococcus aureus*



Isolate # 200

The decrease in Fe(III) reduction in Isolate #200 with carbon monoxide treatment was markedly influenced by the growth conditions (Fig. 31). Cells grown under conditions that favour cytochromes' syntheses were intensely inhibited by carbon monoxide in their iron-reducing capability. When grown in  $C_4F_1$  medium or synthetic medium plus iron, cultures were inhibited 52-56%. In contrast, only 30% inhibition was registered with cells grown under conditions of iron starvation. Under this condition, the cells synthesized non-detectable levels of cytochromes. With cells grown in synthetic medium lacking iron, carbon monoxide inhibition was low probably because the cytochrome content was also very low. Since carbon monoxide blocks electron transfer at the terminal step of electron transport chain, the inhibition of Fe(III) reduction by this compound is a conclusive evidence of the role of Fe(III) as a terminal electron acceptor from the cytochromes.

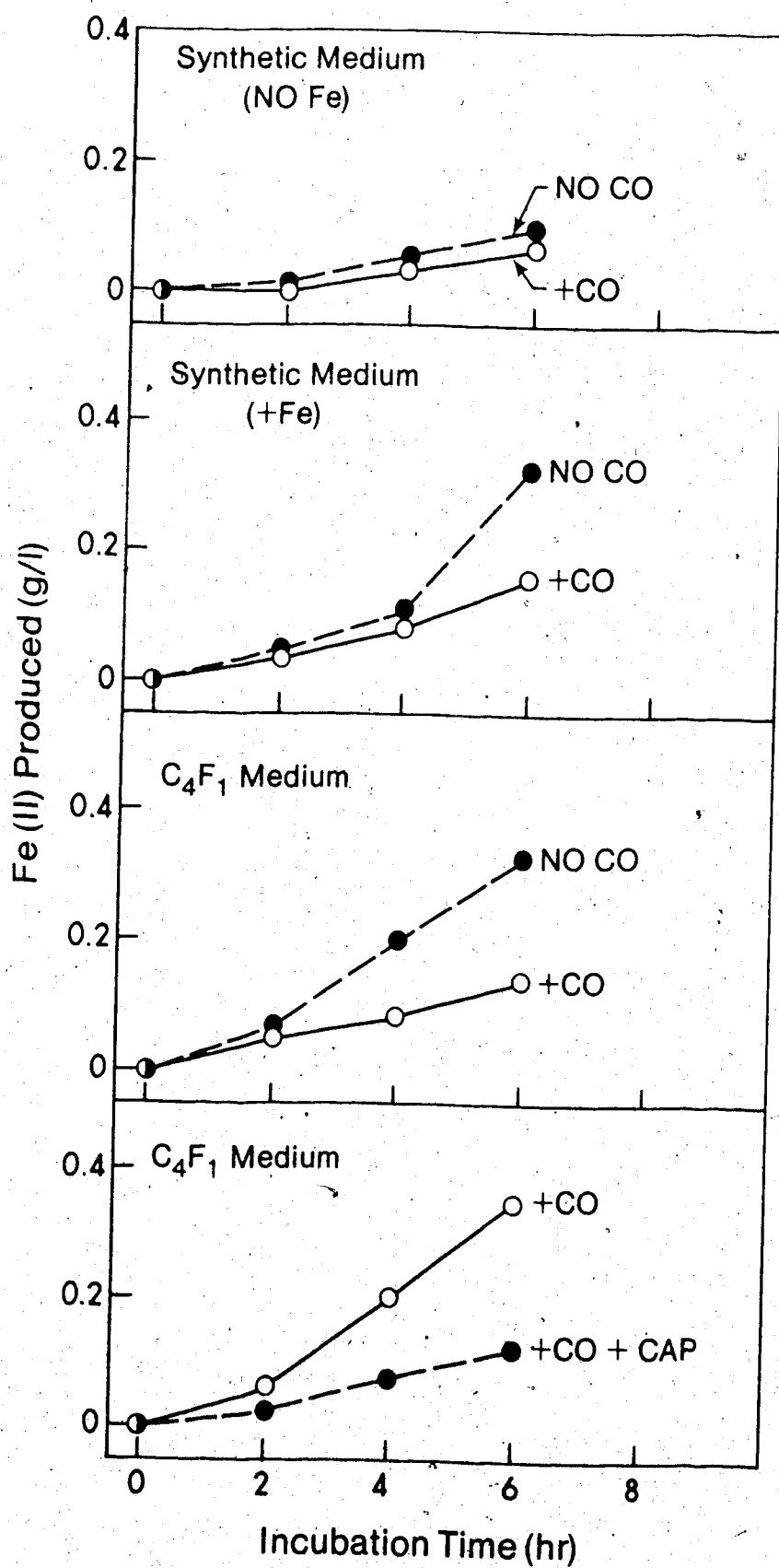
#### Reduction of ferric iron in the presence of potential electron acceptors

It was proposed (Ottow, 1970) that in *Aerobacter aerogenes* or other nitratase-positive bacteria,  $NO_3^-$  might serve as an alternative electron acceptor during the reduction of Fe(III). This proposal was put forward to explain the decline in Fe(III) reduction in the presence of  $NO_3^-$ . Because of this observation, it was necessary to investigate the effect of the other potential electron acceptors (organic and inorganic) on Fe(III) reduction. Such an investigation would help determine whether inhibition of Fe(III) reduction is specific to  $NO_3^-$  or a common phenomenon in the presence of potential electron acceptors.



Fig. 31. Effect of CO treatment of cells of Isolate #200 on Fe(III)

reduction. Chloramphenicol (CAP) concentration = 0.3 mg/ml.



### Organic potential electron acceptors

Three redox dyes and fumarate were chosen for this investigation. Methylene blue, indochlorophenol and phenazine methosulphate, chosen because of their non-toxicity were used in their oxidized states. In this condition, these redox dyes can only function as electron acceptors, not donors as would be the case if they were used in their reduced states. The use of fumarate as an electron acceptor in some bacteria was demonstrated by Miller and Wakerley (1966).

The data in Fig. 32 show that all the redox dyes inhibited Fe(III) reduction. The degree of inhibition varied, however, with phenazine-methosulphate, indochlorophenol and methylene blue being effective, in that order. Fumarate, on the other hand, increased Fe(III) reduction. There was a decline after 3 hr, the cause of this decline is not known. Fumarate alone can be used as an electron source for Fe(III) reduction by Isolate #200. So the favourable effect of fumarate would possibly be due to the ability of the organism to utilize fumarate in addition to the primary electron donor (lactate) for Fe(III) reduction.

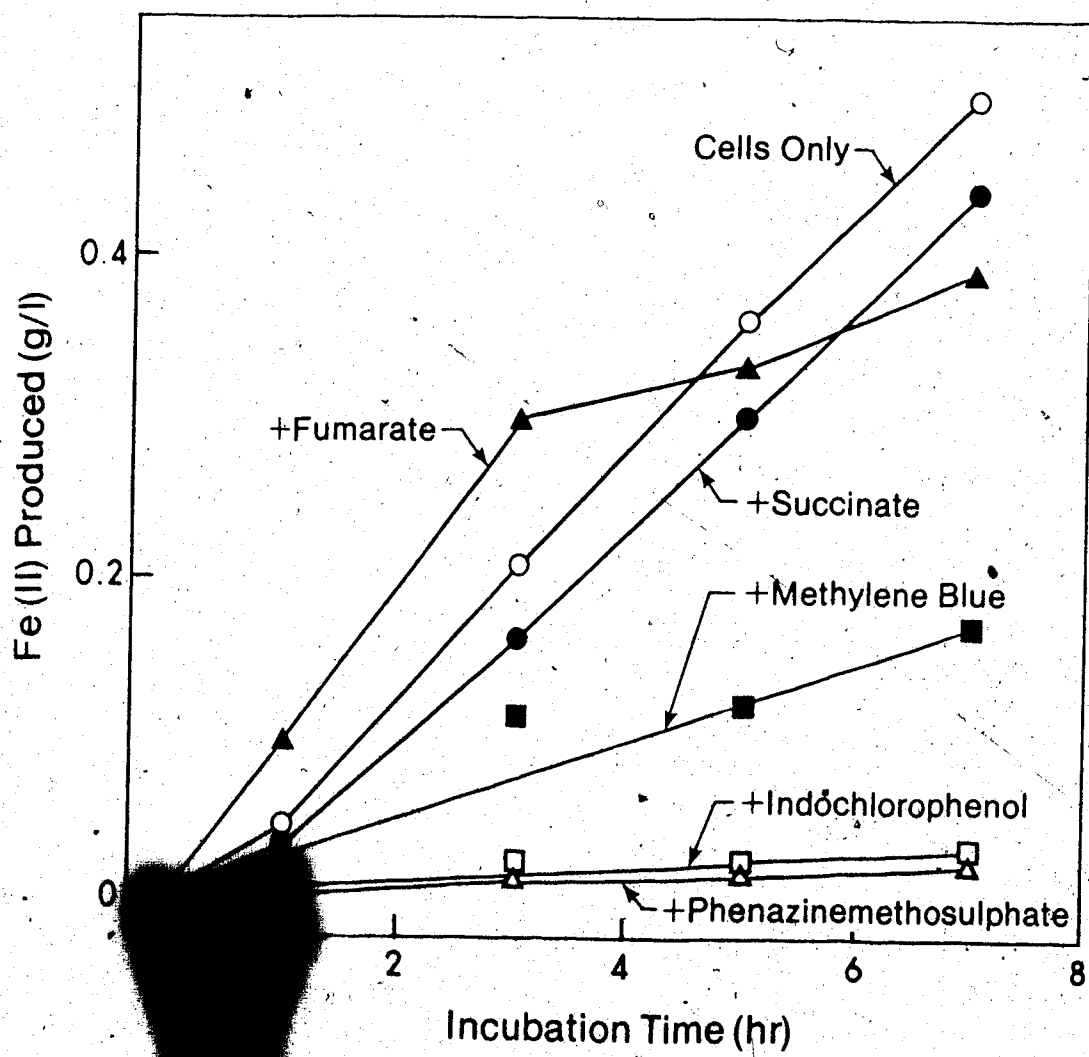
### Inorganic potential electron acceptors

The influence of inorganic compounds capable of accepting electrons (*i.e.* being reduced) is shown in Fig. 33. Permanganate, sulphite, thiosulphate and dichromate all affected detrimentally the ability of Isolate #200 to reduce Fe(II) to Fe(III). No Fe(II) was produced in the presence of dichromate. On the other hand, reduction of Fe(III) in the presence of permanganate started only after prolonged incubation. Once started, Fe(III) reduction was fast and proceeded at a rate (slope of

Fig. 32. Effect of potential organic  $e^-$  acceptors on Fe(III) reduction.

(The  $e^-$  acceptors were in their oxidized states.)

Each potential  $e^-$  acceptor supplied at the concentration of  
1  $\mu\text{mole/ml}$ .



curve) comparable to that obtained in the presence of cells only (control). During the incubation the violet colour of the solution disappeared. In a more detailed study (Fig. 33) the relationship between the disappearance of  $\text{KMnO}_4$  and  $\text{Fe(III)}$  reduction was explored. It was found that  $\text{MnO}_4^-$  was reduced first before  $\text{Fe(III)}$ . Isolate #200 was found capable of reducing  $\text{KMnO}_4$ , albeit slower, in the absence of any added  $\text{Fe(III)}$ . Sodium sulphite inhibited  $\text{Fe(III)}$  reduction more than sodium thiosulphate and the effect of thiosulphate was noticeably small. After about 7 hr incubation, when sulphide was being produced in the  $\text{S}_2\text{O}_3^{2-}$ -containing medium, the amount of  $\text{Fe(II)}$  produced increased. This was due to an additional reduction of  $\text{Fe(III)}$  by the  $\text{S}^{2-}$  produced.

Chlorate ( $\text{ClO}_3^-$ ) and nitrate did not adversely affect  $\text{Fe(III)}$  reduction until after 3 hr incubation. The effect of  $\text{NO}_3^-$  was remarkable. Within the first hour of incubation, more  $\text{Fe(II)}$  was actually produced in the presence than the absence of  $\text{NO}_3^-$  (*i.e.* cells only). This observation was unexpected since many workers had reported only an inhibition (Ottow, 1970; Lascelles and Burke, 1978; Kamura *et al.*, 1973). After incubating for 7 hr the amount of  $\text{Fe(II)}$  in solution declined and appeared to have been removed from the reaction mixture.

It was apparent that all the potential electron acceptors (except fumarate) tested were ultimately able to decrease  $\text{Fe(II)}$  produced, compared to the control. The effect of  $\text{ClO}_3^-$  was observed after 3 hr incubation. This observation was consistent with the report of Southamer (1967) that chlorite ( $\text{ClO}_2^-$ ), the reduction product of  $\text{ClO}_3^-$ , inhibited bacterial action. Presumably, inhibitory concentration of  $\text{ClO}_2^-$  was not formed until after 3 hr incubation. On the other hand, the decline in  $\text{Fe(II)}$  concentration in the presence of  $\text{NO}_3^-$  could not be

Fig. 33. Effect of potential inorganic electron acceptors on Fe(III) reduction by Isolate #200.

Each potential  $e^-$  acceptor was supplied at the rate of 10  $\mu\text{moles/ml}$  except  $\text{MnO}_4^-$  (5  $\mu\text{moles/ml}$ ).

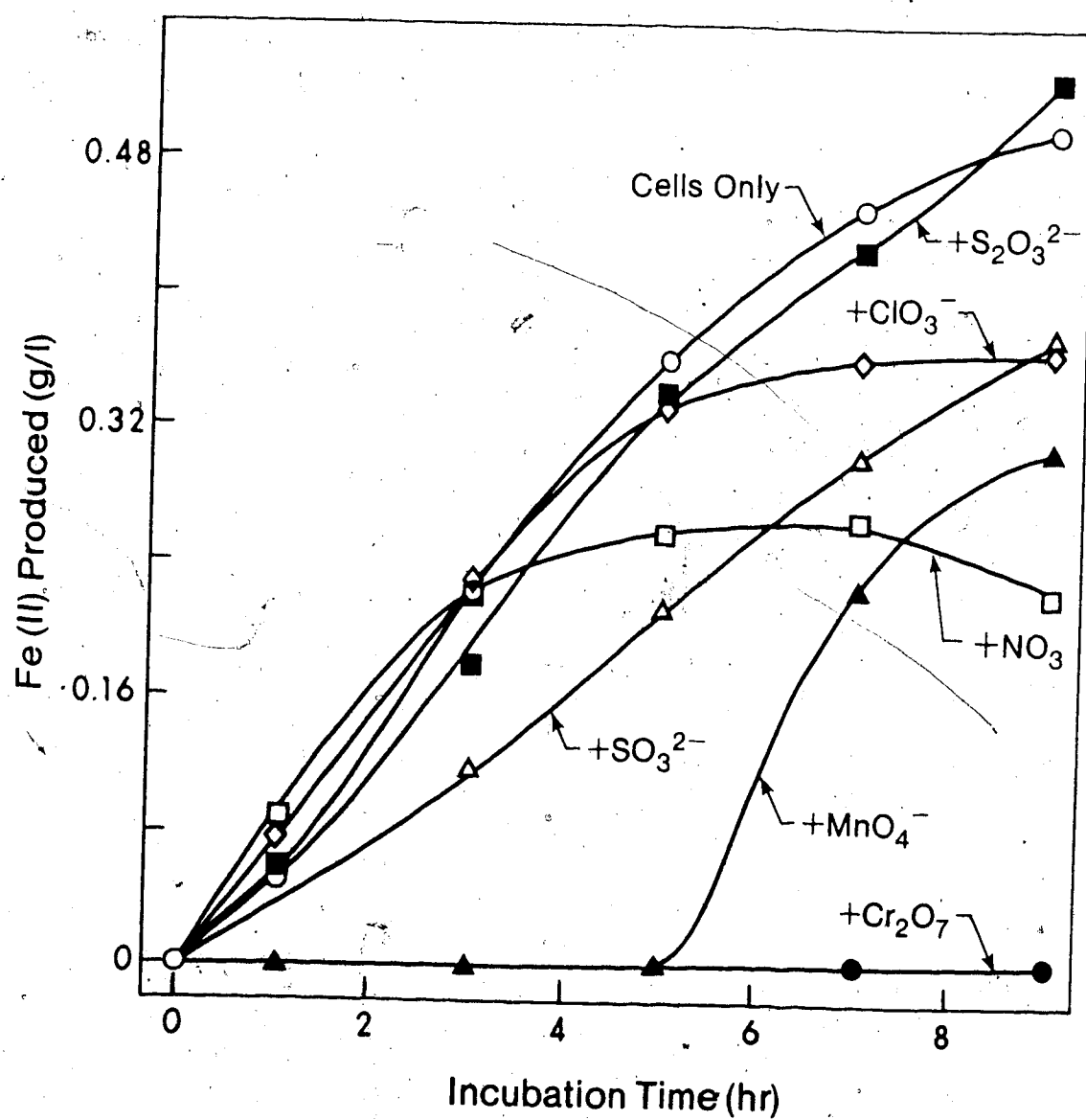
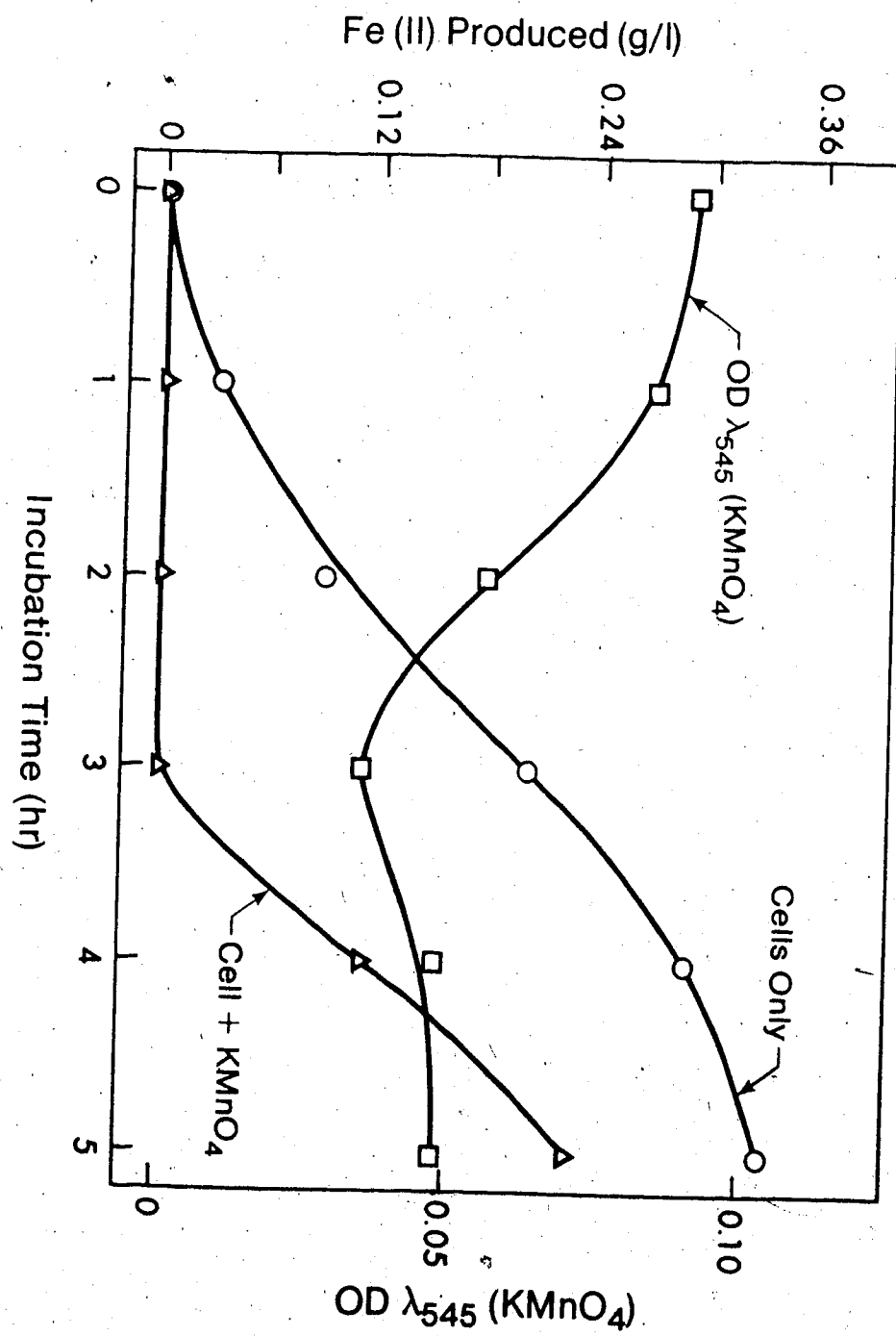
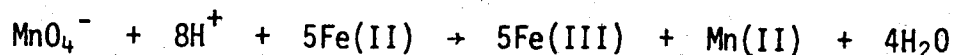




Fig. 34. Relationship between  $\text{KMnO}_4$  disappearance and  $\text{Fe(III)}$  reduction by Isolate #200.



adequately explained by the inhibition theory. Mere inhibition of the reduction process would be expected to leave Fe(II) already produced in the reaction mixture constant, and not decline (or appear to be removed from the solution) as was the case in Fig. 33. Therefore, a mechanism other than inhibition of the bacterial reduction process should be sought to explain the  $\text{NO}_3^-$  effect. Dichromate and permanganate are very strong oxidizing agents and, therefore, will show a strong tendency to accept electrons (*i.e.* to be reduced). These compounds equally showed the greatest inhibitory effect on Fe(III) reduction. In view of this, there appeared to be a preferential channelling of electrons to the more oxidizing inhibitors. Hence their inhibitory effect. The observation that no Fe(III) was reduced until all of  $\text{KMnO}_4$  was reduced first enhanced this view. However, the interpretation of  $\text{MnO}_4^-$  effect could be compounded by the fact that  $\text{MnO}_4^-$  can be reduced by Fe(II) alone. If this situation is obtained, then the  $\text{MnO}_4^-$  effect can be interpreted thus: Isolate #200 reduced Fe(III) to Fe(II) which in turn reduced  $\text{MnO}_4^-$  according to the following reaction:



This situation, although it could arise, was by no means exclusive since the organism could reduce  $\text{MnO}_4^-$  even when Fe(III) was not present. From this work, it was evident that other potential electron acceptors could decrease Fe(III) reduction and so the effect of  $\text{NO}_3^-$  could not be considered specific.

#### Reduction of Ferric Iron in the Presence of Nitrate

In the preliminary experiment on the effect of potential electron acceptors on Fe(III) reduction, it was observed that the production of

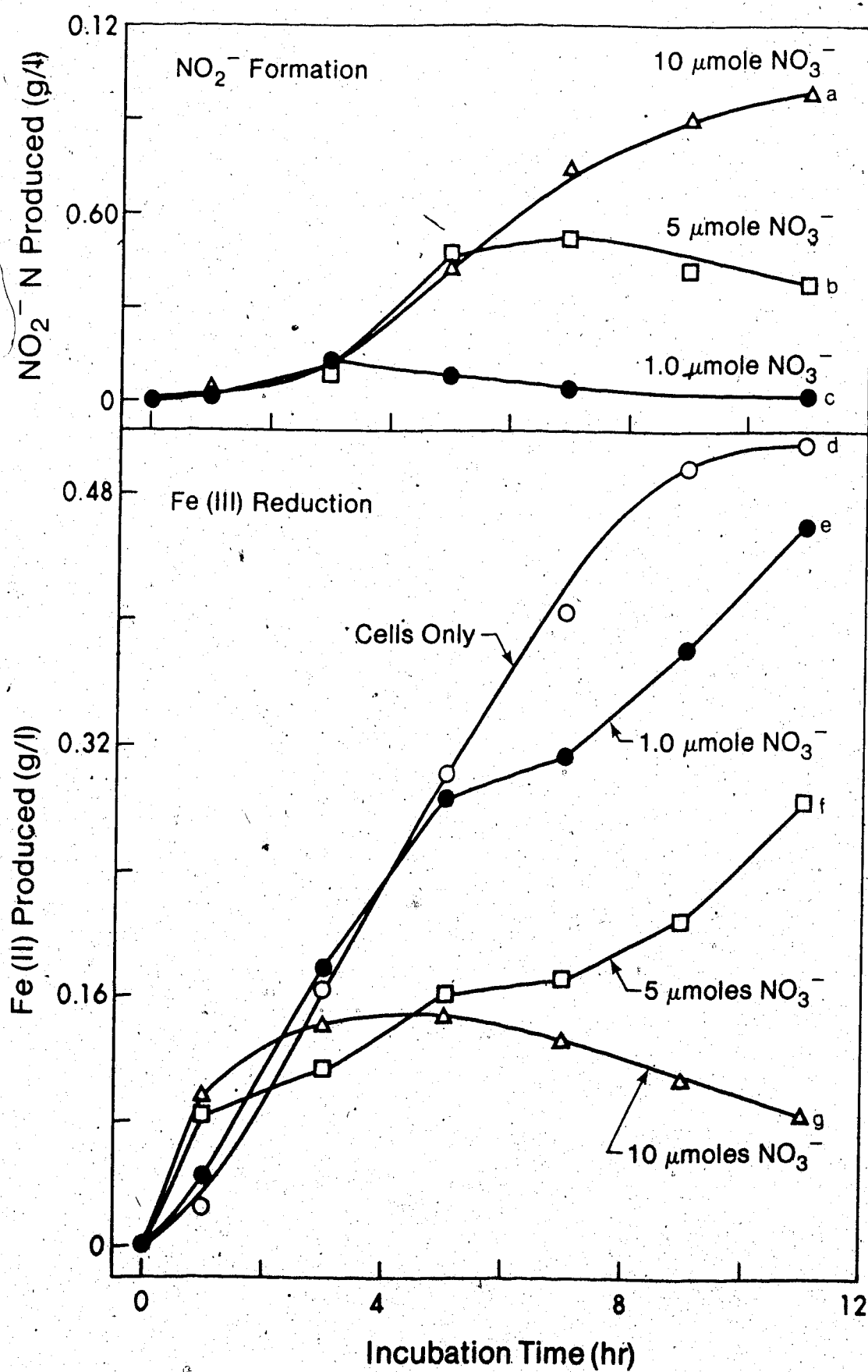
Fe(II) by the organism was greater in the presence than in the absence of nitrate after an hour incubation. This was contrary to reports in the literature (Ottow, 1968, 1970; Ponnamperna and Castro, 1964; Kamura *et al.*, 1973) where  $\text{NO}_3^-$  has been said to inhibit Fe(III) reduction. However, formation of Fe(II) in the nitrate-containing medium was lower than in nitrate-free medium only after prolonged incubation.

#### Effect of nitrate on ferric iron reduction

The effect of different concentrations of  $\text{NO}_3^-$  on Fe(II) production is presented in Fig. 35. More Fe(II) was produced eventually in the absence of nitrate than in its presence. Formations of Fe(II) and  $\text{NO}_2^-$  (Fig. 35) were simultaneous; no one process was abolished in preference to the other. It was also noted that the slopes of graphs of Fe(III) and  $\text{NO}_2^-$  productions differed, showing that these processes were progressing at different rates. For relatively short reaction times (up to at least 1 hr), the presence of  $\text{NO}_3^-$  increased the capacity of the cells to reduce Fe(III) to Fe(II). During the first hour of incubation, more Fe(II) was produced with increase in concentration of  $\text{NO}_3^-$ . The increase in the amount of Fe(II) produced was statistically significant (99.5% confidence limit) regardless of the concentration of  $\text{NO}_3^-$  employed, when compared with the amount of Fe(II) produced in the absence of  $\text{NO}_3^-$ .

At lower concentrations of  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  production (Fig. 35) was not sustained on prolonged incubation but started to decrease after an early peak. Contrary to this observation,  $\text{NO}_2^-$  production at high  $\text{NO}_3^-$  concentration rose continually throughout the incubation time. It was observed that the early peaking and subsequent decrease in measurable

Fig. 35. Fe(III) reduction and  $\text{NO}_2^-$  formation in the presence of various concentrations of  $\text{NO}_3^-$ .

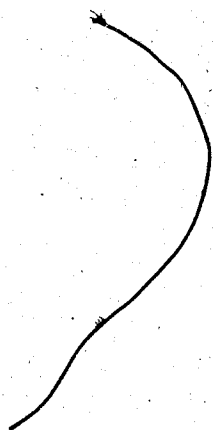


$\text{NO}_2^-$  at the lower  $\text{NO}_3^-$  concentrations were accompanied by the recovery of Fe(II) production of the culture. Under this condition, the yields of Fe(II) tended towards those obtained in the absence of  $\text{NO}_3^-$ . However, a sustained decrease in measurable Fe(II) was observed at high  $\text{NO}_3^-$  concentration where a sustained  $\text{NO}_2^-$  production was equally observed. Thus, the decrease in Fe(II) production was associated with increased  $\text{NO}_2^-$  production. In a separate experiment no nitrous oxide ( $\text{N}_2\text{O}$ ) was detected by gas-liquid chromatography at this time; it took up to 5 days for small amounts of  $\text{N}_2\text{O}$  to be formed. The decrease in measured  $\text{NO}_2^-$ , therefore, could not be attributed to denitrification, but probably to a chemical process which utilized the available  $\text{NO}_2^-$ .

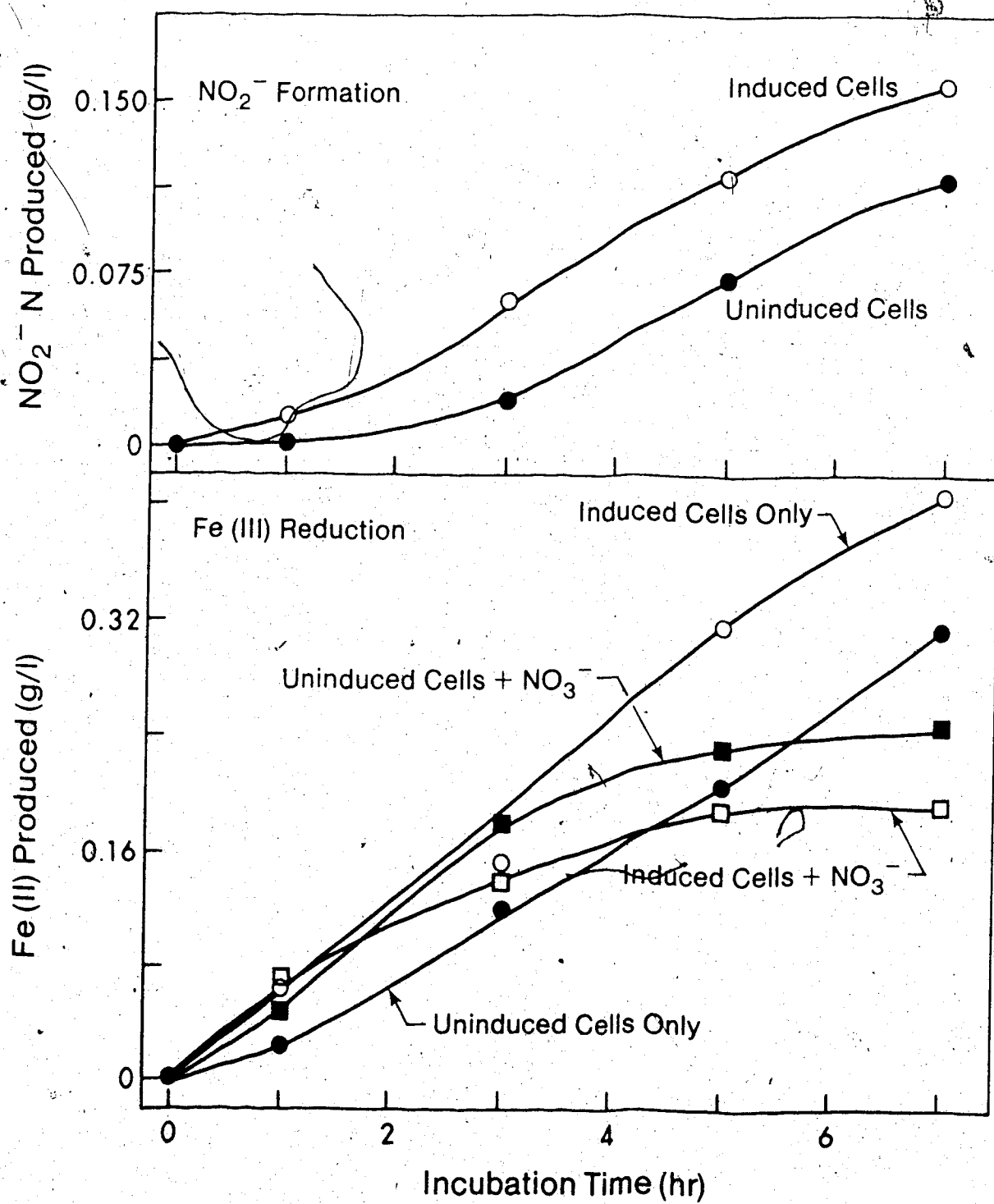
#### Reduction of ferric iron by nitrate reductase-induced cells

Nitrate reductase was induced in the inoculum when incubated anaerobically in the presence of  $\text{NO}_3^-$  (Fig. 36a). Pre-induced cells showed immediate formation of  $\text{NO}_2^-$  in the presence of  $\text{NO}_3^-$ , where uninduced cells showed a lag period which was required for the induction of nitratase. Induced cells produced more  $\text{NO}_2^-$  than uninduced cells at any one time during the incubation (Fig. 36a). On the other hand, nitratase-pre-induced cells produced much less Fe(II) in the presence of  $\text{NO}_3^-$  than the uninduced cells, except during the first hour of incubation (Fig. 36). Low amounts of assayable Fe(II) was again associated with the ability of culture to produce and accumulate  $\text{NO}_2^-$  in the reaction medium. When no  $\text{NO}_3^-$  was added to the culture, pre-induced cells produced more Fe(II) than uninduced cells. This observation could be due to a better general physiological state of pre-induced cells compared to uninduced ones.

Fig. 36. Fe(III) reduction and  $\text{NO}_2^-$  formation by nitrate reductase-  
preinduced and uninduced cells of Isolate #200 in the presence/  
absence of  $\text{NO}_3^-$ .







### Ferric iron reduction in the presence of HQNO and nitrate

The effect of  $\text{NO}_3^-$  alone, and  $\text{NO}_3^-$  plus HQNO on Fe(II) formation and  $\text{NO}_2^-$  production by Isolate #200 is shown in Fig. 37. When no  $\text{NO}_3^-$  was added, the rate of Fe(II) production was initially low and increased until after 7 hr when it started to decrease. However, throughout the incubation period the total amount of Fe(II) produced in solution increased. In the presence of  $\text{NO}_3^-$ , there was a much faster initial Fe(II) production rate, with the result that more Fe(II) was produced in the first three hours, than in the absence of  $\text{NO}_3^-$ . However, the early rise in Fe(II) production was followed by a decline as was observed previously. A similar trend was observed in Fe(II) production in the presence of HQNO which inhibited Fe(III) reduction. The quantity of Fe(II) produced in the presence of  $\text{NO}_3^-$  plus HQNO was lower than in the absence or presence of  $\text{NO}_3^-$  alone. If, however, the decrease in the quantity of Fe(II) measured in Fig. 37 was the result of removal of Fe(II) from the solution due to an equilibrium reaction like precipitation which occurred at high Fe(II) concentration, such decrease would not be observed in the presence of HQNO. This is because HQNO inhibited Fe(II) formation and Fe(II) would be present in solution only in small amounts to prevent loss due to precipitation. Moreover, no precipitate was evident in solution which at this stage appeared less greenish and more yellowish-brown.

### *In vitro* reaction of nitrite and ferrous sulphate

The data presented in Fig. 38 show that the quantity of Fe(II) present in solution decreased with the quantity of  $\text{NO}_2^-$  added as  $\text{NaNO}_2$ .

Fig. 37. Fe(III) reduction and  $\text{NO}_2^-$  formation by cells of Isolate #200 in the presence of  $\text{NO}_3^-$  and an  $\text{e}^-$  transport inhibitor (HQNO).

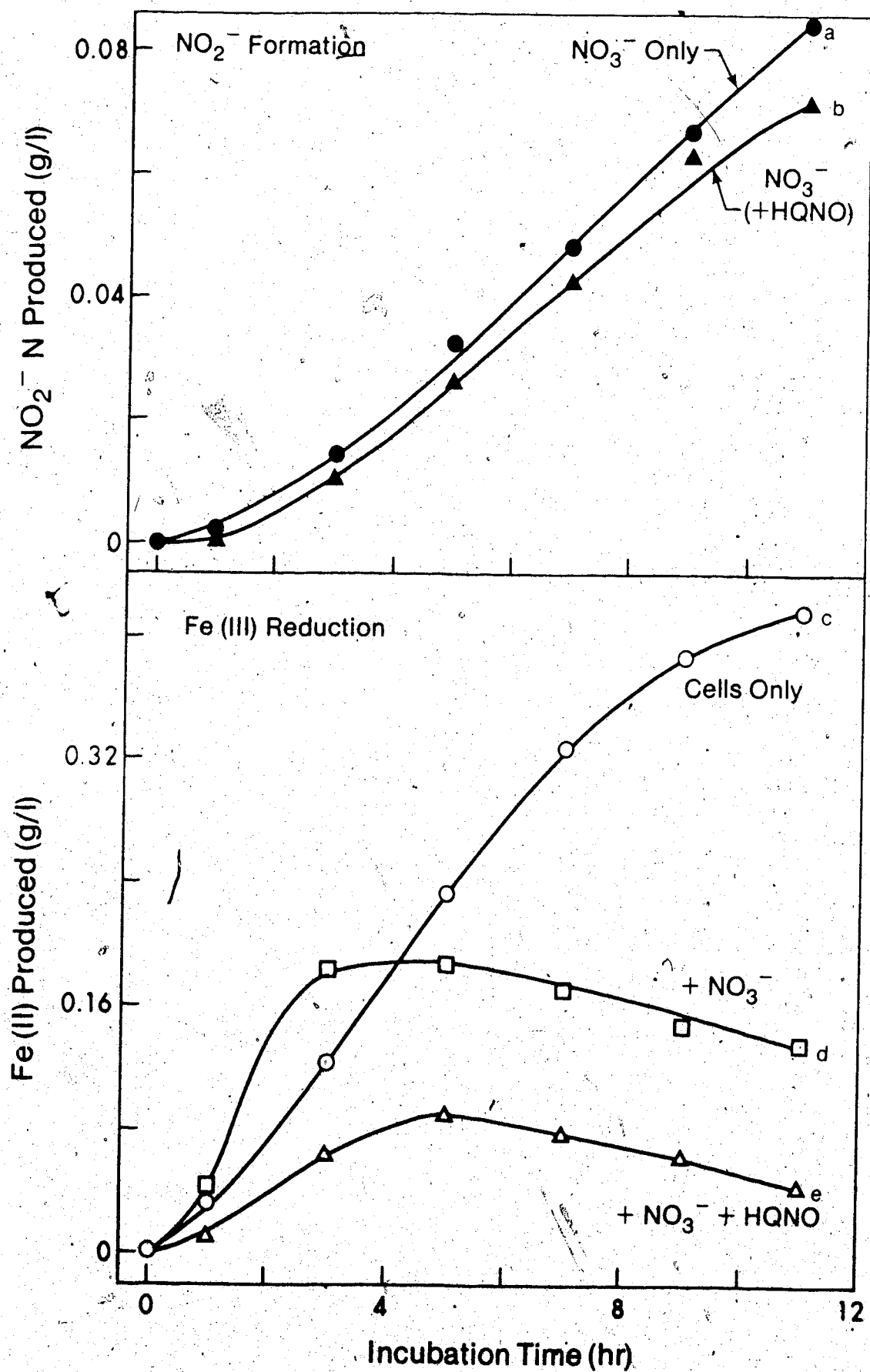
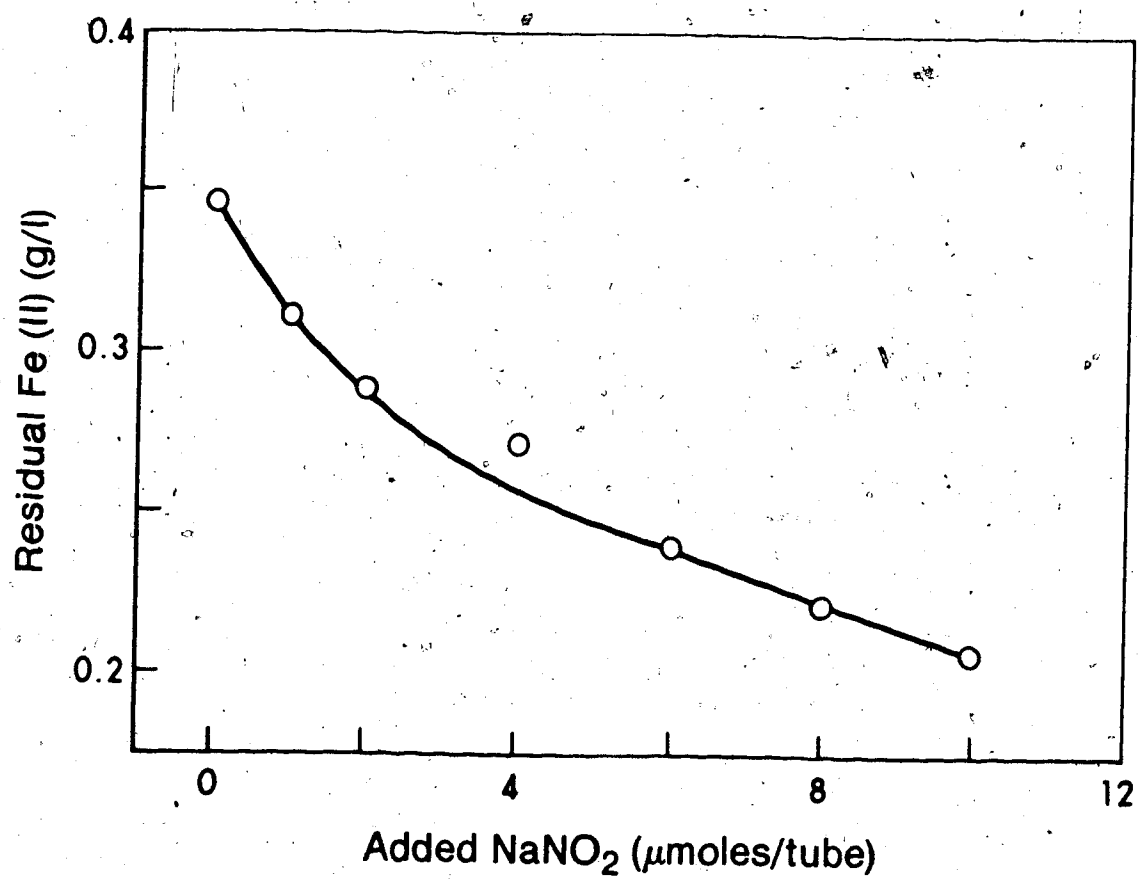


Fig. 38. *In vitro* oxidation of Fe(II) by  $\text{NaNO}_2$ .

Incubation was for 30 min at  $30^\circ\text{C}$ .



In the absence of  $\text{NO}_2^-$ , a much greater quantity of  $\text{Fe(II)}$  was present in solution than in the presence of  $\text{NO}_2^-$ . Furthermore, when reaction mixtures were reacted with potassium thiocyanate, more intense reddish-brown coloration was observed in solutions containing high amounts of  $\text{NO}_2^-$ . This observation indicated that more  $\text{Fe(III)}$  was formed by the reaction of  $\text{NO}_2^-$  and  $\text{Fe(II)}$  than when no  $\text{NO}_2^-$  was added.

Although it has been reported in Literature (Ottow, 1968, 1970; Ottow and von Kloptopek, 1969; Ponnampuruma and Castro, 1964) that  $\text{NO}_3^-$  inhibited microbial  $\text{Fe(III)}$  reduction, nitrate did not initially inhibit  $\text{Fe(III)}$  reduction by Isolate #200. It was observed that during short incubation period the presence of  $\text{NO}_3^-$  actually enhanced  $\text{Fe(III)}$  reduction. No inhibition was observed until large amounts of  $\text{NO}_2^-$  had been produced and accumulated in the reaction mixture. The data presented suggest that the decrease in the amount of measurable  $\text{Fe(II)}$  in the presence (0.1 to 10 g) of  $\text{KNO}_3$  was not due to impairment or inhibition of  $\text{Fe(II)}$  reduction process by  $\text{NO}_3^-$ . It was due rather to an extraneous, secondary reaction which resulted in the re-oxidation of  $\text{Fe(II)}$  already in solution. If  $\text{NO}_3^-$  acted merely as an inhibitor, it would be expected that the quantity of  $\text{Fe(II)}$  already produced should remain constant indefinitely. In this case, the amount of  $\text{Fe(II)}$  in solution actually decreased (Figs. 33, 35 and 37) showing that it was being transformed. This transformation was thought to be due to re-oxidation by  $\text{NO}_2^-$ . A prevention of the accumulation of  $\text{NO}_2^-$  above critical levels (*i.e.* at low  $\text{NO}_3^-$  concentration) will prevent this re-oxidation process. This is evident from the data presented in Fig. 35 d, e, f.

In most organisms, nitrate reductase is inducible (Stouthamer,

1976) and only after the induction of the enzyme can  $\text{NO}_3^-$  be reduced to  $\text{NO}_2^-$ . The observation by Ottow (1970) that *nit*<sup>-</sup> mutants of several bacteria produced the same amount of Fe(II) in the presence or absence of  $\text{NO}_3^-$  led him to suggest that Fe(III) served as an alternative electron to  $\text{NO}_3^-$ . Data already shown in this work did not support this theory. But Ottow's observation on *nit*<sup>-</sup> mutants can be adequately explained by the re-oxidation theory: since nitrate reductase is necessary for the formation of  $\text{NO}_2^-$  from  $\text{NO}_3^-$ , no  $\text{NO}_2^-$  can be produced in *nit*<sup>-</sup> mutants (lack nitratase) or wild type that lacks the enzyme. Consequently, there would be no Fe(II) re-oxidation in *nit*<sup>-</sup> mutants, hence the amount of Fe(II) formed would be the same in the presence or absence of  $\text{NO}_3^-$ . In addition, if the decrease in Fe(III) reduction (*i.e.* decline in Fe(II) formation) in the presence of  $\text{NO}_3^-$  was due to  $\text{NO}_2^-$  formed, a greater extent of this decline would be expected with high concentration of  $\text{NO}_3^-$  because more  $\text{NO}_2^-$  would be produced. One would also expect a smaller measurable Fe(II) content (in the presence of  $\text{NO}_3^-$ ) after long incubation in culture which had been pre-induced for nitrate reductase. This is because pre-induction for the enzyme would assure an immediate production of  $\text{NO}_2^-$  from  $\text{NO}_3^-$ , and, therefore, a greater tendency to accumulate  $\text{NO}_2^-$  and re-oxidize Fe(II). These assumptions have been consistent with the result obtained in this work. For example, at low  $\text{NO}_3^-$  concentrations it would be expected that the amount of  $\text{NO}_2^-$  produced would be limited because  $\text{NO}_3^-$  availability was limited and, therefore, the re-oxidation of Fe(II) would not be sustained. In the experiment in which 0.1 and 0.5 g  $\text{KNO}_3$ /litre were used, the quantity of  $\text{NO}_2^-$  produced peaked after 3 and 7 hr, respectively. The subsequent decrease in  $\text{NO}_2^-$  suggested that it was being



used up for re-oxidation of Fe(II). Such direct chemical reaction between Fe(II) and  $\text{NO}_2^-$  was reported by Moraghan and Buresh (1977). More importantly, the decrease in measured  $\text{NO}_2^-$  was accompanied by the recovery of the ferric iron reduction process, as measured by the Fe(II) in solution. These observations suggest that the inhibitory effect of  $\text{NO}_3^-$  as suggested by Ottow (1970) was actually due to the re-oxidation of the reduced iron (Fe(II)) already in solution by  $\text{NO}_2^-$ . It was noteworthy that before the decrease in the measurable Fe(II) and the increase in  $\text{NO}_2^-$  (i.e. initially) there had been a significantly higher amount of Fe(II) formed in the presence than in the absence of  $\text{NO}_3^-$ .

In a more recent work, Lascelles and Burke (1978) suggested that  $\text{NO}_2^-$  produced inhibited Fe(III) reduction. As has been pointed out previously, if  $\text{NO}_2^-$  acted merely as an inhibitor it would be expected that the amount of Fe(II) produced in the reaction mixture would not change as from the time of inhibition. However, the result shown in Fig. 37 d, e actually showed a decrease from what was originally present. This type of information was not available in the report of Lascelles and Burke (1978) because readings were taken only at the beginning and at the end. What happened in between was not known. Furthermore, from the results of the work on effect of electron transport inhibitors it was demonstrated that the path of electron transport to Fe(III) differed in *S. aureus* and in isolate #200. As a consequence of this fundamental but marked difference in the process in the two organisms, it must be concluded that these two organisms have different mechanisms for Fe(III) reduction. Therefore, even if  $\text{NO}_2^-$  acted as an inhibitor in *S. aureus* this mode of action would not necessarily be applicable to isolate #200 in view of marked difference in the path of

electron transport to Fe(III). Furthermore, in the work reported in this thesis the *in vitro* reaction of  $\text{NO}_2^-$  and Fe(II) demonstrated that  $\text{NO}_2^-$  is capable of oxidizing Fe(II) under the experimental conditions. Moreover, the role of  $\text{NO}_2^-$  in the oxidation of Fe(II) under physiological conditions was reported by Turner and Kienholz (1972); haemoglobin was oxidized to methaemoglobin by  $\text{NO}_2^-$ . In chemical process streams, the corrosion of ferrous metals has been inhibited by the oxidative property of  $\text{NO}_2^-$ . Nitrite oxidizes iron to the ferric compound (Olefjord, 1975; Draper, 1967; Lumsden and Szklarska-Smialowska, 1978). Ashton *et al.* (1973a, 1973b) reported that  $\text{NO}_2^-$  formed by the reduction of  $\text{NO}_3^-$  by *E. coli* cultures caused the oxidation of iron to ferric compounds. These workers reported that in the presence of nitrate in the cultures of nitrite-producing *E. coli* the amount of soluble iron previously present in solution appeared to have been removed.

On the basis of the results reported herein, strengthened by large information in the Literature on the oxidative properties of  $\text{NO}_2^-$  it is concluded that  $\text{NO}_3^-$  *per se* did not inhibit bacterial Fe(III) reduction but appeared to do so only after prolonged incubation because of the re-oxidation of Fe(II) by the  $\text{NO}_2^-$  produced.

ELECTROCHEMICAL STUDIES ON THE CORROSION OF MILD STEEL  
IN CULTURES OF ISOLATE #200

Polarography

Early attempts at investigating the electrochemistry of the corrosion of mild steel in cultures of Isolate #200 employed classical polarography.

With sterile B<sub>10</sub> medium, a reduction wave with a half-wave potential,  $E_{1/2}$ , of -2.37 V and a limiting current plateau of 7.0 mA was obtained. After 5 days of incubation of the inoculated B<sub>10</sub> medium, two reduction waves were obtained; a new wave at  $E_{1/2} = -1.80$  V and a limiting current of 4.2 mA and the previously described wave at  $E_{1/2} = -2.37$  V resulted. This indicated that a new reducible product was formed during the bacterial growth. When incubation was continued for a longer period, well-defined waves could no longer be recorded. This latter behaviour was pronounced with cultures of the organism in Butlin's medium, even after a day's growth.

The inability to record the reaction (reduction) waves whenever bacteria were grown for a few days is thought to be due to surface-active products formed by the bacteria.

Polarization Studies

The studies on the polarization characteristics of mild steel in cultures of Isolate #200 were carried out in four different media. These media include a synthetic (defined) medium (Appendix 1e), B<sub>10</sub> medium (Appendix 1a), Butlin's medium (Appendix 1b) and produced water.

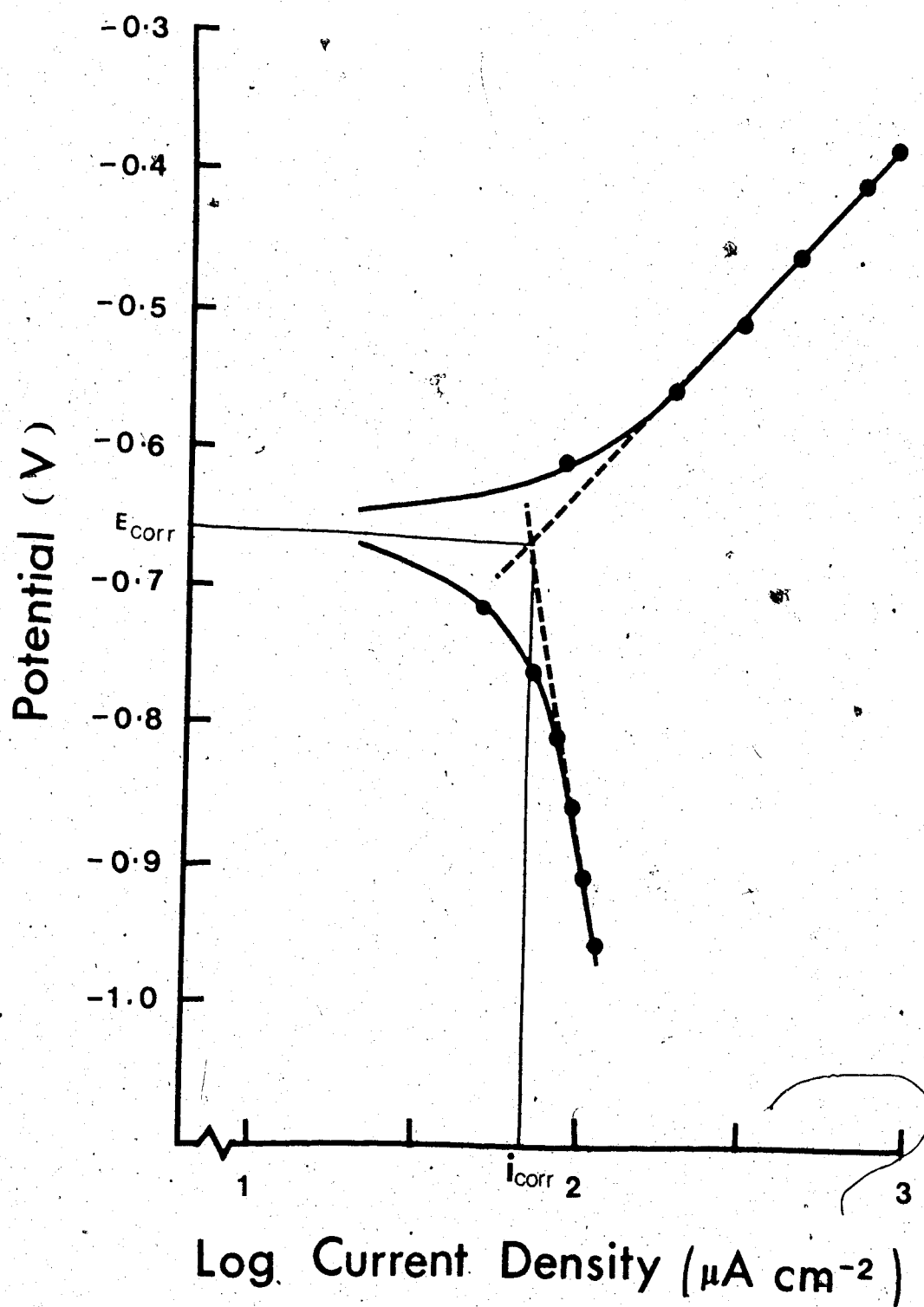
These media were chosen to cover a range of nutritional and environmental variables to which the organism might be exposed in nature. Medium B<sub>10</sub> has a very high ferric iron content (0.47% FePO<sub>4</sub>) to reflect a soil environment rich in iron. Since much of the iron present in soils occurs in the ferric state, this form of iron was employed. Furthermore, it was hoped that the effects of the ability of the organism (Isolate #200) to reduce Fe(III) to Fe(II) would be most easily observed in this medium. Butlin's medium represented a general purpose medium (non-selective environment), while the synthetic medium was used so that the composition would be sufficiently characterized and controlled. The decision to use produced water was a pragmatic one. Produced water was a component of the crude oil-water emulsion from which the organism was isolated and, therefore, represented the natural environment of Isolate #200. The composition of produced water would not be expected to be constant throughout the production life span of an oil-well or oilfield because of the seasonal variation in soil water and microbial population.

After the construction of the polarization cell (Fig. 3), a trial polarization run was undertaken to check the functionality of the cell. Data obtained during the polarization of mild steel coupon in sterile B<sub>10</sub> medium was used to construct a Tafel plot. As shown in Fig. 39, the plot shows well-defined anodic and cathodic Tafel regions, thus indicating that the constructed cell can be reliably used in the subsequent experiments. Extrapolation of the anodic and cathodic branches to intersection gave a potential of -0.665 V in good agreement with the open-circuit voltage of -0.66 V.

The polarization characteristics of mild steel coupons in B<sub>10</sub>

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Fig. 39. Tafel plot constructed from data obtained during polarization of mild steel coupon in sterile B<sub>10</sub> medium in the newly constructed electrochemical cell.



cultures of Isolate #200 grown at room temperature ( $25 \pm 2^\circ\text{C}$ ) are shown in Fig. 40. The anodic polarization curves show that the metal became more active with exposure time in cultures of the organism. The increase in corrodibility was greater after 14 hours of incubation but increased more slowly up to 24 hours. The polarization obtained after 48 hours of incubation coincided with that at 24 hours.

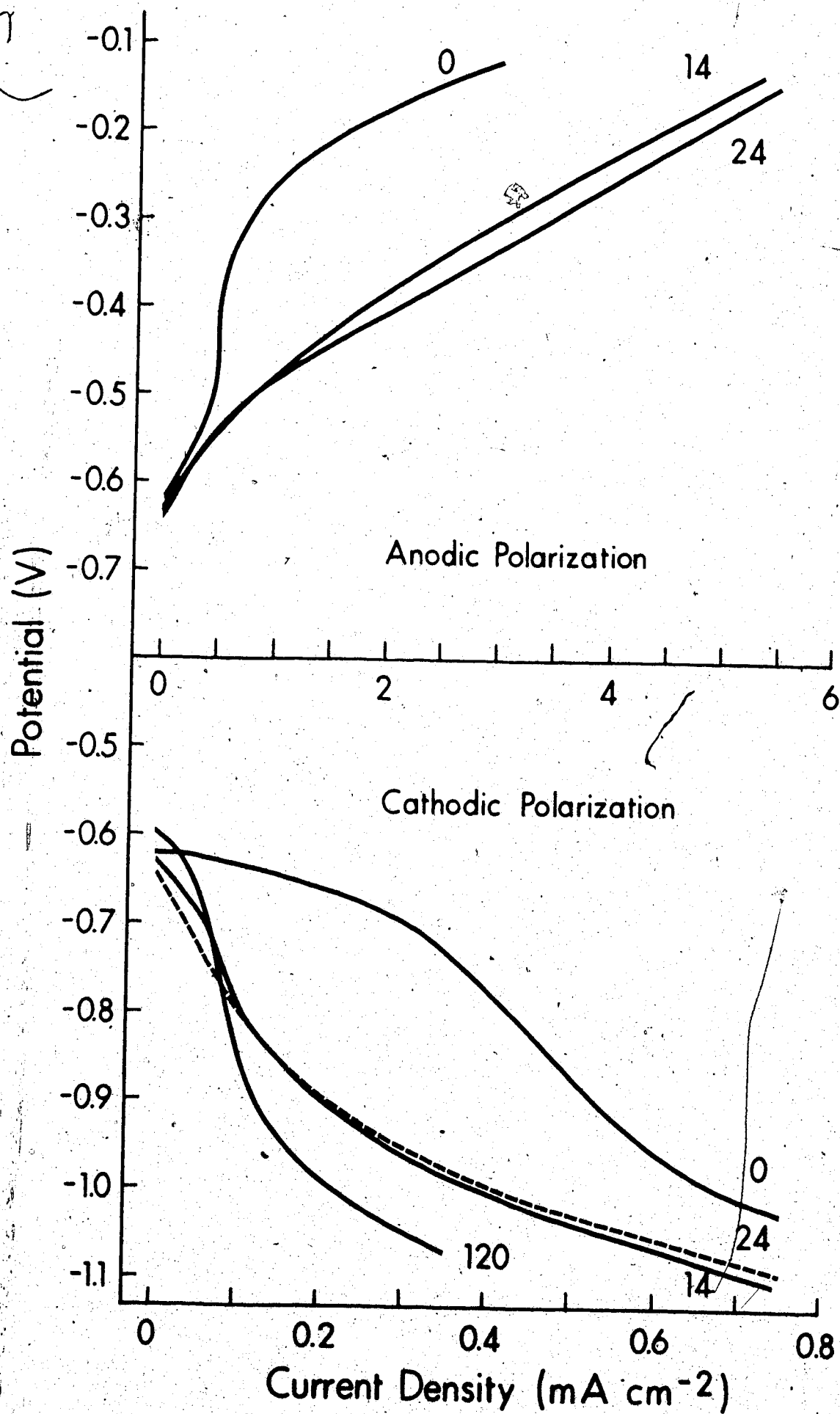
At the onset of the experiment, the inoculated culture medium appeared deep brown but with incubation soon changed to a greenish colour. This indicated the reduction of the ferric compound present ( $0.47\% \text{FePO}_4$ ) to the ferrous  $[\text{Fe(II)}]$  form. Also, after about 24 hours of incubation, a thick sediment was deposited at the bottom of the cell.

The polarization curves of the coupon in sterile  $\text{B}_{10}$  medium are shown in Fig. 41. The coupon became more resistant to corrosion with exposure, which it did not in the inoculated medium. The increase in current per unit potential change ( $dI/dE$ ) was highest at the beginning of the experiment and decreased with time. By the sixth day of incubation, the metal was almost completely polarized. Therefore, it appears that while polarization (inhibition) of the anodic corrosion reaction occurred in  $\text{B}_{10}$  medium in the absence of the organism, the presence of Isolate #200 caused an intense depolarization of the anodic reaction. In the presence of the organism, the decrease in the coupon corrodibility observed after 24 hours coincided with the formation of a gelatinous deposit which covered the lower part of the coupon. This deposit was thought to be clumps of bacterial mass surrounded by ferrous products resulting from the reduction of  $\text{Fe(III)}$ .

The polarization curves showed that the coupon was inhibited cathodically both in the presence and absence of the organism. The

Fig. 40. Polarization curves for mild steel in  $B_{10}$  medium inoculated  
with Isolate #200.






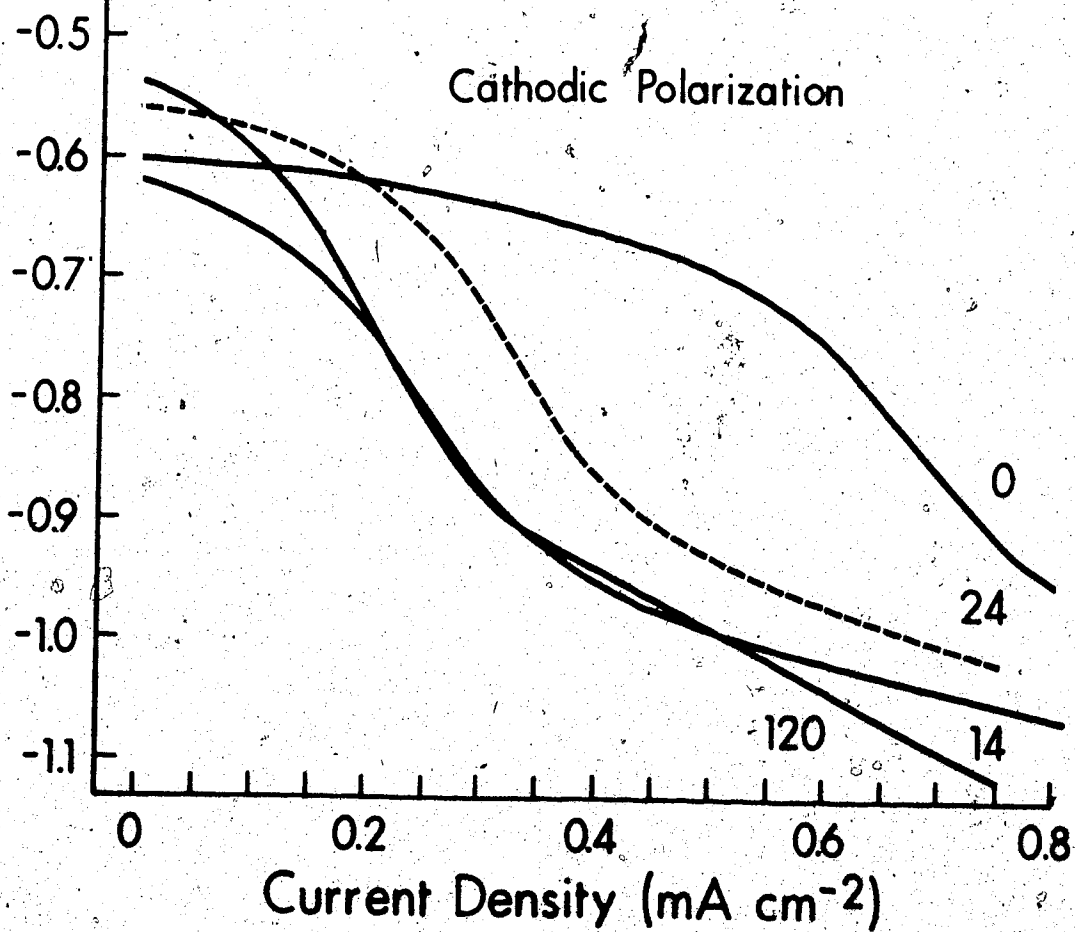
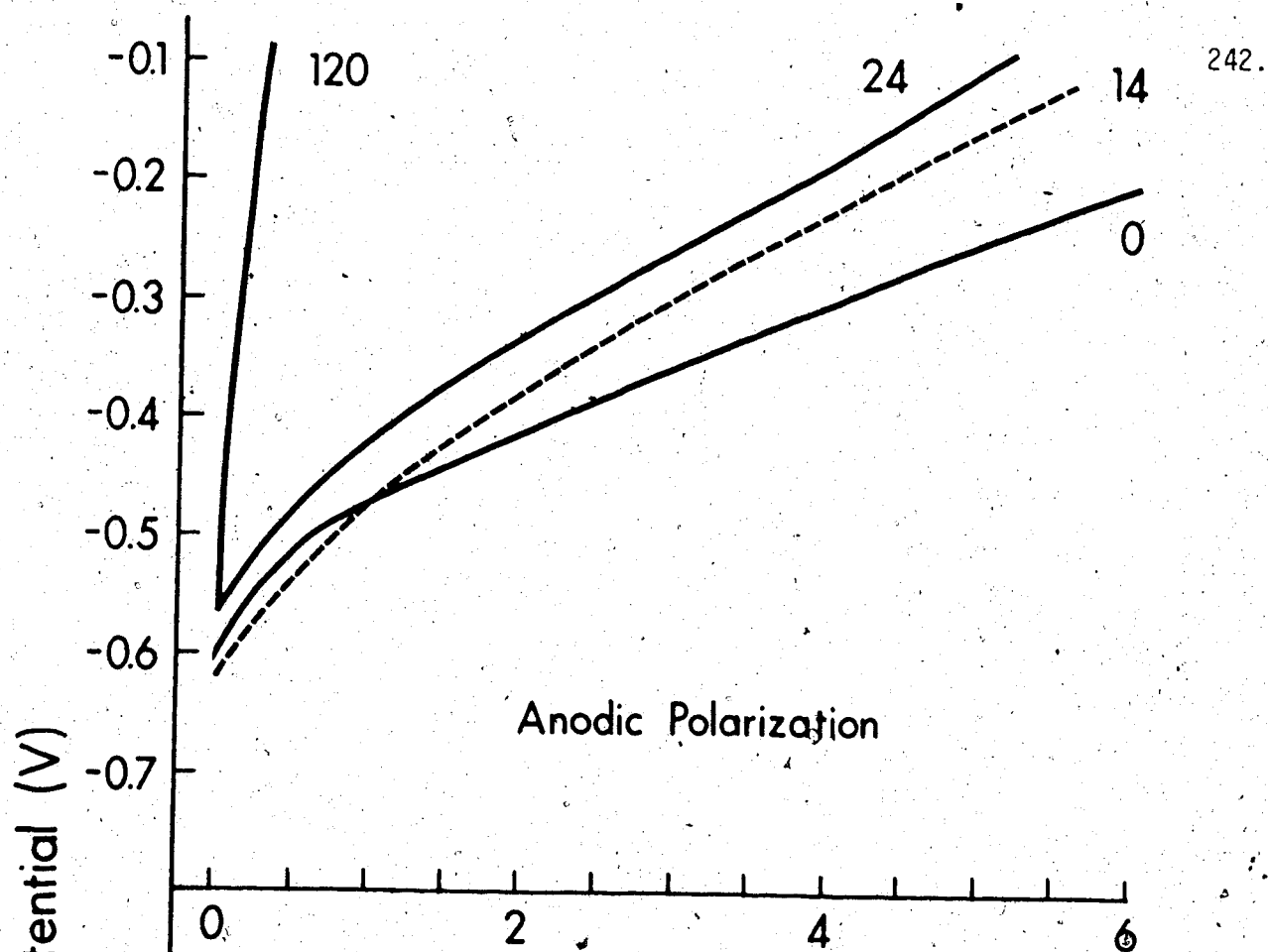


Fig. 41. Polarization curves for mild steel in uninoculated (control)

B<sub>10</sub> medium.

(0, 14, 24, 120 denote incubation time (hr) at 25 ± 2°C)



cathodic and anodic polarization curves in inoculated Butlin's medium are shown in Fig. 42. There was an intense anodic depolarization reaction observed after 14 hours. Prolonged incubation for up to six days yielded a polarization curve which did not differ from that obtained within the first day of bacterial growth. Thus, as in B<sub>10</sub> medium, very active depolarization of the anode process occurred during the active stage of the bacterial growth.

The cathodic polarization revealed that the coupon was cathodically inhibited in inoculated Butlin's medium. In the absence of the organism (Fig. 43), the coupon was inhibited both cathodically and anodically.

In the synthetic medium (Appendix 1e) which contained lactate (1.8 g sodium lactate/litre) as the sole energy source, active anodic depolarization was recorded within 14 hours (Fig. 44). The active anodic depolarization persisted throughout the experimental period of 72 hours. On the other hand, the cathodic polarization curves indicated that the cathodic reaction was progressively inhibited. In the sterile synthetic medium (Fig. 45), both the anodic and cathodic reactions were inhibited.

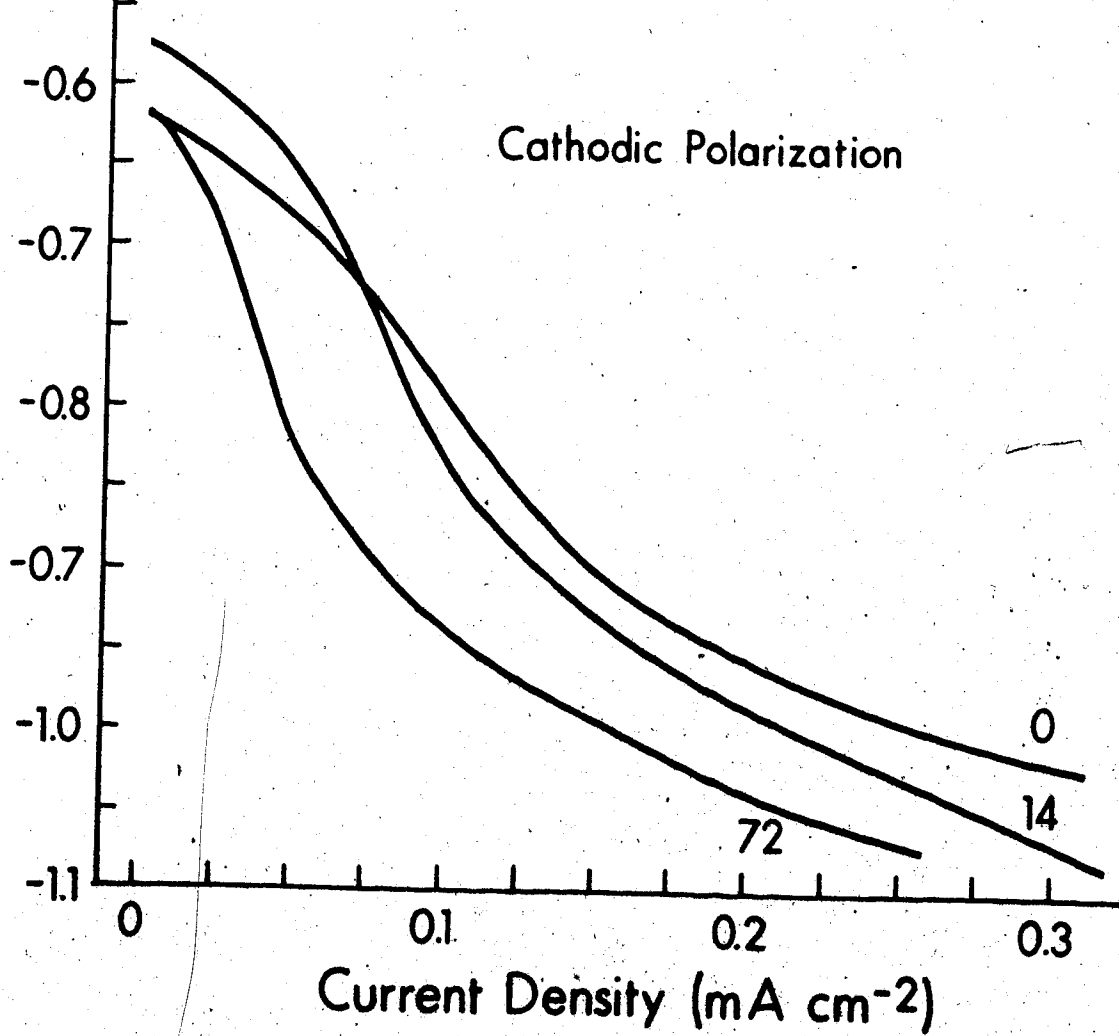
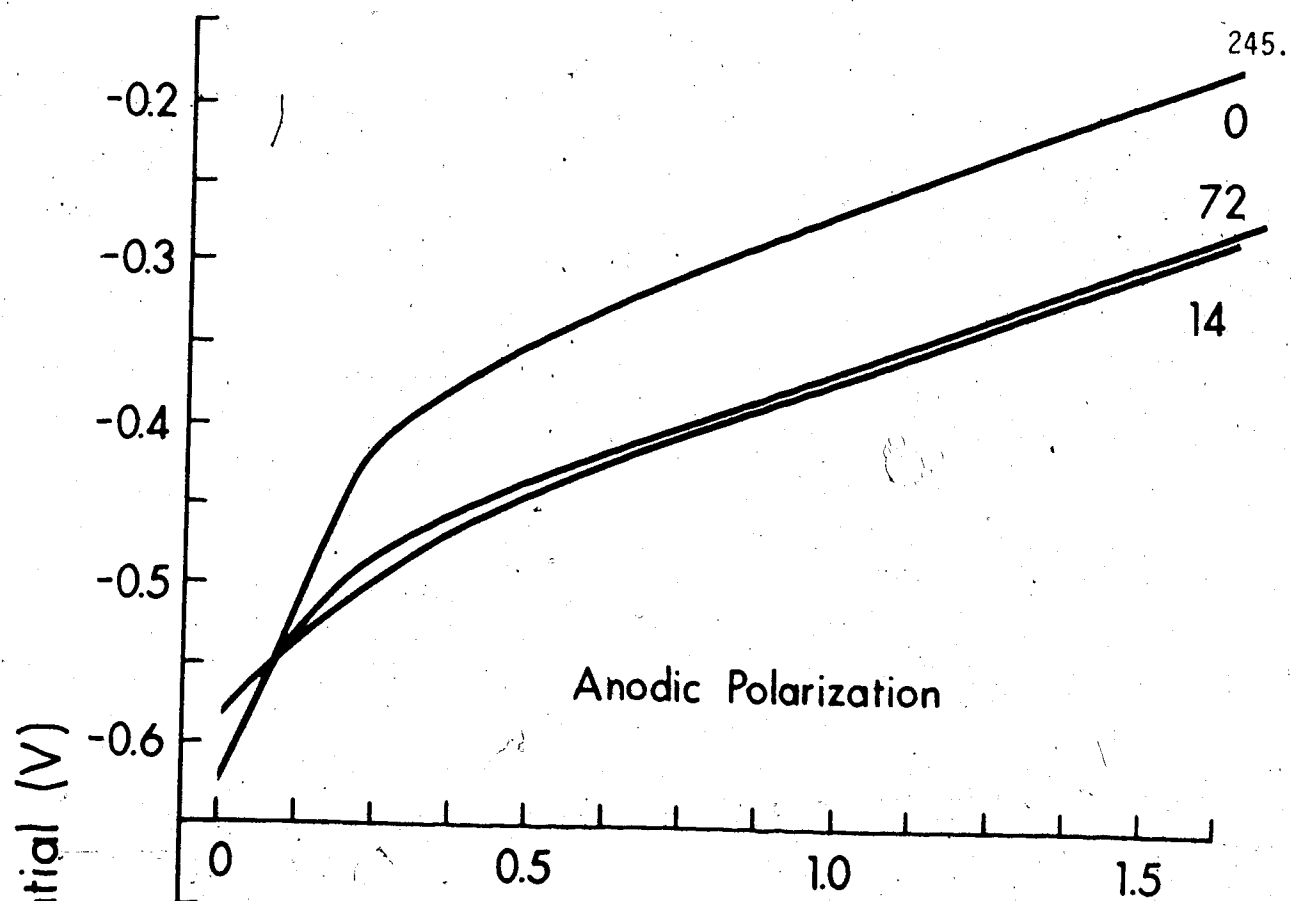
In all of the well-defined media employed, the presence of Isolate #200 caused the depolarization of the anode reaction, a reaction which would normally have been inhibited in the absence of the organism.

#### Produced Water

The trend in the response of the coupon to cathodic and anodic polarizations in produced water was more complex than observed in the other media. The anodic polarization curves (Fig. 46) showed that in

Fig. 42. Polarization curves for mild steel in Butlin's medium inoculated with Isolate #200.

(0, 14, 72 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )






Fig. 43. Polarization curves of mild steel in uninoculated Butlin's medium (control).

(0, 14, 72 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )

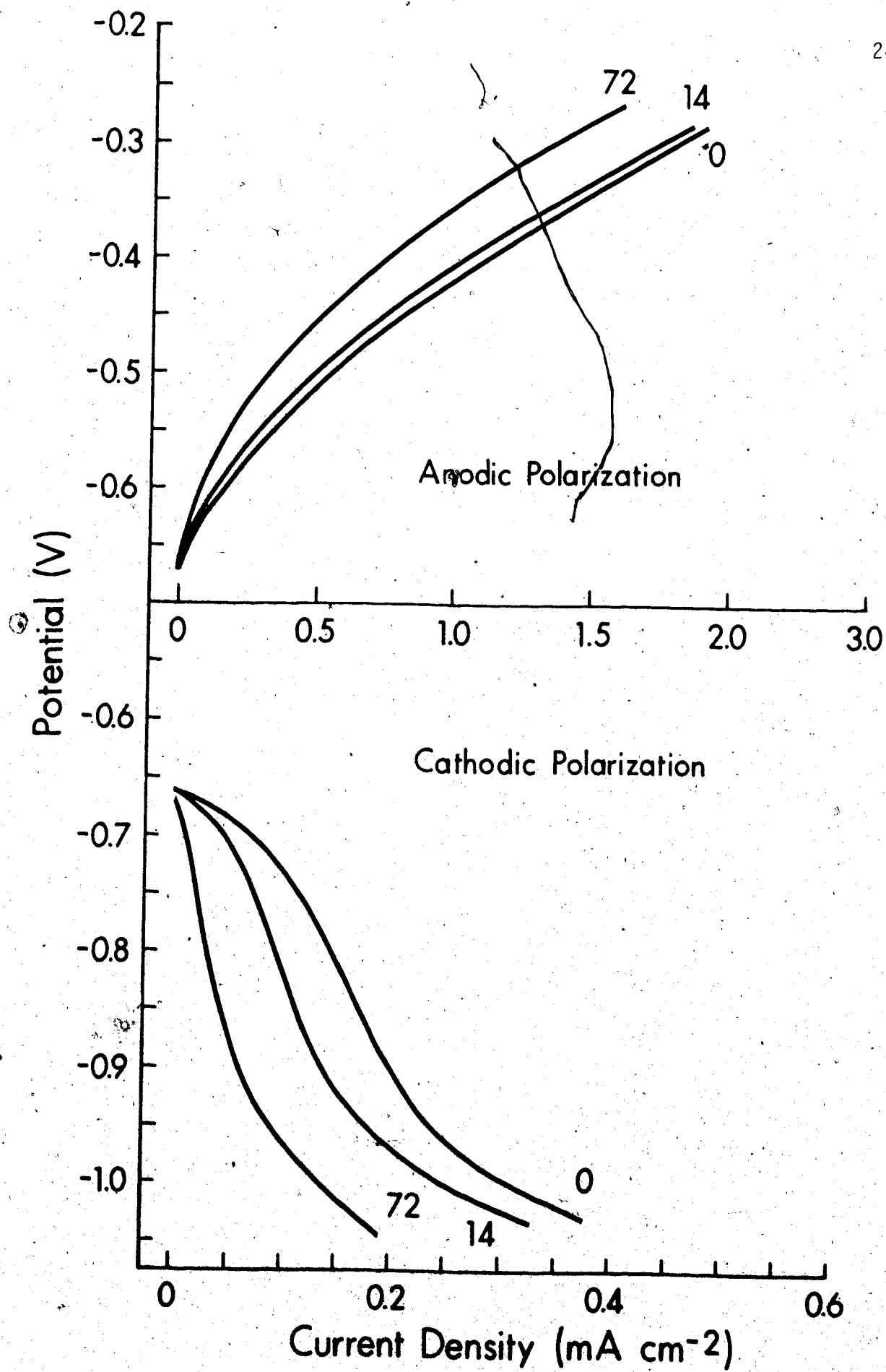




Fig. 44. Polarization curves for mild steel in synthetic medium  
(containing 1,800 mg sodium lactate/l) inoculated with  
Isolate #200.

(0, 14, 72 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )

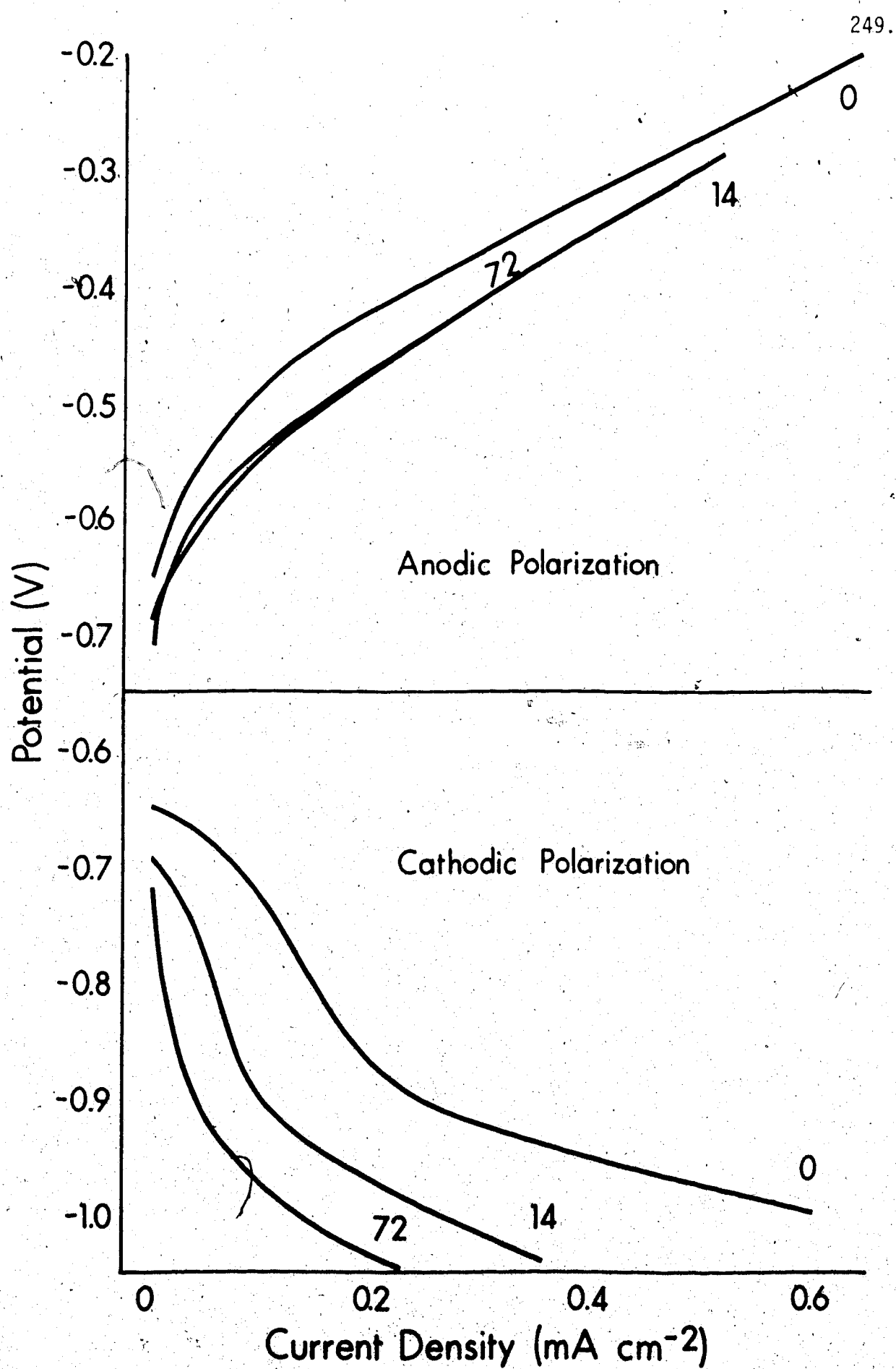
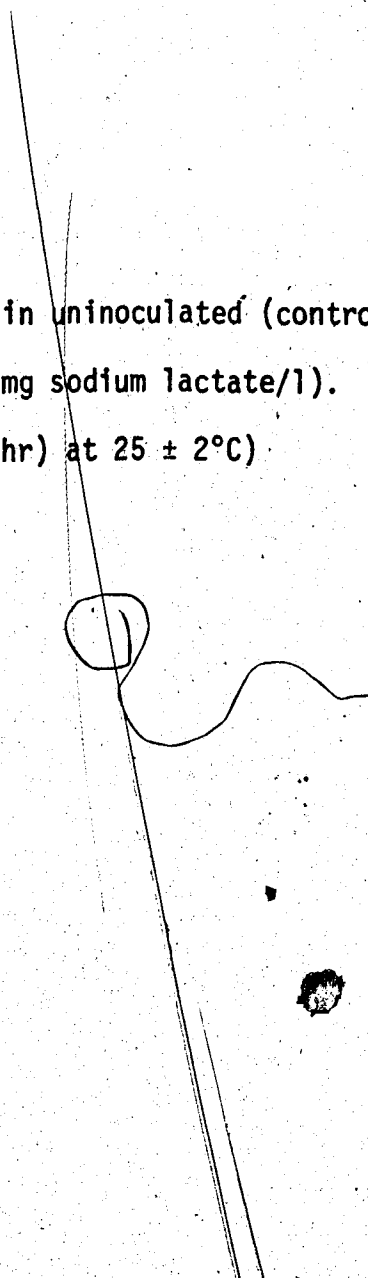


Fig. 45. Polarization curves for mild steel in uninoculated (control)  
synthetic medium (containing 1,800 mg sodium lactate/l).  
(0, 14, 72 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )



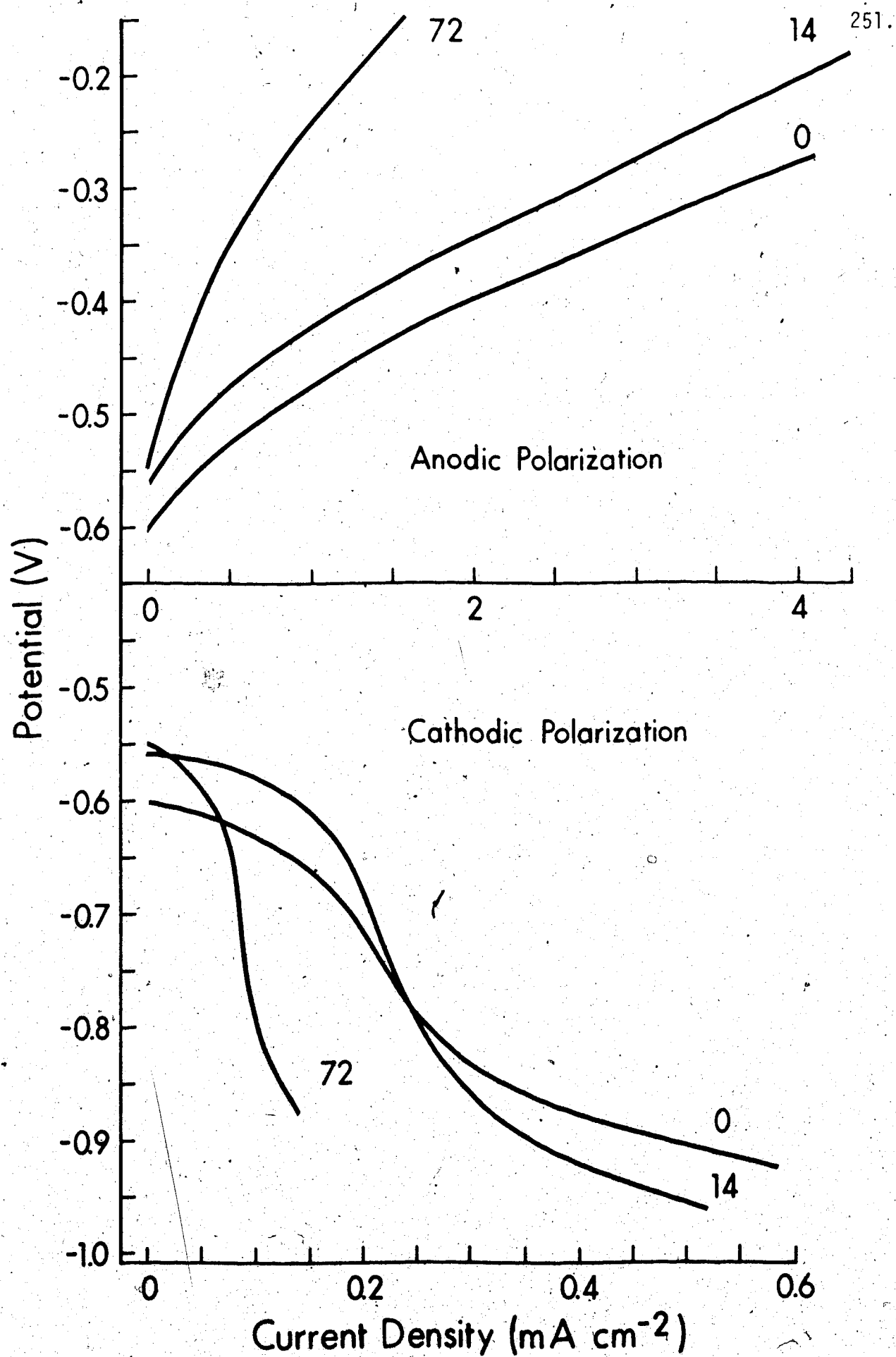
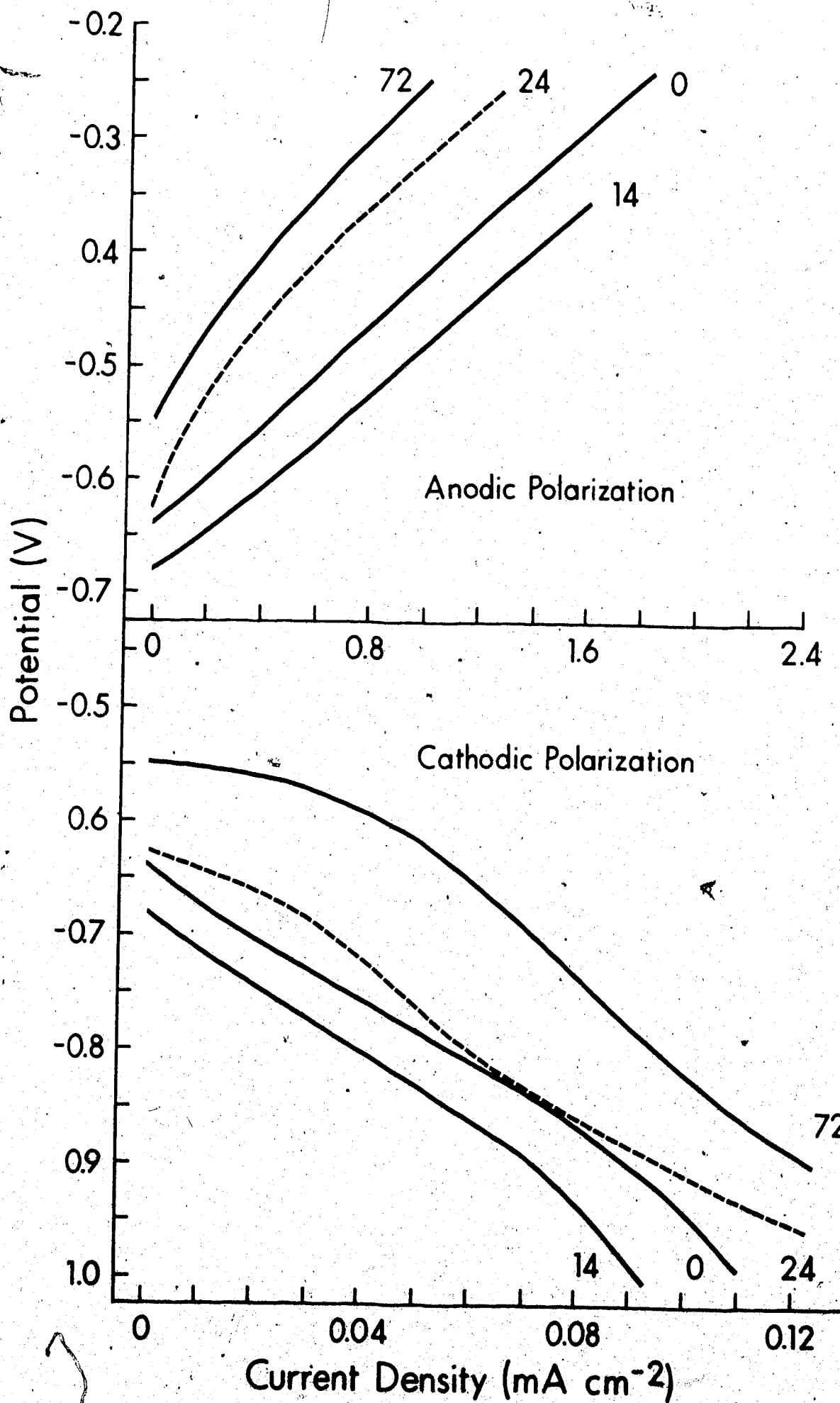


Fig. 46. Polarization curves for mild steel in unautoclaved, uninoculated (*i.e.* with natural flora) produced water.

(0, 14, 24, 72 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )



fresh, uninoculated, unautoclaved produced water there was an initial active depolarization. Subsequently, this was succeeded by a period during which the reaction was stifled. Therefore, it appeared that in unautoclaved, uninoculated produced water, the metal was initially corrodible but became less so with exposure. With the cathodic reaction, an opposite trend was observed: an initial polarization was succeeded by active depolarization process.

The observed depolarization of the anodic reaction coincided with the active growth period normally observed with the organism. However, produced water contains many different microorganisms, including the iron-reducing bacteria used in this experiment. Therefore, the observed succession in the electrode reaction could be due to the interactions of the different organisms present.

However, as shown in Fig. 47, when the produced water was autoclaved to kill off all organisms initially present and then inoculated with only Isolate #200, the same trend in the polarization curves was observed as before: there was an initial anodic depolarization followed by stifling of the reaction. Similarly, with the cathodic reaction, an initial polarization was succeeded by a very active depolarization reaction. The close similarity between the results obtained in unautoclaved non-inoculated produced water and those obtained with the autoclaved produced water inoculated with Isolate #200 indicates strongly that the reaction is caused by the same type of organism. It is now evident that depolarization of the anodic process is consistently expressed whenever Isolate #200 is growing actively, the type of medium notwithstanding. Thus, it can be concluded that under oilfield conditions, depolarization of the anodic process must be a mechanism

expected to contribute to the corrosion of ferrous metal.

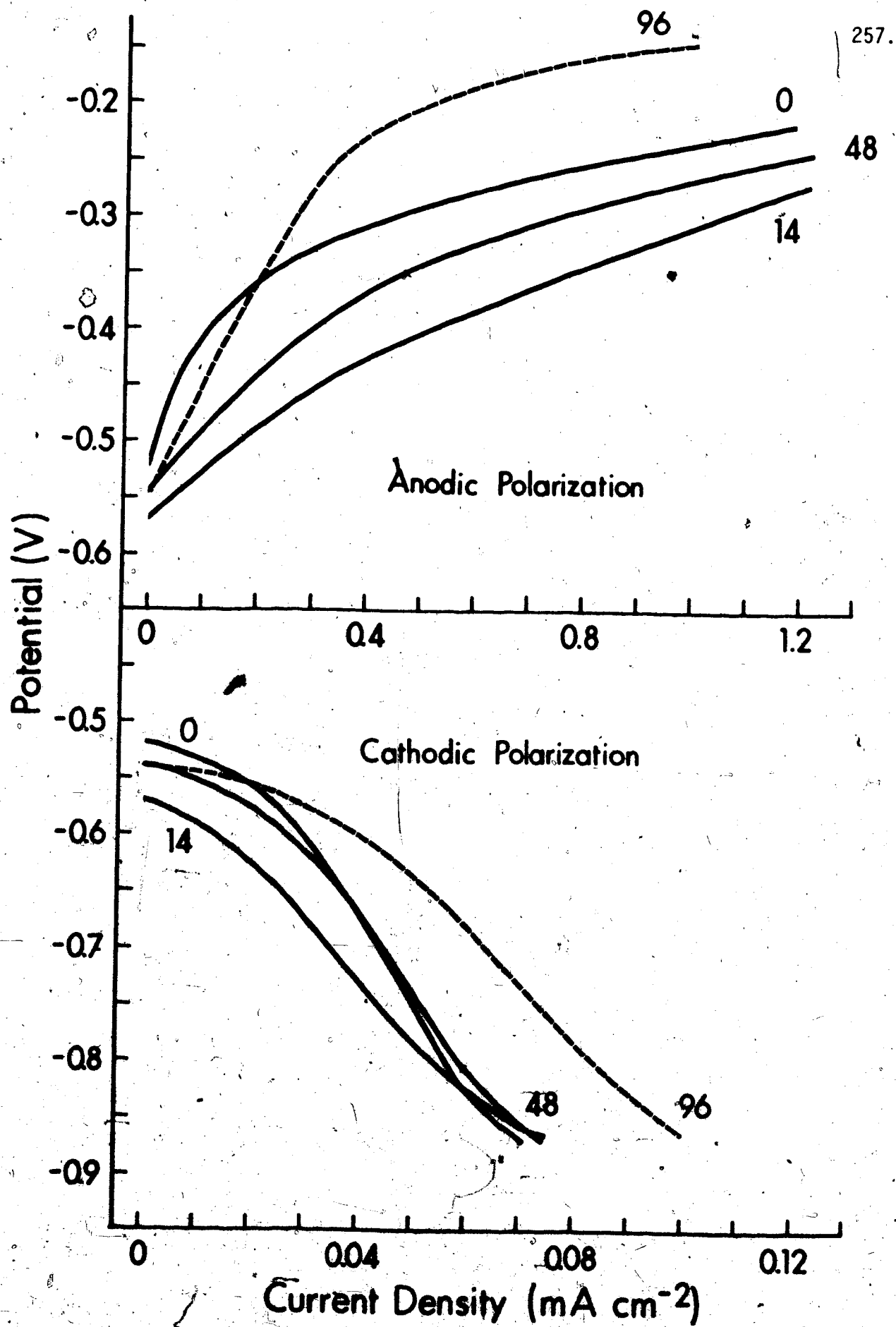
It was thought that the energy source in produced water available to the organism was limited, and, therefore, the activity of the cells would be reduced. The observed loss of anodic depolarization capacity after 14 hours of incubation might be due to the depletion of utilizable energy and a change to a different metabolic process which resulted in the subsequent cathodic depolarization. If, however, the induction of cathodic depolarization was caused by substrate limitation, then it should be abolished by the addition of a sufficient amount of some utilizable energy source such as lactate. Also, the supply of a sufficient energy source in produced water should be able to sustain the bacterial activity so that anodic depolarization would be sustained, too, and not be short-lived as previously observed. As a corollary, if only a limited energy source was added to synthetic (defined) medium the organism should be expected to lose its anodic depolarization capacity and show cathodic depolarization instead.

To test these hypotheses, 600 mg and 300 mg sodium lactate per litre were separately incorporated in the synthetic medium. In another experiment, 900 mg sodium lactate was added to a litre of sterile produced water to boost its total energy content. The polarization characteristics of the mild steel in cultures of Isolate #200 in these media are shown in Figs. 48, 49, 51, 52 and 59. The data shown in Figs. 48 and 49 show that the coupon was still polarized, in the presence or absence of the organism, when 600 mg of lactate was supplied as the sole energy source. However, when the energy source was further decreased to 300 mg lactate per litre (Fig. 50), a slight cathodic depolarization activity was observed especially at higher potentials



Fig. 47. Polarization curves for mild steel in autoclaved produced water inoculated with Isolate #200.

(0, 14, 48, 96 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )



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Fig. 48. Polarization curves for mild steel in synthetic medium  
(containing 600 mg sodium lactate/l) inoculated with Isolate  
#200.

(0, 14, 24, 72, 96 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )

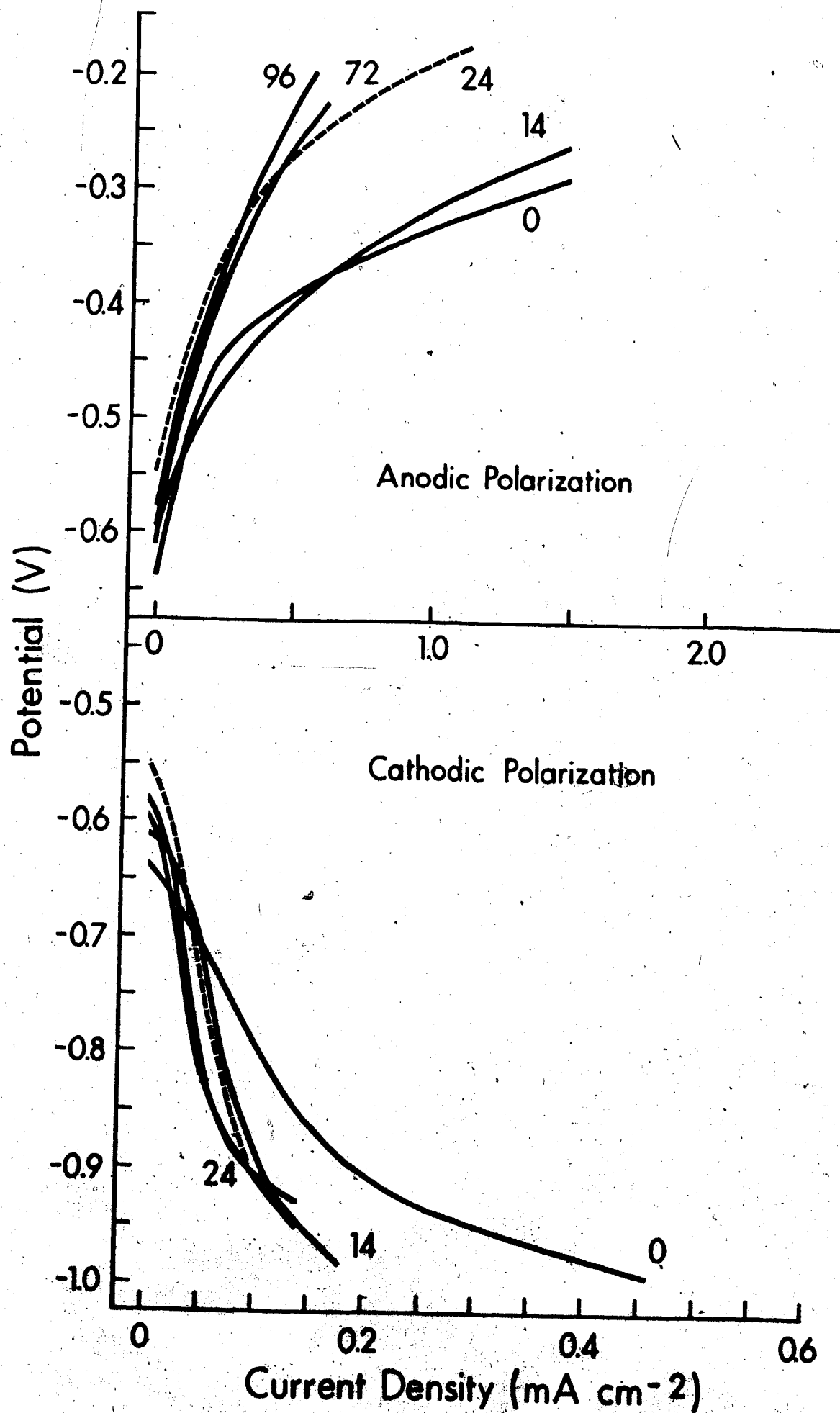


Fig. 49. Polarization curves for mild steel in uninoculated (control) synthetic medium (containing 600 mg of sodium lactate/l). (0, 14, 24, 72, 96 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )

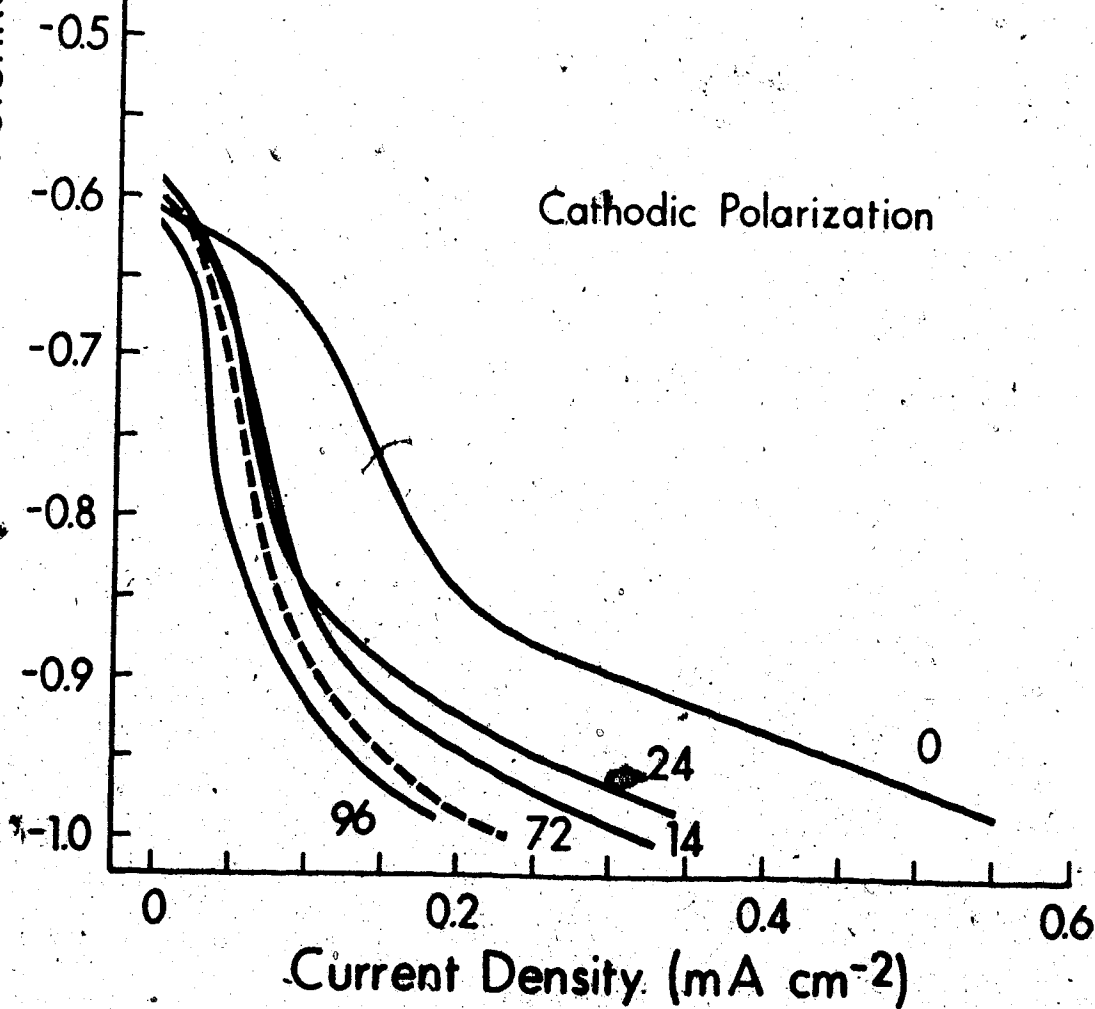
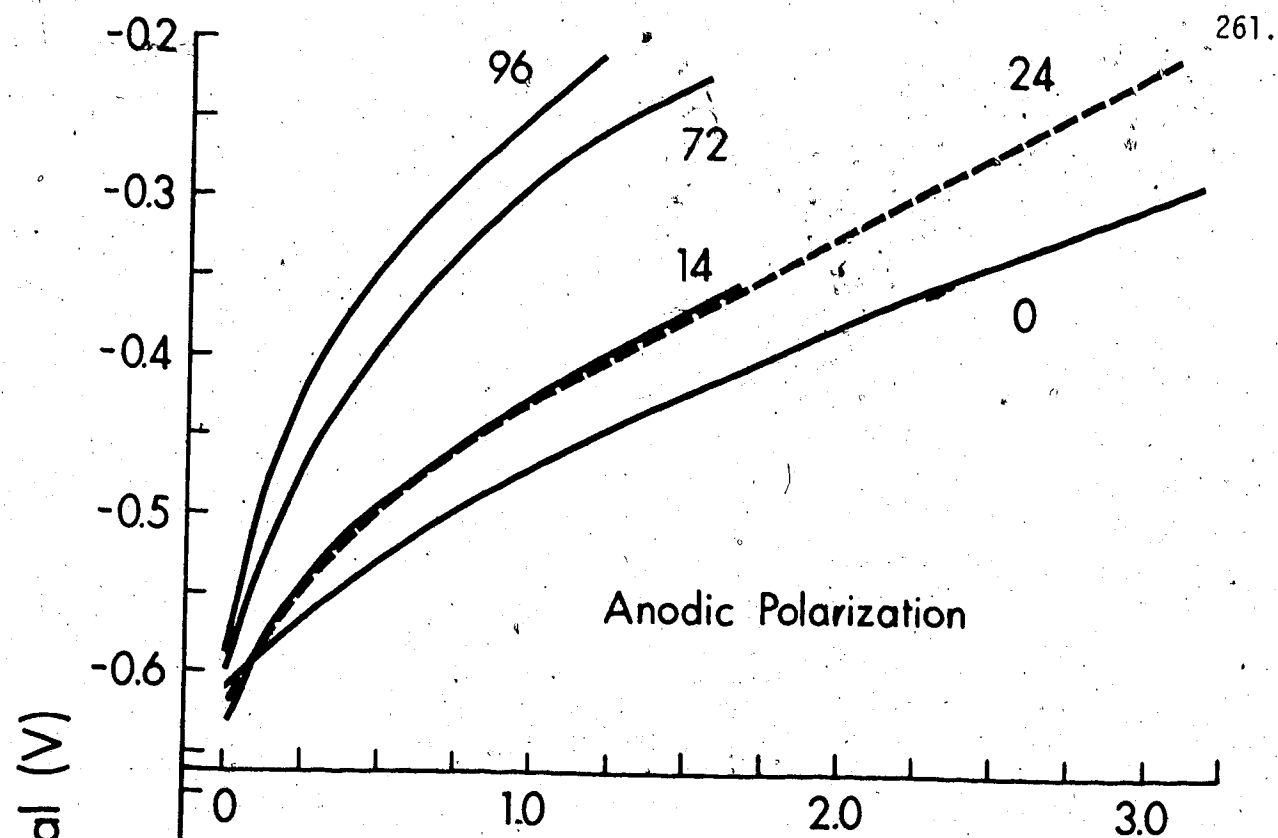


Fig. 50. Polarization curves for mild steel in synthetic medium  
(containing 300 mg sodium lactate/l) inoculated with Isolate  
#200.

(0, 14, 24, 72 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )

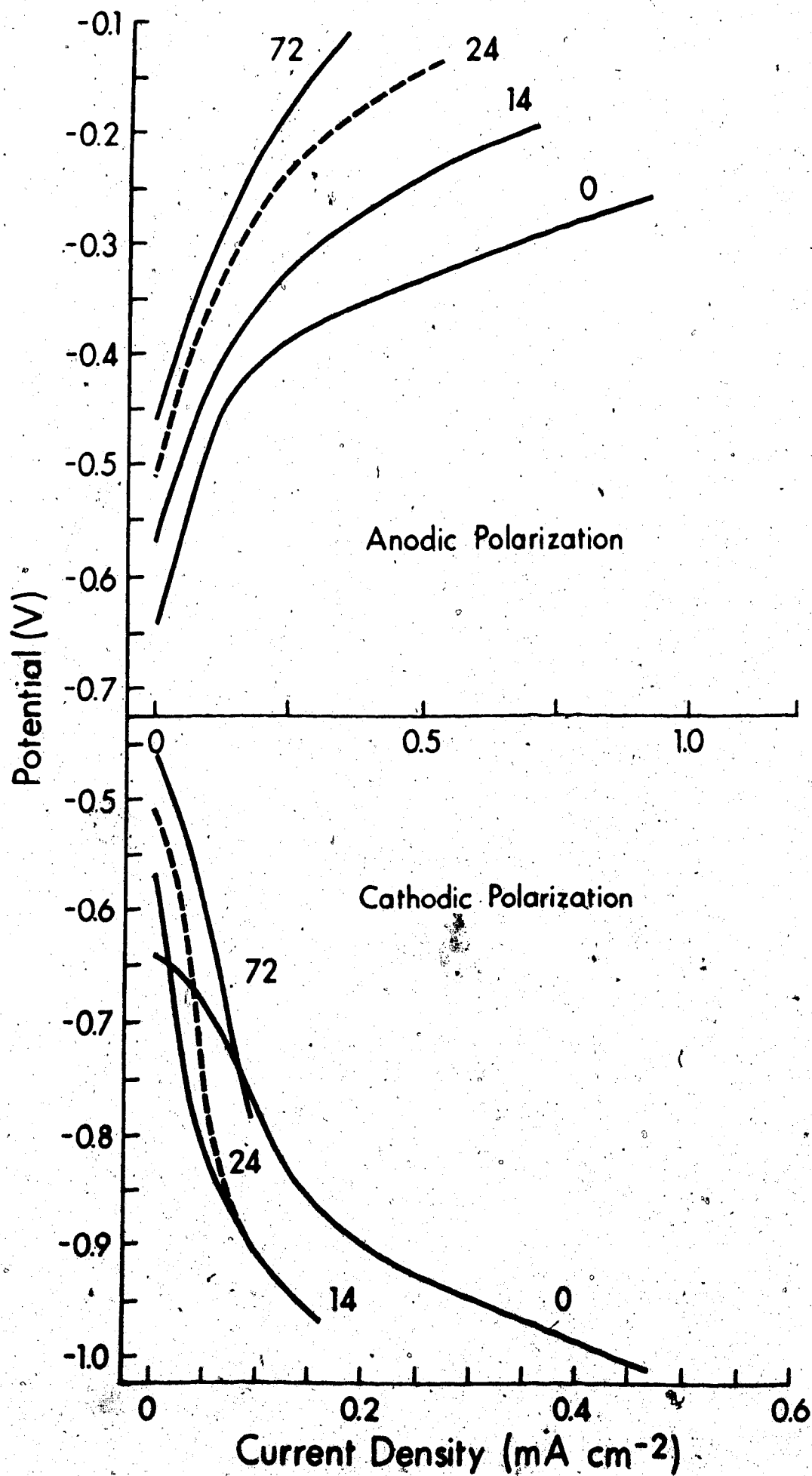




Fig. 51. Polarization curves for mild steel in uninoculated (control) synthetic medium (containing 300 mg sodium lactate/l).  
(0, 14, 24, 72, 96 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )

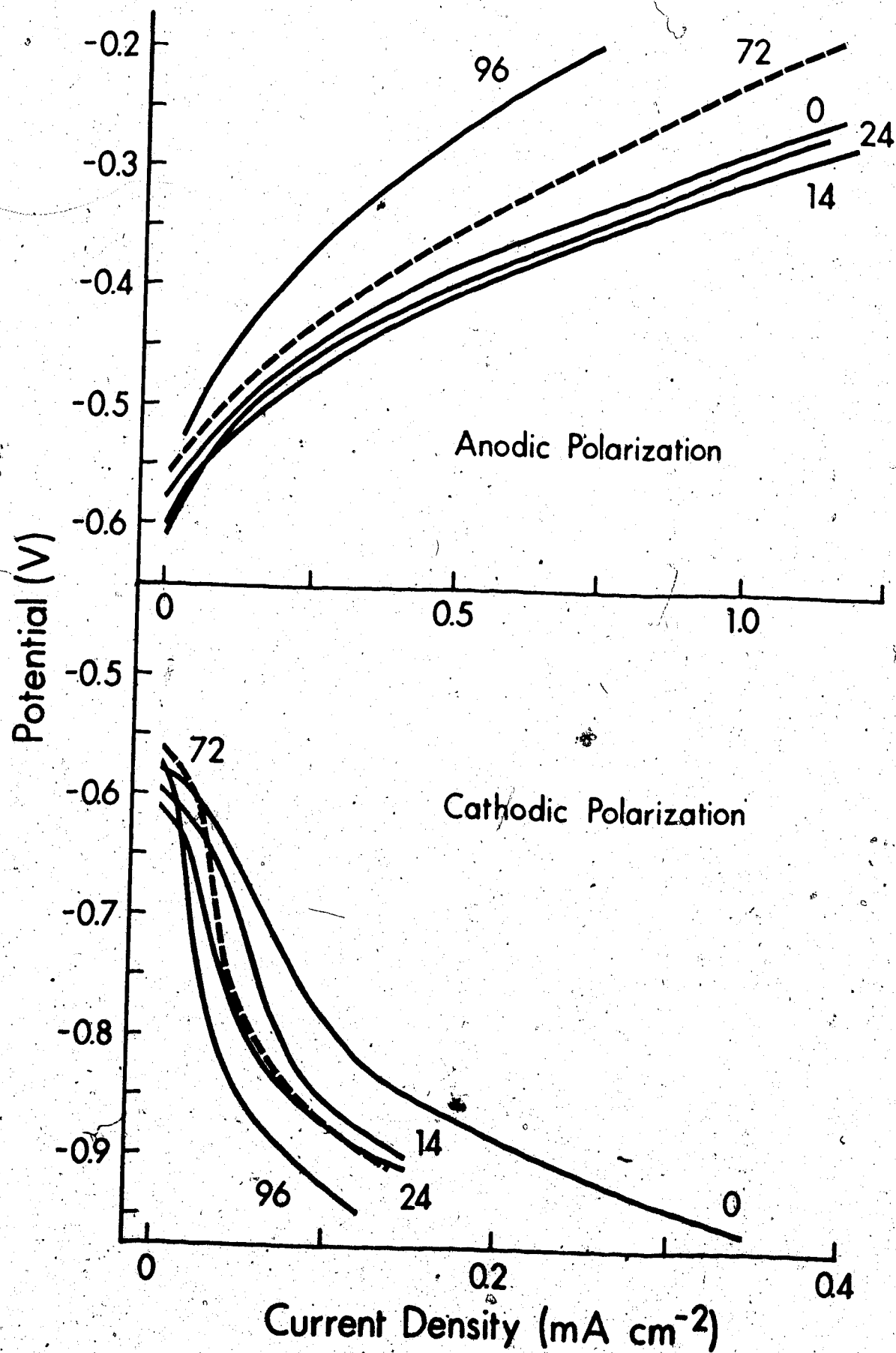
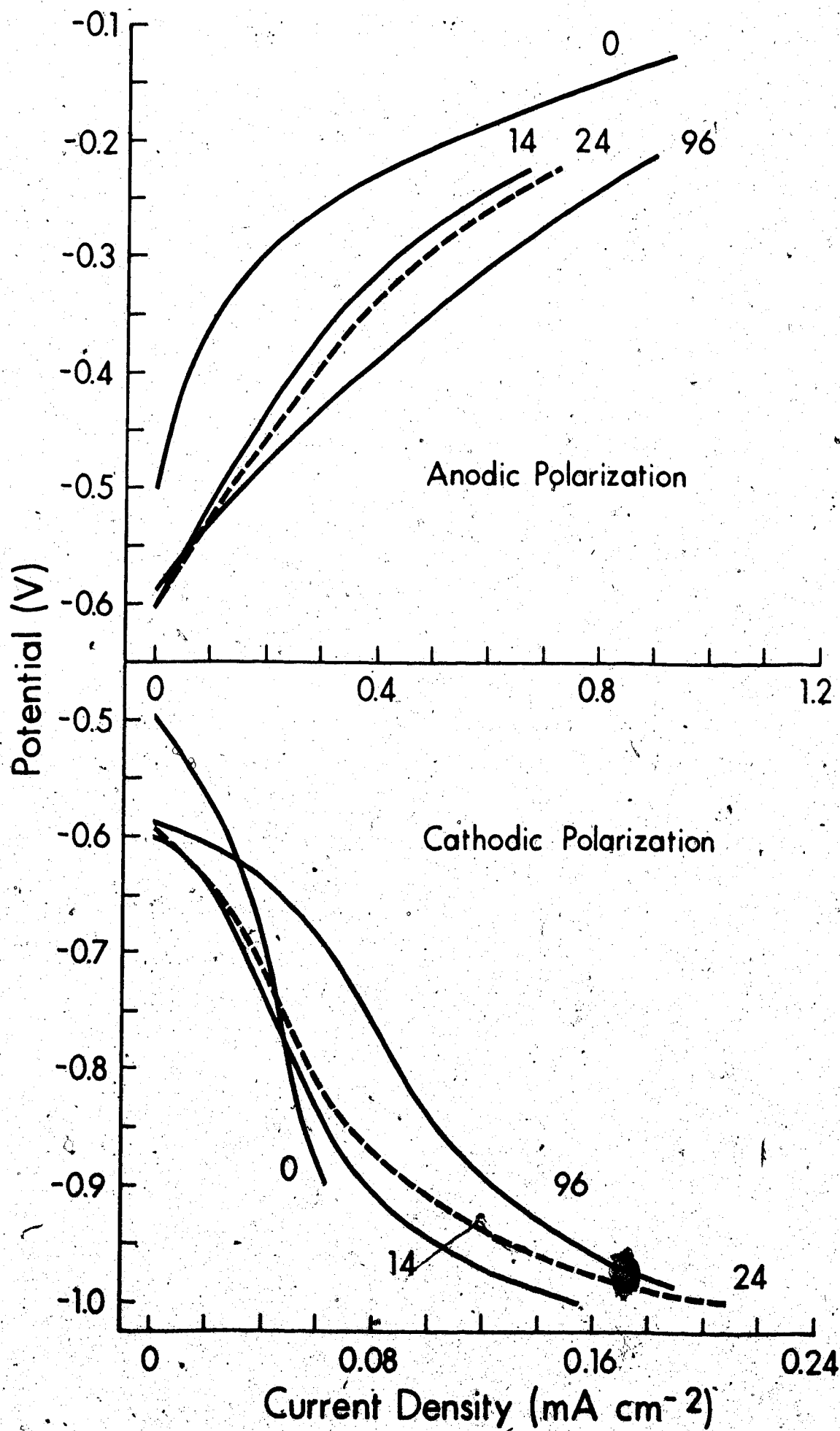


Fig. 52. Polarization of mild steel in produced water modified by the addition of 900 mg sodium lactate/l and inoculated with Isolate #200.

(0, 14, 24, 96 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )



after an initial inhibition. The initial inhibition of the cathodic reaction would, therefore, correspond to a period when the organism was still growing on the available lactate. At the exhaustion of the lactate, the organism probably switched to a different metabolic process which caused the slight cathodic depolarization. In the absence of the organism (Figs. 49 and 51), both electrode processes were inhibited.

When the energy available in the produced water was boosted by the addition of 900 mg lactate per litre, the inhibition of the anodic reaction was abolished (Fig. 52) and active anodic depolarization was sustained. Also, the cathodic depolarization reaction still occurred and was not abolished, as predicted, by the addition of excess energy source. It can, therefore, be concluded that energy limitation in produced water was responsible for the transient anodic depolarization observed earlier. While energy limitation can account for the anodic reaction, it cannot adequately explain the succession of cathodic reactions observed in produced water. Cathodic depolarization was consistently expressed in produced water provided the organism was present. From these experiments it can be ascertained that in produced water medium (natural environment) Isolate #200 is capable of catalyzing both anodic and cathodic depolarization reactions.

The polarization characteristics of mild steel coupon in cultures of Isolate #200 have been studied. In these studies, the organism was grown in four different media. These media were selected to cover, as discussed previously, a range of nutritional and environment variables to which the organism might be exposed in nature.

In all these different media, the presence of the organism (Isolate

#200) caused the depolarization of the anodic process. In produced water, however, both anodic and cathodic depolarization reactions could occur. The operation of both the cathodic and anodic depolarization reactions in inoculated produced water may well explain the severe corrosion reported in the pipeline system carrying the crude oil-water emulsion from which the organism was isolated.

Isolate #200 is essentially an aerobic organism. With the exception of the initial deaeration at the beginning of the polarization runs, no further attempt was made to deaerate the culture. Because of low solubility of  $O_2$  in concentrated solutions such as the culture media employed, these experiments were essentially carried out under microaerobic conditions. The corrosion of the ferrous metal will produce ferrous ions some of which would be oxidized to ferric  $[Fe(III)]$  by the small amount of  $O_2$  present in solution. Ferric compounds are generally insoluble and when deposited on the surface of the metal may prevent further corrosion of the metal by acting as a barrier between the metal and its environment. That the formation of some ferric coating on the surface of corroding metals inhibit the corrosion process is very well known (Logan, 1949; Booth *et al.*, 1963, 1965; Ashton *et al.*, 1973; Olefjord, 1975). Isolate #200 reduces ferric to ferrous ions. In the presence of this organism, as has been amply demonstrated, the anodic reaction was consistently depolarized. It is suggested here that the ability to reduce ferric to ferrous is the single most important factor in the depolarization of the anodic reaction. This theory is supported by the scanning electron micrographs (Plate 3 to 6) which show that in the absence of the organism the surface of immersed coupons were obliterated by a dense crystalline

deposit. In the presence of the organism, however, the surface deposit was parched, exposing large areas of bare metal. The exposure of the metal surface allowed for continued corrosion of the metal, hence the depolarized anodic reaction observed. Since the surface deposition was maximum in the absence of the organism, it is suggested that these deposits are insoluble ferric compounds. The dissolution of these deposits by the organism exposed the bare metal to the medium and allowed continuous anodic dissolution of the metal.

In conclusion, it is suggested that Isolate #200 caused the depolarization of the anodic reaction by preventing the formation of a protective ferric coating of ferric compound(s) on the metal coupon.

#### The Role of Fe(III) Reduction in the Corrosion of Mild Steel

Earlier polarization studies showed that Isolate #200 caused the depolarization of the anodic process. Scanning electron micrographs revealed the different degrees of surface deposition and bacterial attachment on immersed coupons in the presence/absence of the organism. These surface deposits were not readily washed off or dissolved away when exposed to running tap water and were, therefore, considered insoluble. Based on these observations, it was suggested that Isolate #200 caused the depolarization of the anodic reaction by dissolving away a protective ferric film or coating, the organism being capable of reducing ferric to ferrous iron.

It was an assumption, however, that the corrosion-retarding surface coating was ferric since no chemical analysis was conducted to determine the nature of the coat. If, as it was suggested, the depolarization of

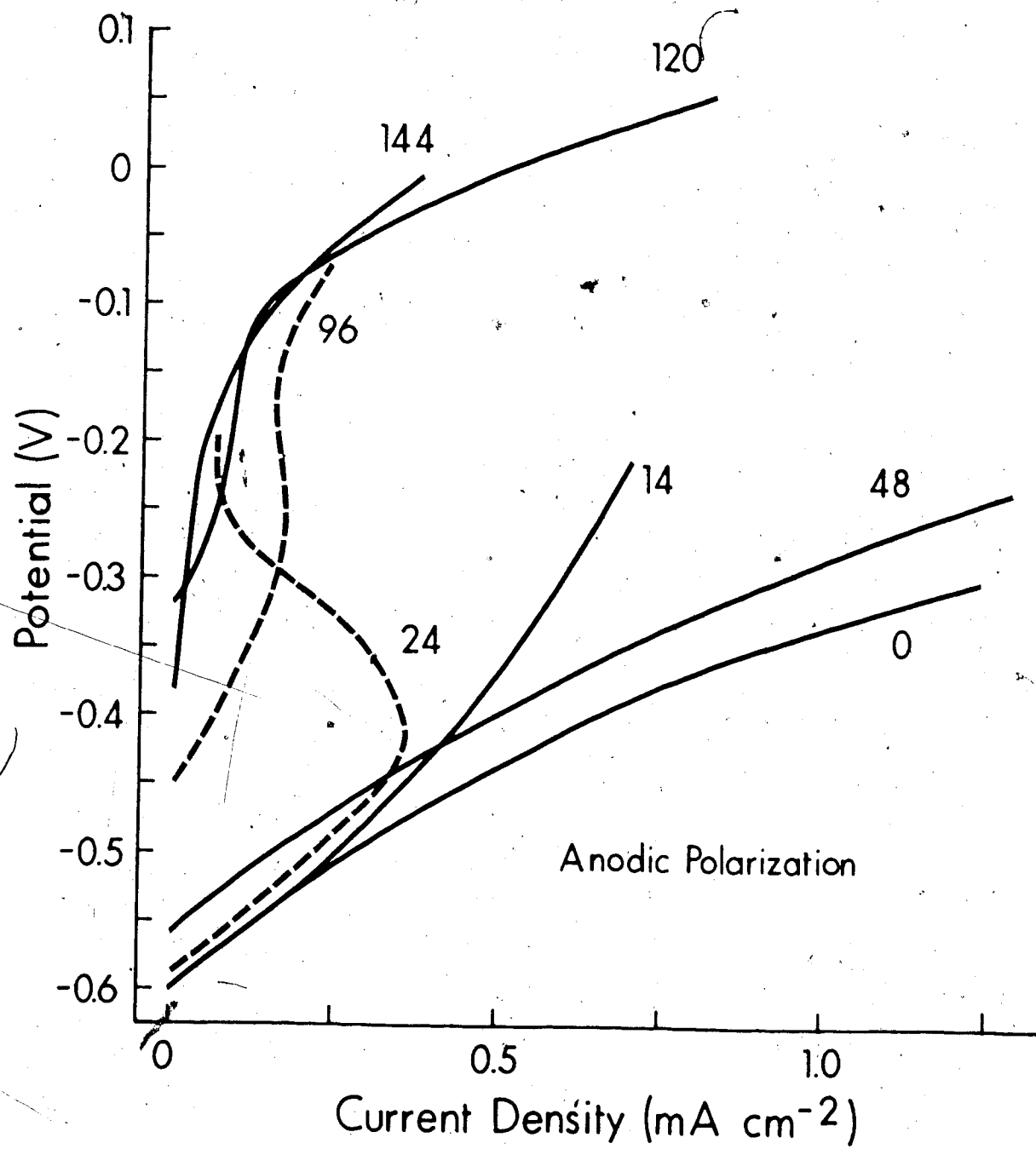
the anodic process was due to the removal of a protective ferric covering, it would be possible to specifically produce a ferric covering on the coupon and observe the depolarization/polarization reactions in the presence/absence of the organism. Protective ferric covering can be conveniently achieved by incorporation of nitrite ( $\text{NO}_2^-$ ) in culture medium. Nitrite is a very strong oxidizing agent which can oxidize Fe(II) to Fe(III) (Moraghan and Buresh, 1977) and its oxidative activity has been widely adopted in the inhibition of corrosion of ferrous metals (Fontana and Greene, 1967; Draper, 1967; Olefjord, 1975; Lumsden and Szklarska-Smialowska, 1978). It is also possible to generate  $\text{NO}_2^-$  *in situ* by the addition of  $\text{NO}_3^-$  to the culture since the organism reduces  $\text{NO}_3^-$  to  $\text{NO}_2^-$ . The advantage of the latter method is that it should be theoretically possible to observe the changes in corrosion rate as the organism transforms  $\text{NO}_3^-$  to  $\text{NO}_2^-$  and as the ferric products are reduced to the soluble ferrous iron. The inhibitory effect of  $\text{NO}_2^-$  is due to the formation of protective ferric film on the metal surface (Draper, 1967; Olefjord, 1975).

#### $\text{B}_{10}$ medium + $\text{NO}_3^-$

The data in Fig. 53 show anodic polarization curves for mild steel in  $\text{B}_{10}$  medium containing  $\text{KNO}_3$  (1 g/l), inoculated with Isolate #200. Evidently, the anodic current decreased continually within the first 24 hours of incubation. Moreover, the mild steel coupon showed a clear case of passivation, with minimum anodic current density of less than  $0.1 \text{ mA cm}^{-2}$  at 24 hours. However, with further incubation the metal lost its passivity and the anodic current soared markedly at 48 hours of incubation. Subsequently, there was a general decline in the anodic



Fig. 53. Anodic polarization curves of mild steel in B<sub>10</sub> medium containing KNO<sub>3</sub> (1 g/l) inoculated with Isolate #200. (0, 14, 24, 48, 96, 120, and 144 denote the incubation time (hr) at 25 ± 2°C)



current.

Comparison of the anodic polarization curves at 24 and 96 hours shows a marked difference in the behaviour of the mild steel. During these two periods the metal showed varied degrees of passivity: at 24 hours the critical passivating potential was  $-0.41$  V but shifted to the more noble potential of  $-0.25$  V after 96 hours. The critical anodic current density at 24 hours was  $0.35 \text{ mA cm}^{-2}$  and double that ( $0.15 \text{ mA cm}^{-2}$ ) obtained after 96 hours of incubation. The shift of the critical passivating potential from a lower to a more positive value in the culture of the organism would indicate that it was becoming more difficult to passivate the metal. The observation that the metal showed an upsurge of anodic corrosion current at 48 hours of incubation also showed that the passive film previously formed at the 24 hours of incubation was destroyed and could not be reformed immediately.

The open circuit potential of the mild steel rose from  $-0.6$  V to  $-0.32$  V, an increase of  $+0.25$  V. The change in the open circuit potential in the positive direction indicated that the mild steel became more noble with time.

#### Butlin's medium + $\text{NO}_3^-$

When nitrate was added to inoculated Butlin's medium the anodic polarization curves of the mild steel in the medium revealed an initial inhibition of the anodic reaction (Fig. 54) within the first 48 hours. Anodic inhibition was succeeded by very active depolarization reactions at the anode. The metal was not passivable in Butlin's medium though it was in the  $\text{B}_{10}$  medium. The difference in the passivability of the


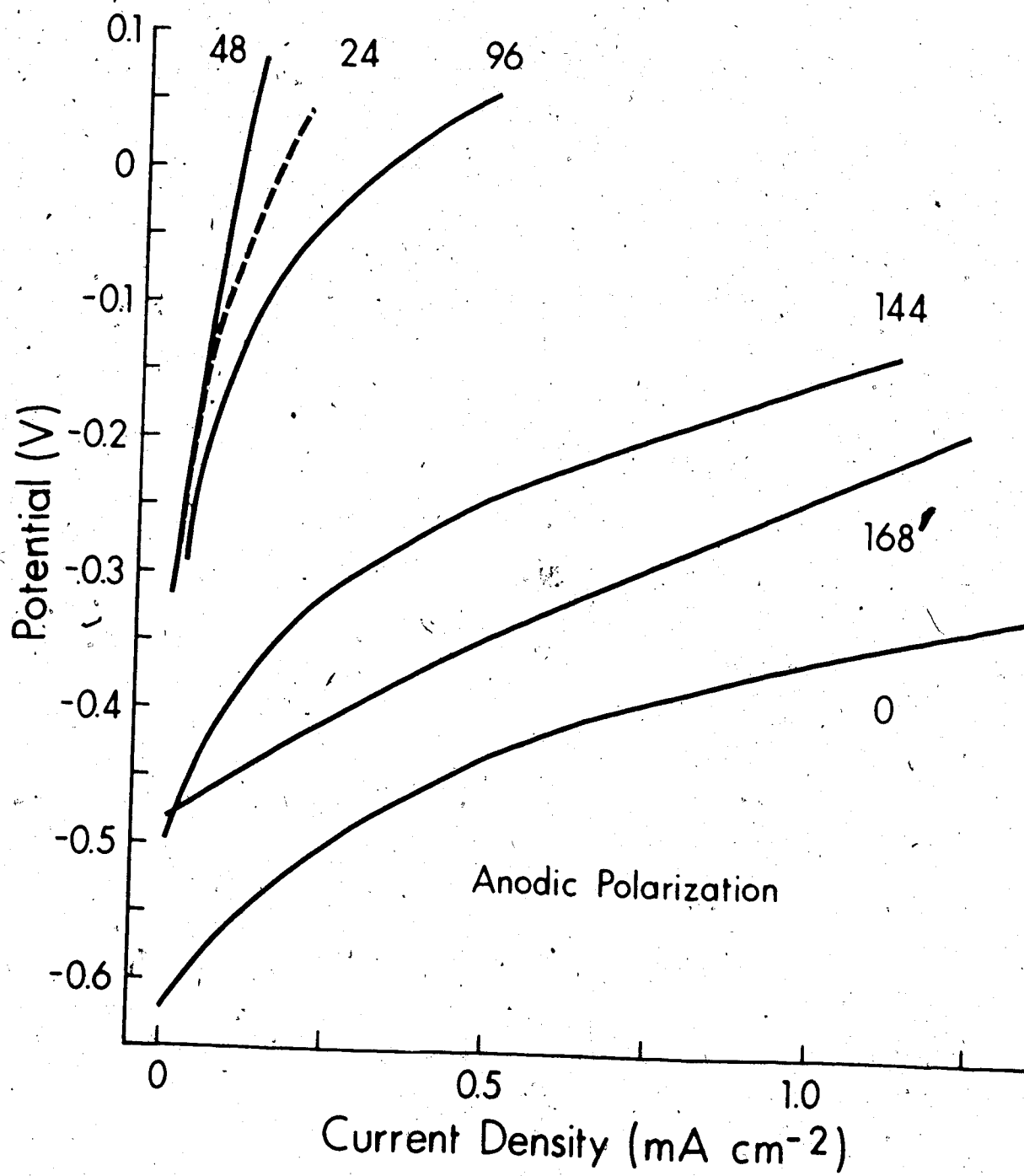


Fig. 54. Anodic polarization curves for mild steel in Butlin's medium containing  $\text{KNO}_3$  (1 g/l), inoculated with Isolate #200. (0, 24, 48, 96, 144 and 168 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )

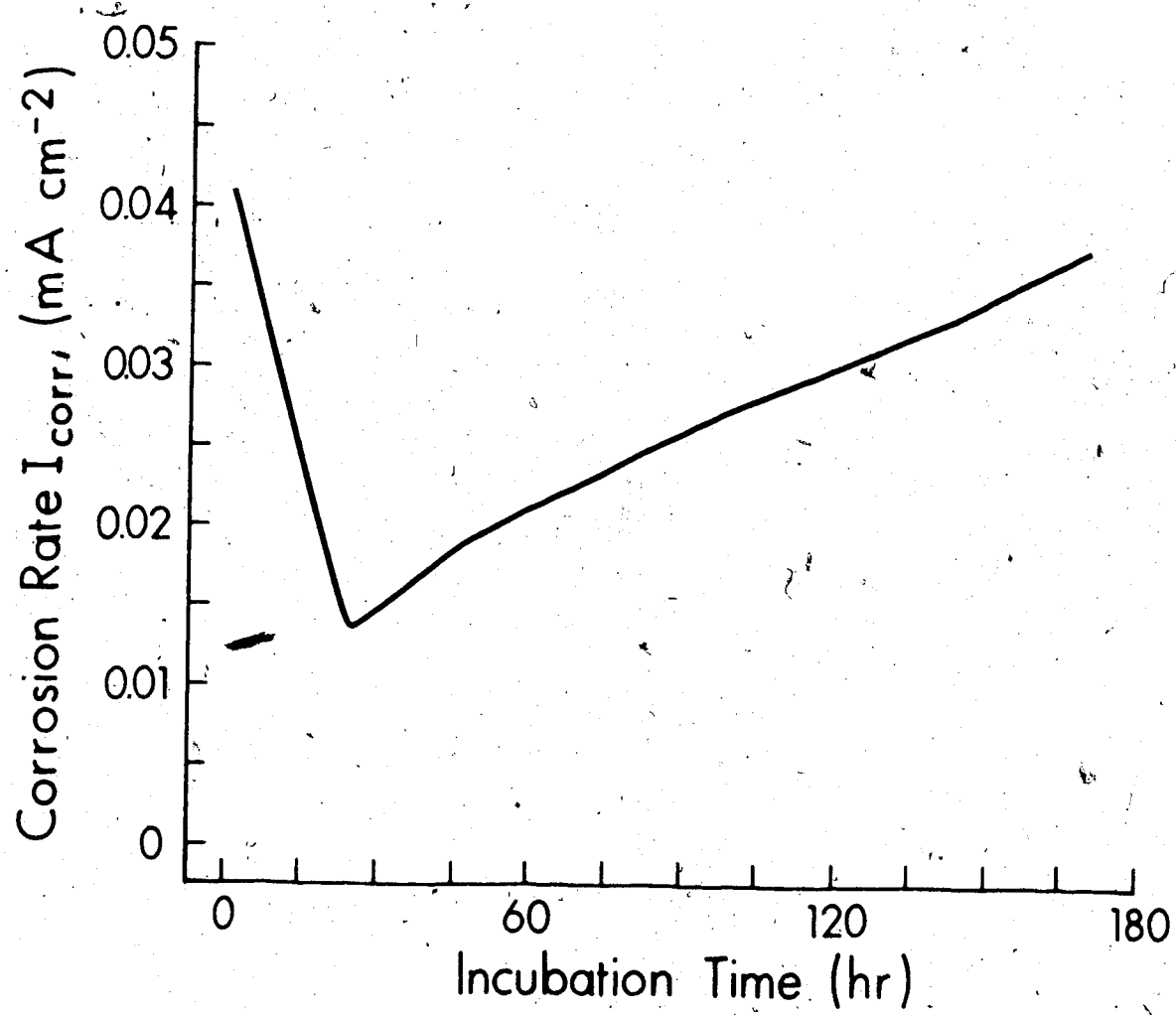


metal in B<sub>10</sub> and Butlin's medium may be accounted for by the total oxidizing power of the media. The oxidizing power of B<sub>10</sub> will be the sum of the oxidizing powers of Fe(III) and the NO<sub>2</sub><sup>-</sup> and would be higher than that of Butlin's medium which contained only the NO<sub>2</sub><sup>-</sup>. Strong oxidizing solutions passivate metals more easily than non-oxidizing solutions (Fontana and Greene, 1967). Therefore, one method by which Isolate #200 can facilitate the corrosion of ferrous metals is by preventing passivation of the metal by reducing the total oxidizing power of the environment because Fe(III) is reduced to the less oxidizing Fe(II).

The changes in the corrosion rate of the coupon in inoculated nitrate-containing Butlin's medium are shown in Fig. 55. Corrosion rates were determined by extrapolation of the anodic and cathodic Tafel slopes and the intercepts were the corrosion currents/cm<sup>2</sup>. As shown in Fig. 55 the corrosion rate was initially high but decreased and rose sharply and almost linearly. The changes in the corrosion rate corresponded to the changes in the anodic current as shown in the anodic polarization curves (Fig. 54). The similarity between the trends in anodic current curves and the total corrosion rate indicated that the changes in the anodic reaction, rather than the cathodic reaction, controlled the overall corrosion of the coupon in the presence of Isolate #200 under the experimental conditions.

The open circuit potential changes observed in the nitrate-containing Butlin's medium differed markedly from those already described for B<sub>10</sub>. The potential initially increased to more noble values (from -0.6 V to -0.32 V) within 2 days and subsequently decreased to more negative values (from -0.32 V to -0.5 V). These changes (from active to noble

Fig. 55. Changes in corrosion rate of mild steel in Butlin's medium containing  $\text{KNO}_3$  (1 g/l) inoculated with Isolate #200. Corrosion rate,  $i_{\text{corr}}$ , was estimated by the extrapolation of the Tafel slopes.





and finally to active) in the potentials of the coupon also corresponded to the observed trend in the corrosion rates, as shown in Fig. 55. Such changes in potential have been reported in bacterial corrosion (Wormwell and Farrer, 1952) and were indicative of the changes in the corrodibility of the metal.

Butlin's medium + NO<sub>2</sub><sup>-</sup>

When nitrite, instead of nitrate was incorporated in the Butlin's medium culture of Isolate #200, the polarization curves obtained are shown in Fig. 56a, b. There was immediate passivation of the coupon on immersion since only very low anodic current was observed at lower potential. It is known that exposure of steel to strong oxidizing solutions (e.g. chromate and nitrite) cause immediate passivation (Fontana and Greene, 1967; Olefjord, 1975). Further increase in potential (see curve, for 0 time) did not increase the anodic current until the potential +0.15 V was reached when there was a sudden rise in the anodic corrosion current. Similar corrosion characteristics have been observed in all passivable metals immersed in oxidizing solutions. The sudden increase in anodic corrosion current corresponded to a shift from passive (low corrosion) to the transpassive state (Fontana and Green, 1967; Steigerwald, 1968; von Fraunhofer, 1974). The transformation from the passive to transpassive states occur because of the breakdown of a protective film produced on the metal. On further incubation, a progressive increase in the corrodibility of the metal ensued. Moreover, the passivity of the metal was lost. This was indicated by the increase in anodic current with increase in imposed potential. If the metal was still in the passive state, increase in

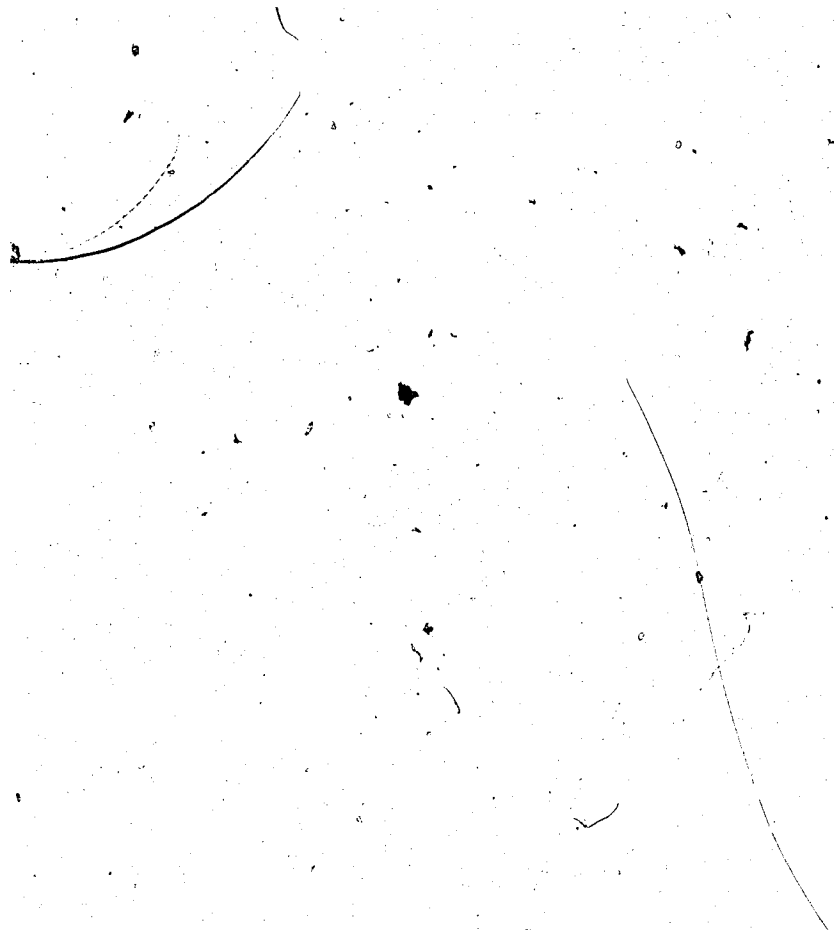


Fig. 56a. Anodic polarization curves for mild steel in Butlin's medium containing  $\text{NaNO}_2$  (0.7 g/l) and inoculated with Isolate #200.

(0, 24, 48, 96 and 168 denote the incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )

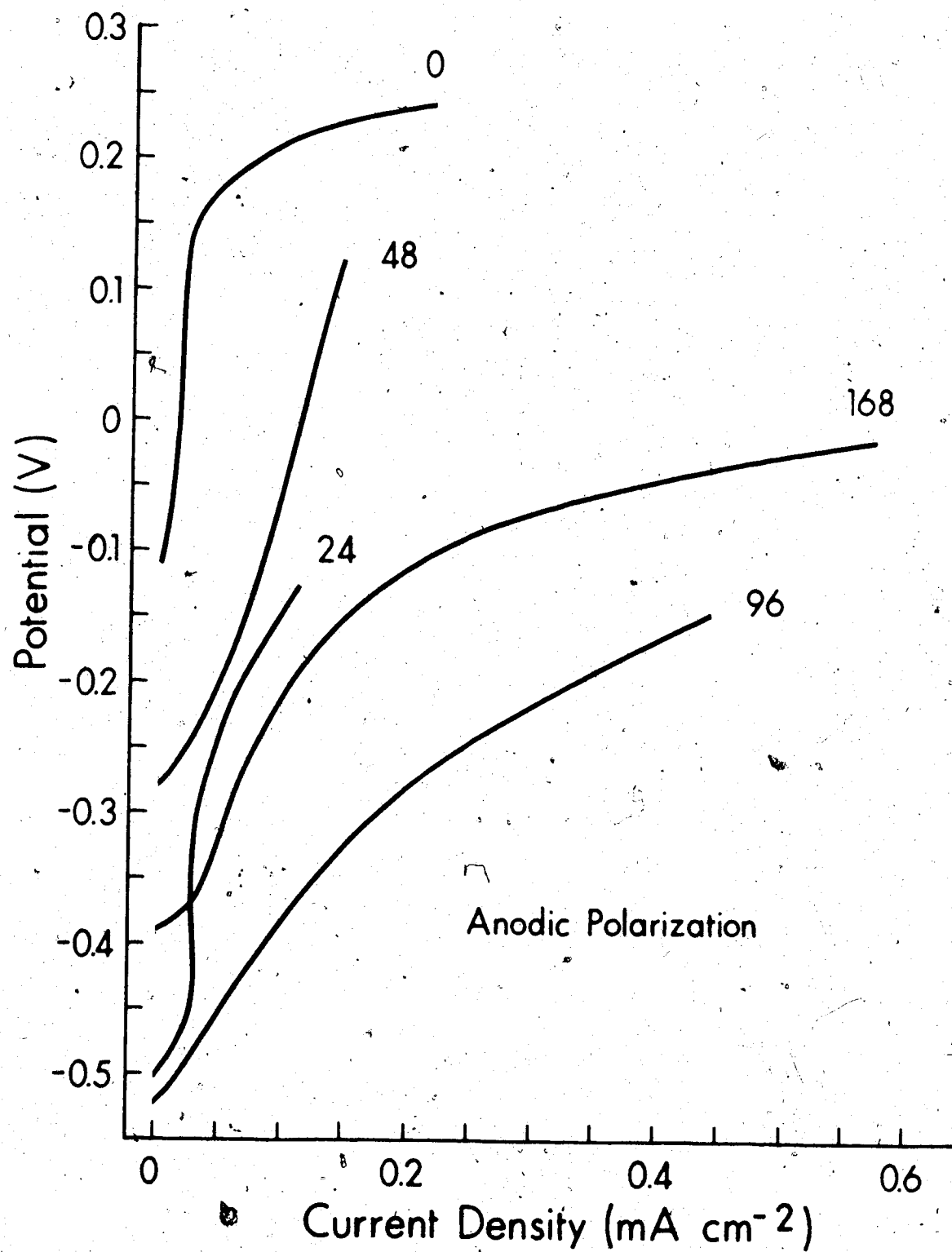
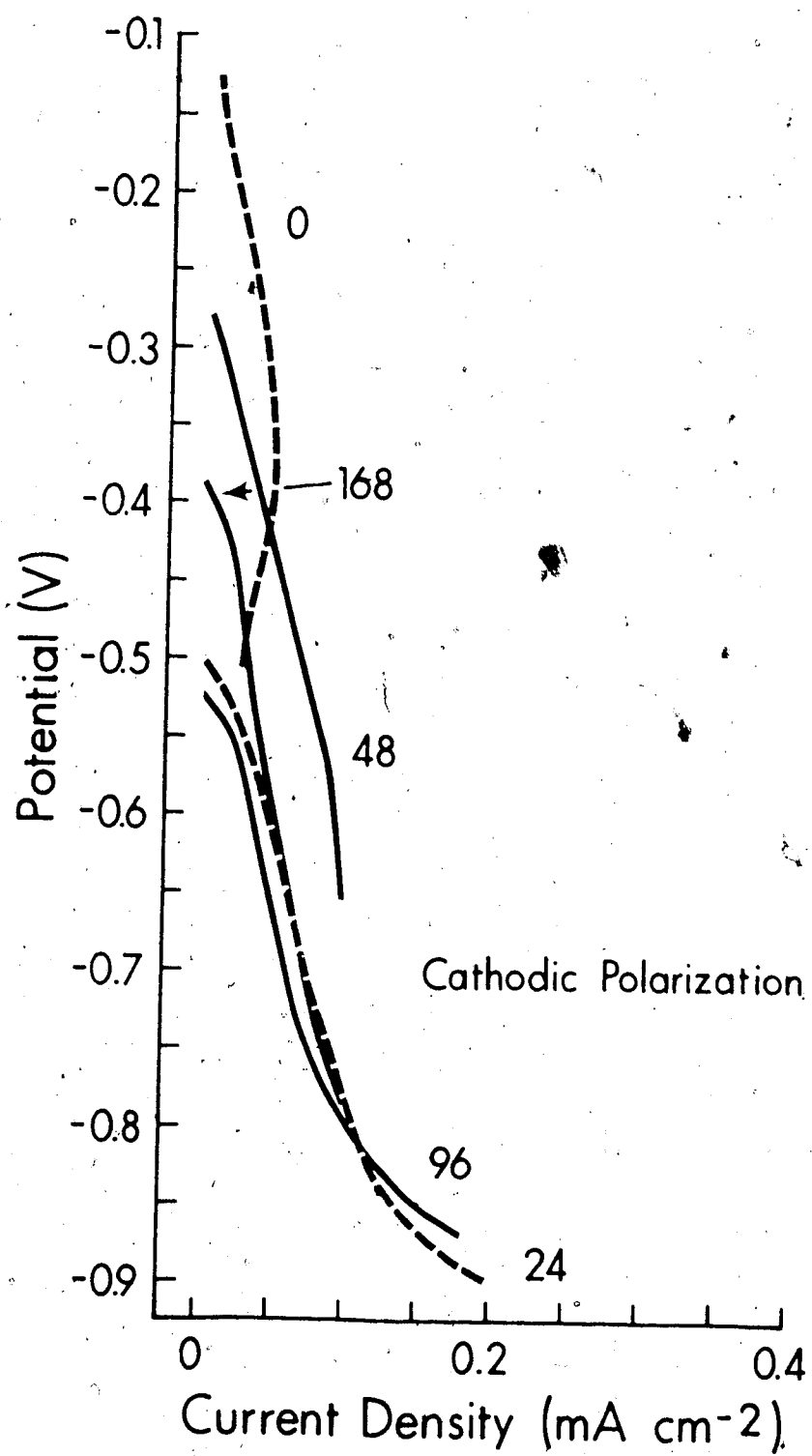


Fig. 56b. Cathodic polarization curves for mild steel in Butlin's medium containing  $\text{NaNO}_2$  (0.7 g/l) and inoculated with Isolate #200. (0, 24, 48, 96 and 168 denote the incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )



the potential imposed would not result in a rise in anodic current until the transpassive zone, as shown by the zero time curve. A similar active anodic depolarization was observed in B<sub>10</sub> medium containing nitrite (Fig. 58).

In the absence of the organism (Figs. 57 and 59) the ferrous metal remained passive in the nitrite-containing media. In fact, the anodic current progressively decreased with exposure. Therefore, the integrity of the protective ferric oxide film was destroyed in the presence of the organism, hence the observed depolarization and the accompanying increase in the anodic current.

The open circuit potential decreased sharply from -0.1 V to -0.52 V in the presence of the organism (Fig. 56). This marked the transition from a noble state to a more active state. Contrarily, in the absence of Isolate #200, the potential of the coupon remained in the noble state.

It has been attempted to demonstrate the relationship between reduction of ferric compound and ferrous metal corrosion. Nitrite is a very strong oxidizing agent which is used as an oxidizing inhibitor of ferrous metal corrosion. The inhibitive action stems from the oxidation of the metal surface to form an insoluble ferric oxide film,  $\gamma\text{-Fe}_2\text{O}_3$  (Olefjord, 1975; Drapter, 1967). Provided the integrity of the ferric film is maintained, the metal remains immune to the action of any corrosive environment. From the work reported here, it has been shown that the protectiveness of the ferric film was destroyed in the presence of ferric iron-reducing bacteria and intense corrosion resulted.

The incorporation of nitrate in the growth medium has enabled one

Fig. 57. Polarization curves of mild steel in uninoculated (control)

Butlin's medium containing  $\text{NaNO}_2$  (0.7 g/l).

(0, 24, 48 and 168 denote incubation time (hr) at  $25 \pm 2^\circ\text{C}$ )

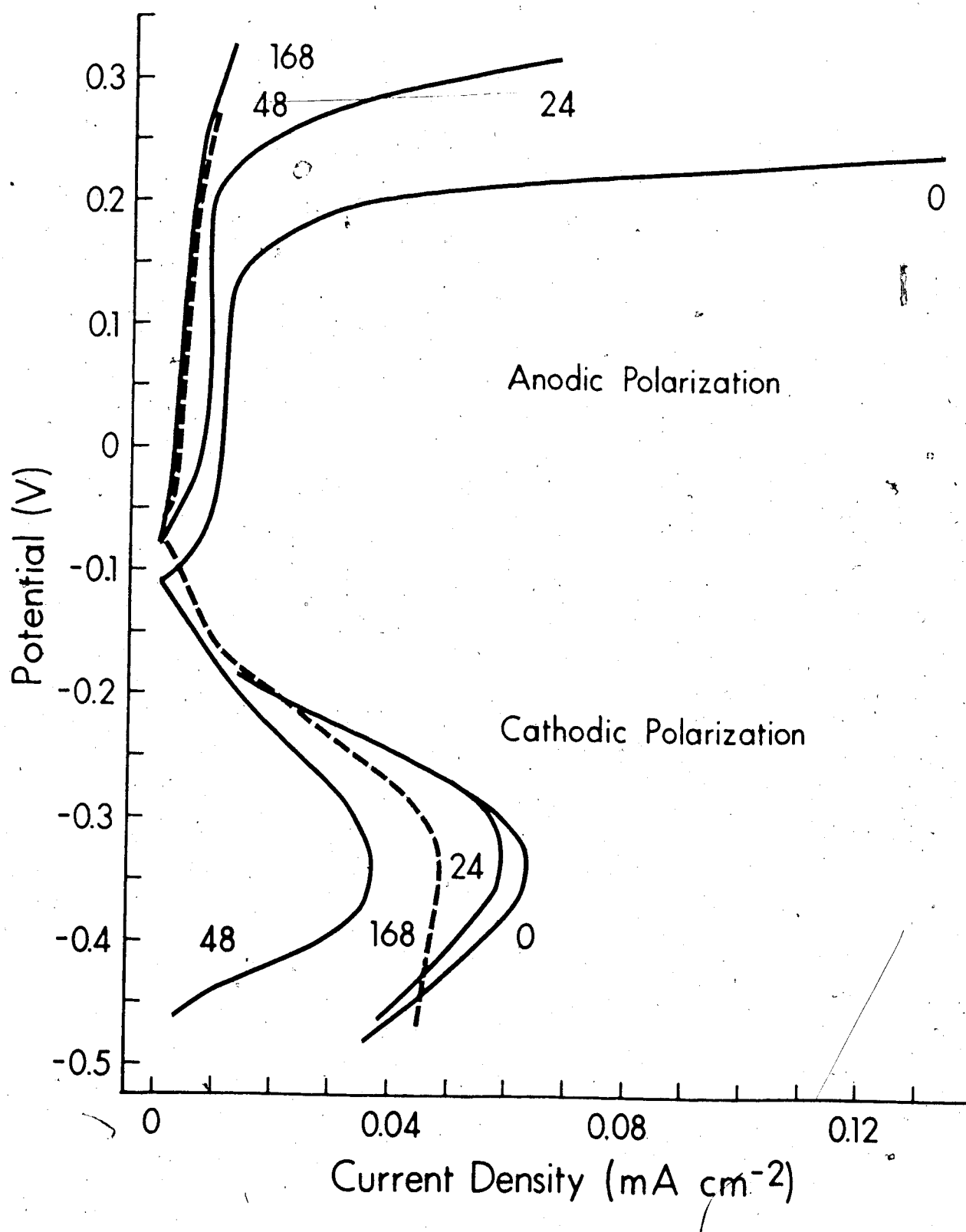




Fig. 58. Anodic polarization of mild steel in B<sub>10</sub> medium containing NaNO<sub>2</sub> (0.7 g/l), and inoculated with Isolate #200. (0, 14, 24 and 48 denote incubation time (hr) at 25 ± 2°C)

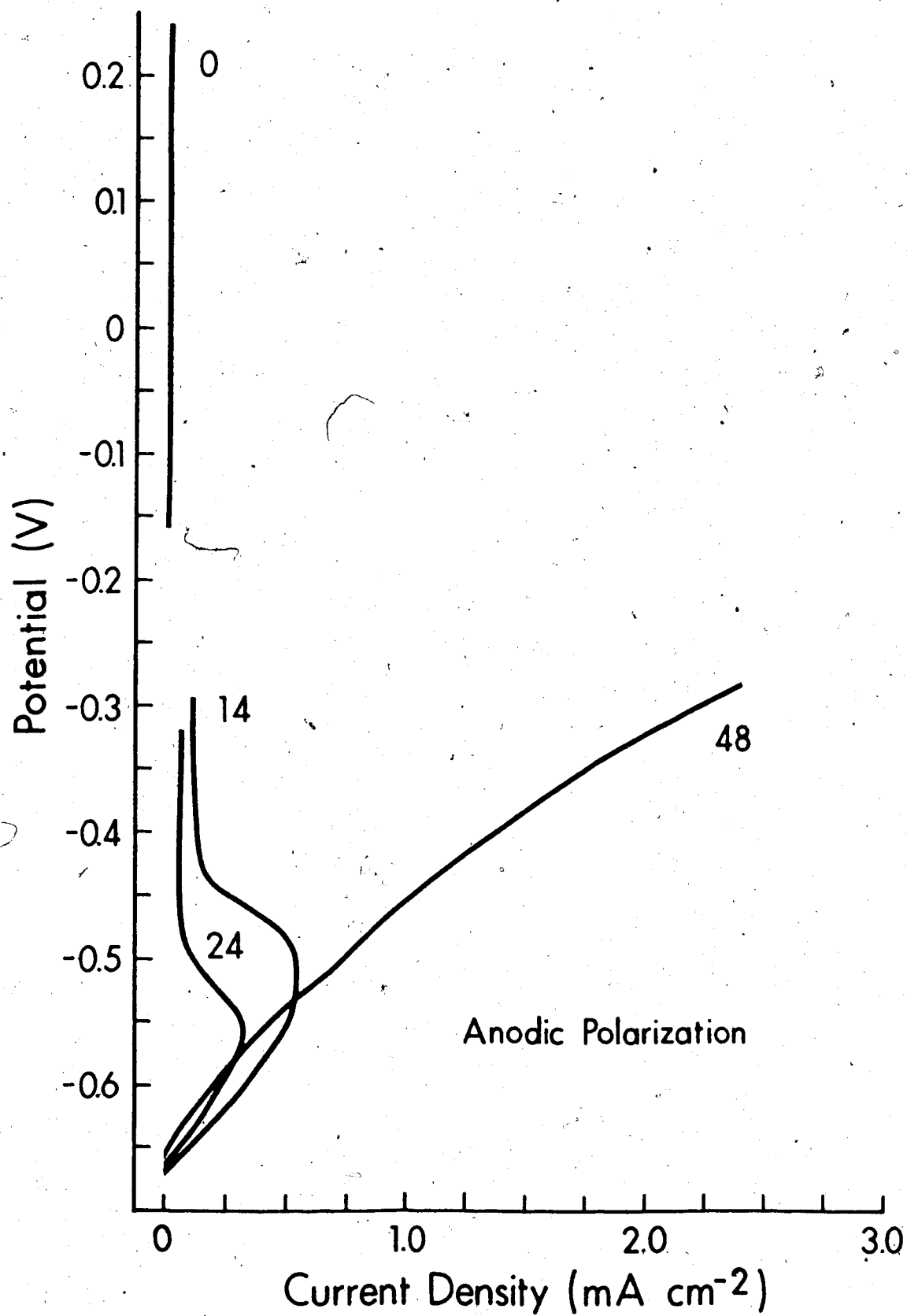
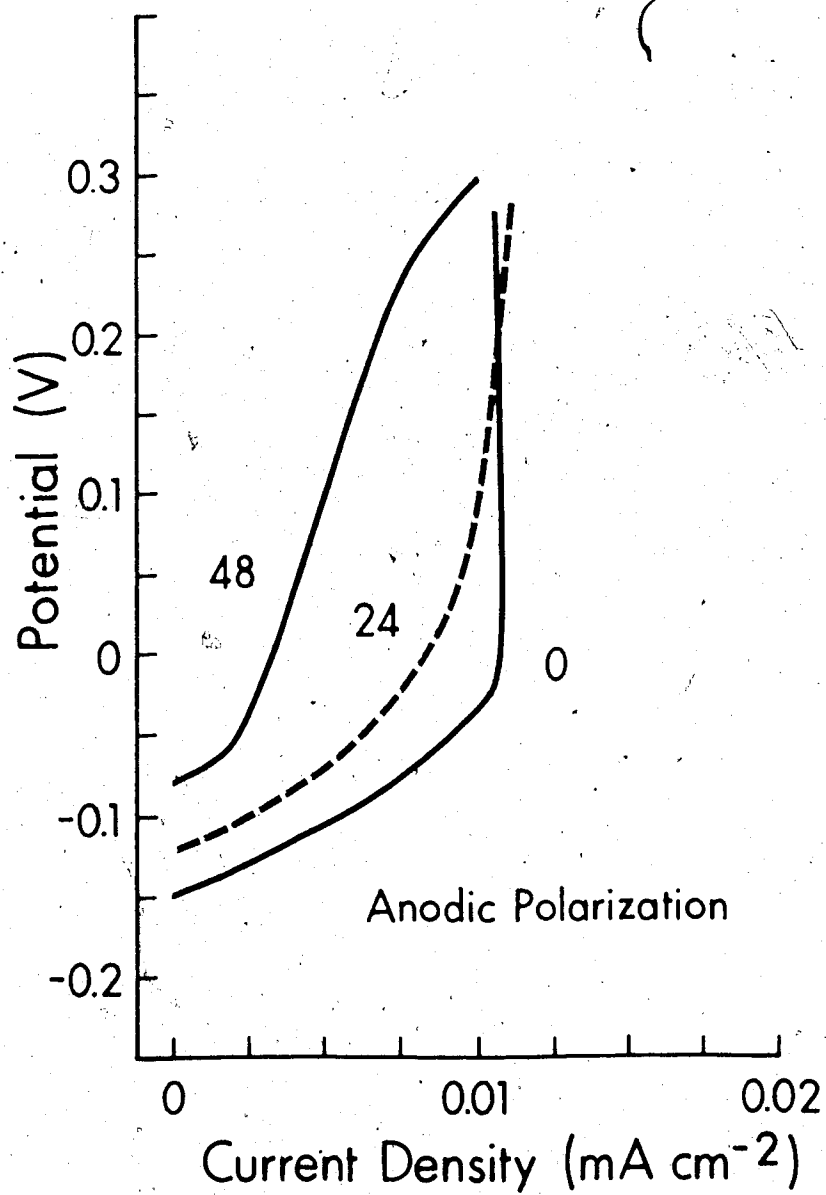


Fig. 59. Anodic polarization curves for mild steel in uninoculated  
(control) B<sub>10</sub> medium containing NaNO<sub>2</sub> (0.7 g/l)  
(0, 24 and 48 denote incubation time (hr) at 25 ± 2°C)



to observe the changes in corrosion rate in the presence of nitrate and the effect of *in situ* generation of nitrite (Fig. 55). The corrosion rate (Fig. 55) was initially high because nitrate is corrosive (Fontana and Greene, 1967), but the subsequent production of nitrite resulted in the oxidation of ferrous iron to ferric iron and produced the characteristic protective  $\gamma\text{-Fe}_2\text{O}_3$  film. Thus, the intense polarization of the anodic process evident between 24 and 48 hours (Fig. 54) was due to nitrite production from nitrate by bacteria. At this stage, the culture solution turned yellowish indicating the preponderance of Fe(III) compounds in the medium. The subsequent depolarization of the anodic reaction (Fig. 56) after 48 hours and an accompanying colour change (from yellow to green) indicated the conversion of ferric to ferrous compounds and the loss of the protective ferric film. Since depolarization reaction occurred only in the presence of Isolate #200 (compared to control - Fig. 56), and this organism reduced Fe(III) to Fe(II) compounds, it can be concluded that the reduction of ferric to ferrous ions was the cause of the depolarization of the anodic process and the concomitant increase in the corrosion rate.

Logan (1945) reported that the corrosion of underground pipes was retarded by the oxidation of corrosion products to form films or thick deposits. Such deposits either reduced the potential difference between the anode and cathode, or posed an electrical resistance which reduced the corrosion current. Also, the retardation of corrosion of iron in estuarine waters of the Thames was reported by Booth *et al.* (1963, 1965). Chemical analysis of the retarding surface film showed that it was composed of the ferric compounds,  $\alpha\text{-Fe}_2\text{O}_3\cdot\text{H}_2\text{O}$ ,  $\gamma\text{-Fe}_2\text{O}_3\cdot\text{H}_2\text{O}$  and  $\text{Fe}_3\text{O}_4$ . A coating of a mixture of ferric and ferrous phosphates

preserved buried ancient nails from corrosive soils (Booth *et al.*, 1962). Removal of such protective ferric compounds would lead to more intense corrosion. It is concluded, therefore, that ferrous metal corrosion as engendered by Isolate #200 is caused by the ability of the organism to dissolve any insoluble protective ferric coating on the surface allowing for further corrosion (oxidation) of the bared metal.

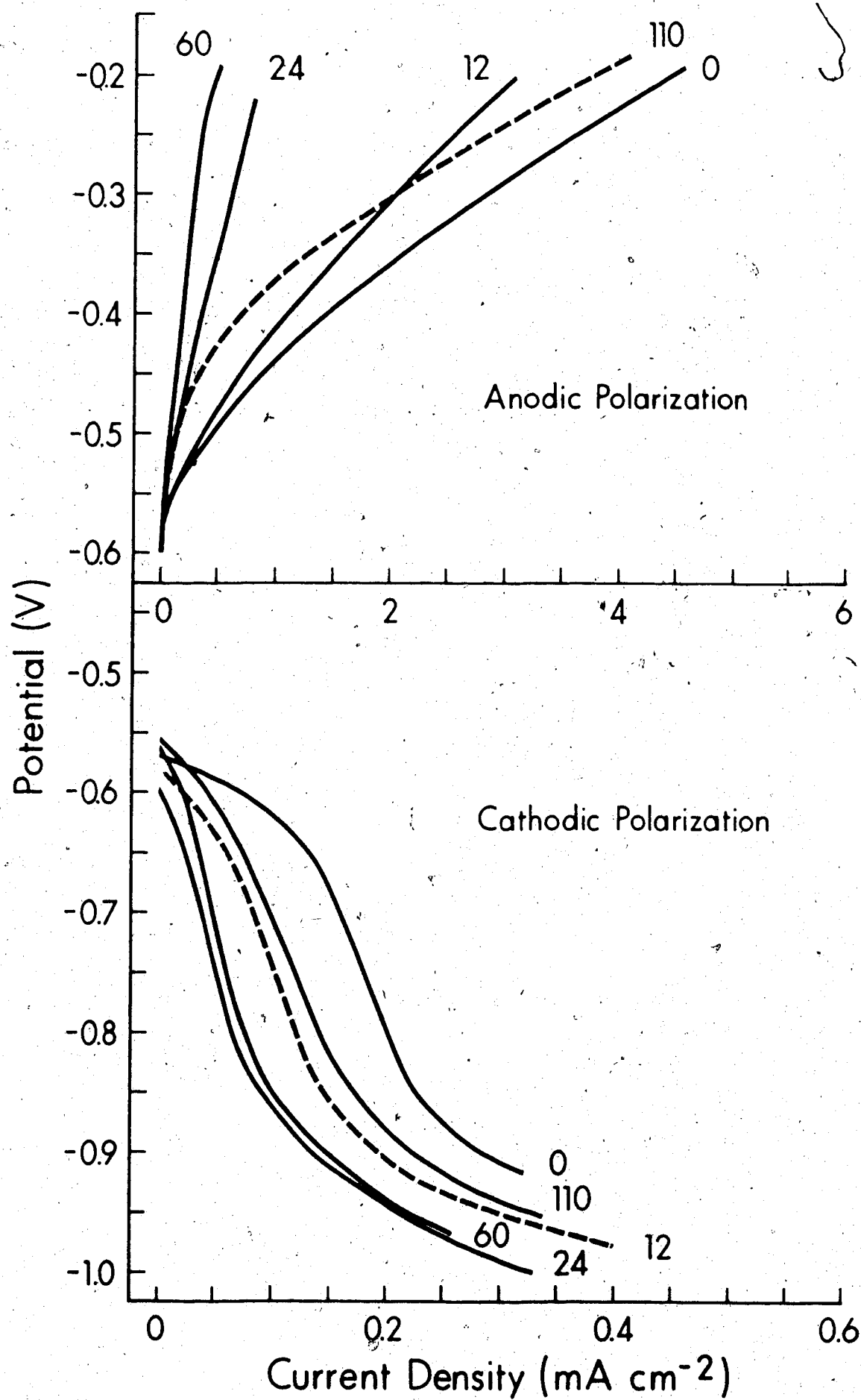
Polarization Characteristics of Mild Steel in B<sub>10</sub> Culture  
of Isolate #200 Containing Reducible Sulphur Compound

This study was undertaken to investigate the effect of simultaneous production of  $S^{2-}$  and Fe(II) on the corrodibility of mild steel. The ability to produce  $S^{2-}$  and reduce Fe(III) are characteristics considered very important in the corrosion of ferrous metals.

B<sub>10</sub> medium +  $S_2O_3^{2-}$

Figure 60 shows the polarization of mild steel in B<sub>10</sub> medium containing sodium thiosulphate as the reducible sulphur compound. Thiosulphate was chosen because it is more stable than  $SO_3^{2-}$ . During the early culture period there was polarization of the anode. However, after about 60 hours of incubation the anodic current started to increase continuously up to the end of the experiment - 5 days. The initial polarization of the anode was thought to be due to the early  $S^{2-}$  formation which reacted with the coupon to form a temporarily adherent FeS coating. Subsequently, the increased production of Fe(II) [from Fe(III)] demobilized any  $S^{2-}$  present in culture solution by forming a precipitate of FeS. The removal of  $S^{2-}$  from the culture would

Fig. 60. Polarization curves of mild steel in  $B_{10}$  medium containing  $Na_2S_2O_3$  ( g/l) and inoculated with Isolate #200. (0, 12, 24, 60, and 110 denote incubation time (hr) at  $25 \pm 2^\circ C$ )

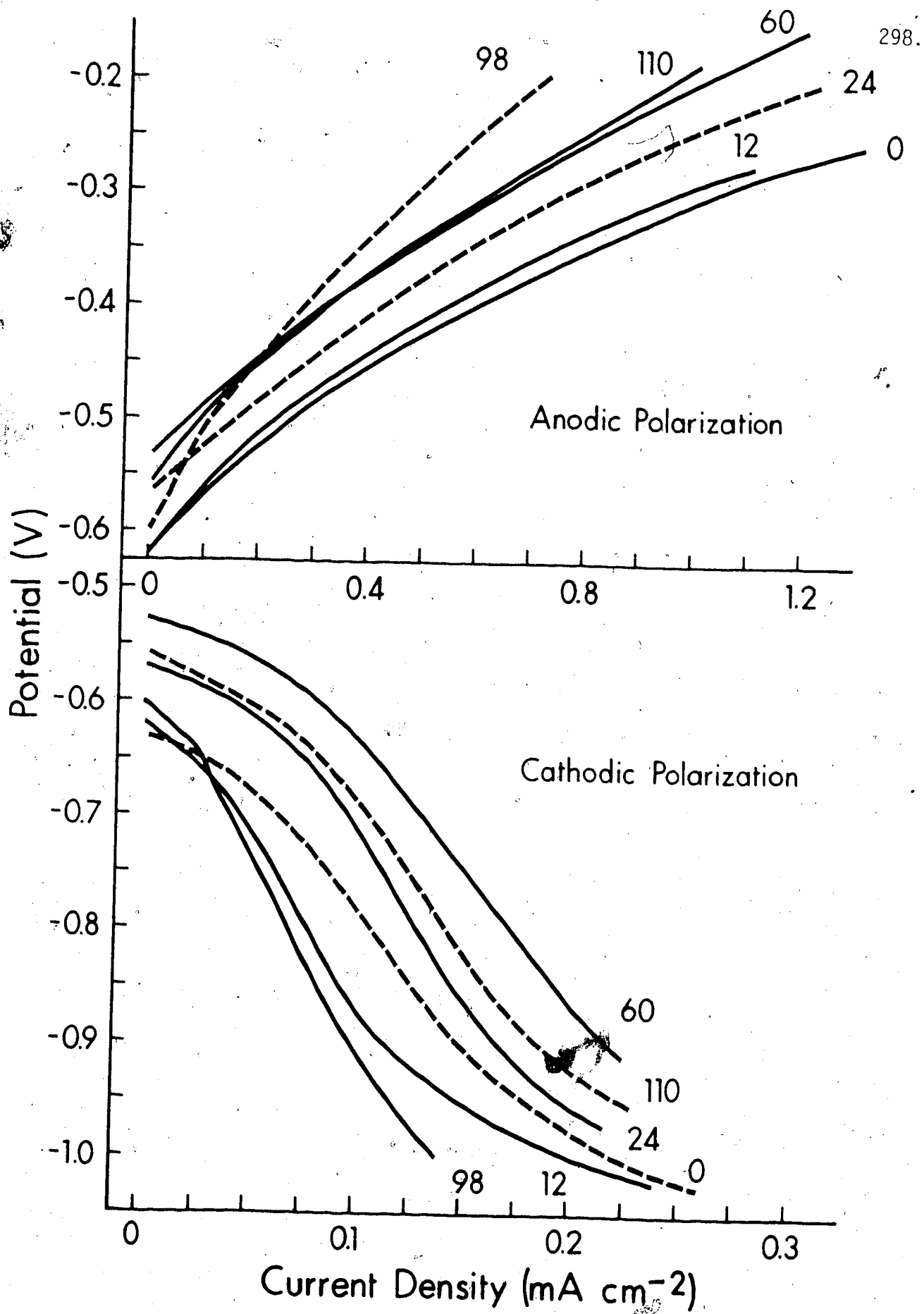




prevent the continued growth of the FeS coating on the coupon and so no protectiveness would be accorded. That this was the case was supported by the observation that coupons immersed in a culture of Isolate #200 in B<sub>10</sub> containing S<sub>2</sub>O<sub>3</sub><sup>2-</sup> were covered by a loose, non-adherent black mass which sloughed off on withdrawal of the coupon from the culture. The sloughing off of the corrosion product revealed the bright metal. Booth *et al.* (1966, 1967) reported on the influence of Fe(II) on the corrosion of mild steel. High levels of Fe(II) in cultures of *Desulfovibrio* prevented the formation of protective FeS coating on the coupon. At the same time the high amount of FeS formed caused depolarization of the cathode. Thus, the slight cathodic depolarization later observed in the B<sub>10</sub> + S<sub>2</sub>O<sub>3</sub><sup>2-</sup> culture of Isolate #200 would be due to the FeS formed in the medium. Corrosivity of soils has been associated with soluble iron (Miller, 1970). Since iron in nature is mostly in the form of insoluble ferric oxide (Gotoh and Patrick, 1974) transformation to the soluble ferrous form would render any such environment corrosive to iron. Miller (1970) reported that soils with Fe(II) content of about 333 µg/g soil or above proved very corrosive to iron and steel but at lower concentrations were non-corrosive.

In the absence of the organism (control) (Fig. 61) the anode was polarized but a slight cathodic depolarization occurred. The cathodic depolarization in the absence of the organism was thought to have been caused by FeS arising from S<sup>2-</sup> formed by reduction of S<sub>2</sub>O<sub>3</sub><sup>2-</sup> at the electrode.

Fig. 61. Polarization curves for mild steel in uninoculated (control)  
B<sub>10</sub> medium containing Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> ( g/l)  
(0, 12, 24, 60, 98 and 110 denote incubation time (hr)  
at 25 ± 2°C)



B<sub>10</sub> without Fe + S<sub>2</sub>O<sub>3</sub><sup>2-</sup>

As a general control to the effect of simultaneous production of S<sup>2-</sup> and Fe(II), B<sub>10</sub> medium was modified in such a way as to be lacking in Fe(III). In this way only the effect of S<sup>2-</sup> production could be assessed.

The anodic and cathodic polarization curves for mild steel in the modified B<sub>10</sub> [no Fe(III)] are shown in Fig. 62. There was a slight stimulation of anodic process within the first 12 hours but the coupon rapidly became polarized. Copious production of H<sub>2</sub>S was very apparent after 12 hours of incubation. This was deduced by the intense characteristic (rotten-egg) smell of H<sub>2</sub>S. It is noteworthy that no smell emanated from the normal B<sub>10</sub> culture. Presumably the formation of FeS in the normal B<sub>10</sub> culture prevented the release of H<sub>2</sub>S. It was this release of H<sub>2</sub>S and the subsequent reaction with the coupon that was thought to have caused the initial stimulation of anodic dissolution. The stimulatory effect of S<sup>2-</sup> on anodic dissolution of mild steel was earlier reported by Wanklyn and Spruit (1952), Wormwell and Farrer (1952) and Hoar and Farrer (1961). Although Booth and Tiller (1960) reported a general decrease in the anodic reaction, these workers did observe an initial stimulation of the anodic process before the subsequent decline.

Unlike the anodic process, there was a sustained depolarization of the cathode. Depolarization of the cathode must have been caused by the FeS formed from the initial sulphide-stimulated anodic dissolution observed. In the absence of the organism (uninoculated control - Fig. 63) both the anode and the cathode were polarized.

Fig. 62. Polarization curves for mild steel in B<sub>10</sub> medium without added FePO<sub>4</sub>, containing Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> ( g/l) inoculated with Isolate #200.  
(0, 12, 24, 60 and 108 denote incubation time (hr) at 25 ± 2°C)

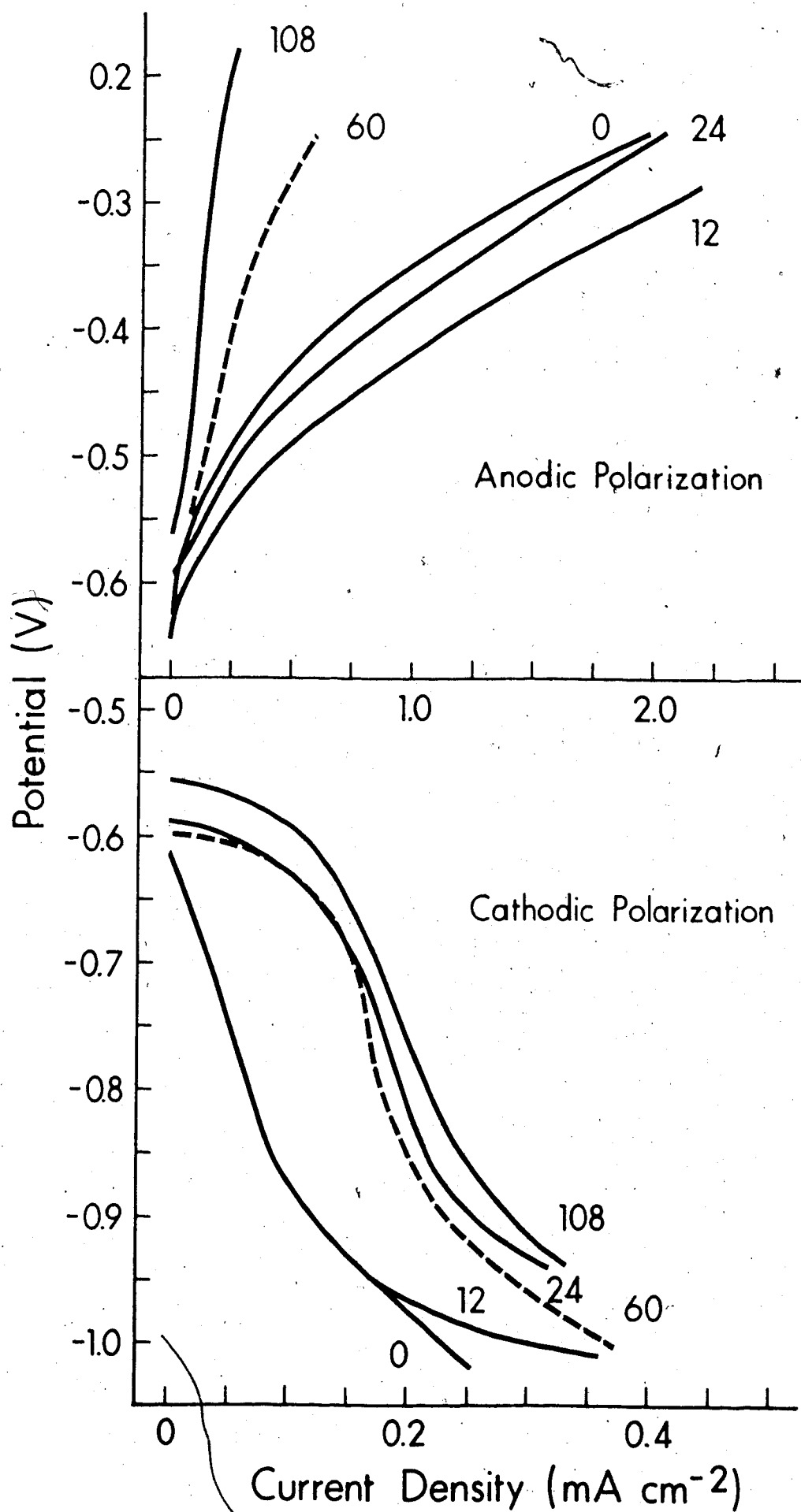
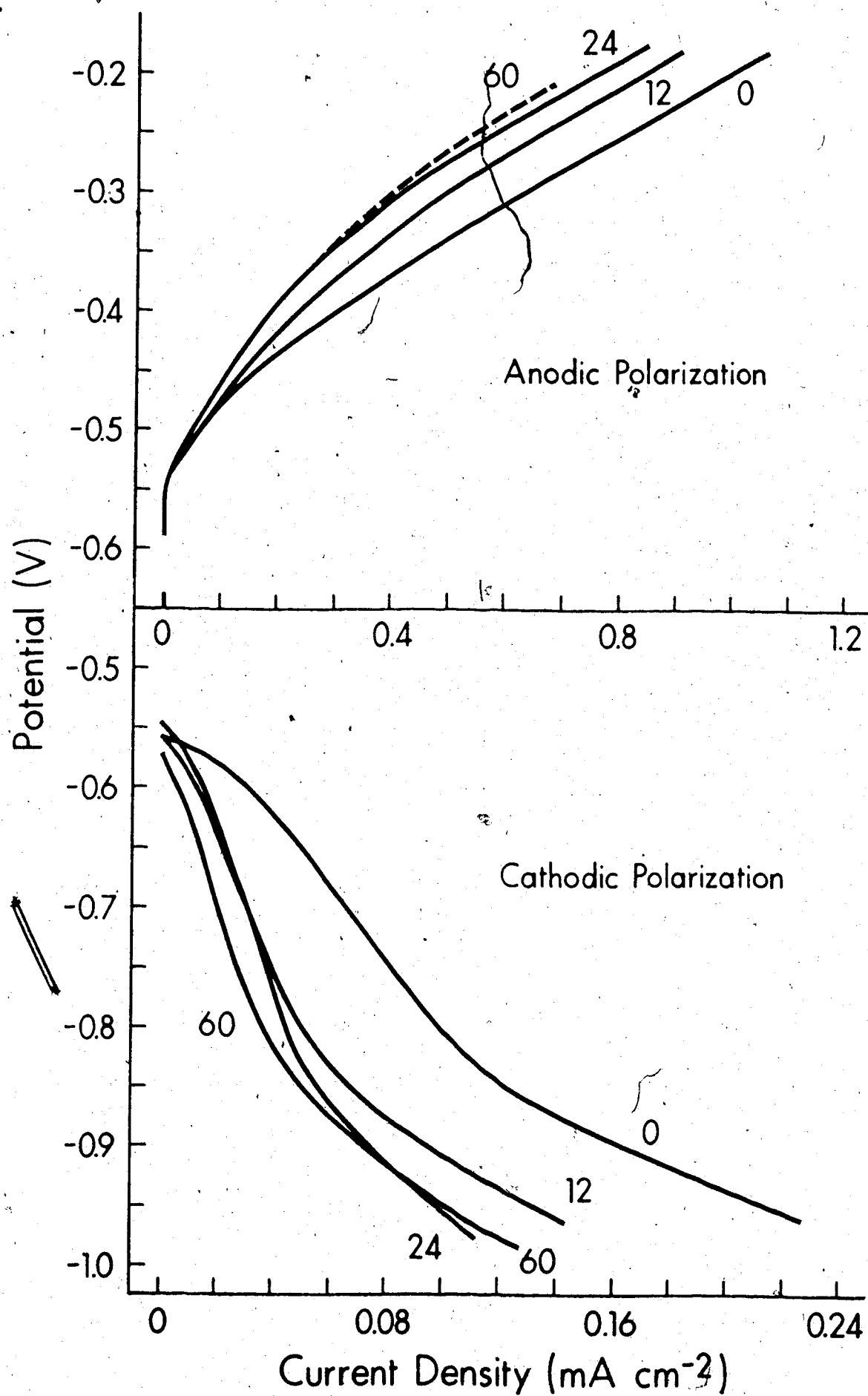


Fig. 63. Polarization curves for mild steel in uninoculated (control)  
B<sub>10</sub> medium without added FePO<sub>4</sub>, containing Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> ( g/l).  
The polarization curve at 108 hr coincided with that at 24 hr.  
(0, 12, 24 and 60 denote incubation time (hr) at 25 ± 2°C)





It can be concluded from this study that the simultaneous production of  $S^{2-}$  and Fe(II) prevented the eventual formation of protective FeS coating. Consequently, the anode was not completely polarized and an initially stifled reaction was allowed to continue. Furthermore, the formation of large amounts of FeS caused some cathodic depolarization. The combined effects of destabilization of FeS coating of the coupon and cathodic depolarization of FeS formed by bacterial activities would undoubtedly increase the corrosion of the coupon.

Corrosion of Mild Steel Specimens (Coupons) Exposed  
to Cultures of Isolate #200

Physiological characteristics of Isolate #200 reported earlier in this work suggested that Isolate #200 can modify the environment of iron/steel and accelerate its corrosion. Moreover, polarization characteristics of mild steel in cultures of Isolate #200 showed that this organism caused anodic depolarization of the steel specimens. The present aspect of investigation was aimed at determining the extent of corrosion that can occur in cultures of Isolate #200.

Static and Semi-continuous Cultures

In static and semi-continuous cultures, coupons (specimens) exposed for two weeks in cultures of Isolate #200, where there was no  $S^{2-}$  production, were devoid of appreciable surface coating. In  $B_{10}$  medium, the specimens were covered by adherent brownish-black film. No such film was evident in coupons exposed in Butlin's medium. In cultures where  $S^{2-}$  was produced ( $S_2O_3^{2-}$ -containing media) the mild steel specimens were covered by a black coating. A qualitative chemical test showed that the black corrosion product contained  $S^{2-}$ . No pits were formed on the specimens during the 2-week immersion period.

In uninoculated flasks, the coupons were covered by rust (brownish deposit) which flaked off easily. In both Butlin's and  $B_{10}$  media without  $S_2O_3^{2-}$ , the specimens exposed for up to 21 weeks were covered by thicker rusty material than was observed during the 2-week

exposure. An intact, brownish-black film underlay the rusty coating. During the long exposure (21 weeks), uninoculated  $B_{10}$  medium-containing flasks turned black, indicating chemical reduction of  $S_2O_3^{2-}$  to  $S^{2-}$ . Few pits were observed on the coupons under this condition.

The corrosion of the mild steel specimens in static and semi-continuous cultures of Isolate #200 are shown in Tables 17 and 18. The coupons in the uninoculated flasks of  $B_{10}$  medium showed higher corrosion rates than those in the inoculated flasks. This effect was more pronounced with the exposure time. The high corrosion rates evident in uninoculated flasks was thought to be due to dissolved  $O_2$  which made the solution oxygenated. This interpretation is supported by the observation that the corrosion product observed was rust, hydrated ferric oxide. Isolate #200 is an aerobic organism and will effectively remove much of the dissolved  $O_2$  in the culture for cell respiration. Consequently, no such corrosion by dissolved  $O_2$  would contribute to the observed corrosion rate as obtained in the cultures of this organism. Therefore, the uninoculated flask actually constituted a different system, whose corrosivity should be expected to be different from that in cultures of Isolate #200.

The corrosion rates obtained (Tables 17 and 18) were higher when  $S^{2-}$  was produced than in its absence. In  $B_{10}$  cultures containing  $S_2O_3^{2-}$ , after 2 weeks exposure a corrosion rate of  $8 \text{ mg dm}^{-2} \text{ day}^{-1}$  (mdd) was obtained as against 5 mdd obtained in the absence of  $S^{2-}$  generation. A similar trend was observed in inoculated Butlin's medium containing  $S_2O_3^{2-}$ . Therefore, the corrosive effect of Isolate #200 must stem, at least in part, from its ability to produce  $S^{2-}$ .

Under the natural environment conditions, Isolate #200 would be

TABLE 17. Corrosion of mild steel exposed to static cultures of isolate #200.

Experimental condition	Corrosion rate			
	2 weeks' exposure		21 weeks' exposure	
	mdd <sup>1</sup>	mpy <sup>2</sup>	mdd	mpy
<i>B<sub>10</sub> medium</i>				
Inoculated	5.12±0.52	0.84±0.01	0.64±0.13	0.11±0.02
Uninoculated	13.47±2.44	2.20±0.40	1.35±0.14	0.22±0.02
<i>B<sub>10</sub> medium + S<sub>2</sub>O<sub>3</sub><sup>2-</sup></i>				
Inoculated	7.70±0.68	1.26±0.11	0.84±0.39	0.14±0.07
Uninoculated	13.92±1.19	2.28±0.22	2.08±0.50	0.34±0.08
<i>Butlin's medium</i>				
Inoculated	n.d.	-	2.58±0.13	0.42±0.02
Uninoculated	n.d.	-	3.73±0.24	0.61±0.04
<i>Butlin's medium + S<sub>2</sub>O<sub>3</sub><sup>2-</sup></i>				
Inoculated	4.54±0.84	0.74±0.14	5.11±0.58	0.84±0.09
Uninoculated	1.20±0.68	0.20±0.11	6.50±0.38	1.06±0.06
<sup>1</sup> mg/dm <sup>2</sup> /day				
<sup>2</sup> mil-penetration/year				

TABLE 18. Corrosion of mild steel exposed to 'semi-continuous' cultures of isolate #200

Experimental condition	Corrosion rate			
	2 weeks' exposure		21 weeks' exposure	
	mdd <sup>1</sup>	mpy <sup>2</sup>	mdd	mpy
<i>B<sub>10</sub></i> medium				
Inoculated	7.27±2.16	1.19±0.35	0.38±0.13	0.63±0.02
Uninoculated	17.31±0.39	2.83±0.06	3.65±0.73	0.06±0.12
<i>B<sub>10</sub></i> medium + S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>				
Inoculated	19.56±0.68	3.20±0.11	1.45±0.30	0.24±0.05
Uninoculated	18.91	2.93±0.23	7.04±1.05	1.15±0.17
<i>Butlin's</i> medium				
Inoculated	3.10±1.10	0.51±0.18	2.76±0.19	0.45±0.03
Uninoculated	0.17±0.30	0.03±0.07	3.29±0.91	0.54±0.15
<i>Butlin's</i> medium + S <sub>2</sub> O <sub>3</sub> <sup>2-</sup>				
Inoculated	4.06±0.39	0.66±0.07	5.88±1.18	0.96±0.19
Uninoculated	0.69±0.46	0.11±0.07	7.72±1.64	1.26±0.27
<sup>1</sup> mg/dm <sup>2</sup> /day				
<sup>2</sup> mil-penetration/year				

able to produce  $S^{2-}$  from  $SO_3^{2-}$  and  $S_2O_3^{2-}$  which are normal intermediate products of  $SO_4^{2-}$  reduction by sulphate-reducing bacteria.

Comparison of the corrosion rates of the coupons in semi-continuous and static cultures of Isolate #200 showed that the rates were higher in the semi-continuous cultures. Thus, in the absence of  $S^{2-}$  production, a corrosion rate of 7 mdd was observed in semi-continuous cultures as against 5 mdd obtained in static culture, after 2 weeks' exposure. This difference in corrosion rates was more pronounced in cultures in which  $S^{2-}$  was produced: 20 mdd in semi-continuous system and 8 mdd in static cultures.

When the exposure period was extended from 2 to 21 weeks, the average corrosion rate declined even though the total weight loss per coupon was still higher than that obtained after 2 weeks' exposure. This indicated that most of the corrosion (weight loss) occurred within the early stage of the exposure. This interpretation is consistent with the earlier observation that intense anodic depolarization occurred early during the culture incubation. Subsequent anodic depolarization, after 3 days of incubation, was very slight. It was pointed out that the higher corrosion rate observed after 2 weeks' exposure was due to the greater cell activity obtainable at the early incubation period before nutrient limitation. This idea is supported by the observation that corrosion rate was higher in semi-continuous cultures than the static cultures. In the semi-continuous system, the medium was replenished weekly.

*Continuous culture:* In the continuous culture system, pitting of the mild steel specimens occurred under conditions of  $S^{2-}$  generation (i.e. in  $S_2O_3^{2-}$ -containing medium). Plate 13 shows pitting of the




Plate 13. Pitting of mild steel coupons exposed for 9 weeks in Butlin's  
+  $S_2O_3^{2-}$  medium culture of Isolate #200. X52.5





coupons exposed for 9 weeks in culture of Isolate #200 in Butlin's medium containing  $S_2O_3^{2-}$ . In such cultures, the black coating covering the coupons was unprotective, being easily sloughed off to reveal the bright pits or bare metal. Most of the pits formed were about 1 mm across but a few were smaller. No pits occurred on control coupons (Plate 14).

In the continuous culture system, the calculated corrosion rates (Table 19) after 9 weeks' exposure varied from 1 mdd to 2 mdd. These rates were significantly less than the values obtained in static and semi-continuous cultures after 2 weeks' exposure and in semi-continuous Butlin's medium plus  $S_2O_3^{2-}$  after 21 weeks. These figures, however, are not directly comparable since the exposure was for different periods. As was evident in the static and semi-continuous culture systems discussed earlier, corrosion rate declined with exposure time.

An interesting observation was that in the continuous culture system,  $S^{2-}$  generation ceased to have any significant effect on the corrosion of the mild steel coupon. Thus, the corrosion rates obtained in Butlin's medium lacking  $S_2O_3^{2-}$  (1.5 mdd) was similar to that obtained (1.7 mdd) in Butlin's medium containing  $S_2O_3^{2-}$ . The only apparent explanation for this effect may be the low residence time of the corrosive product,  $S^{2-}$ , in the corrosion chamber. Any  $S^{2-}$  produced in the culture was soon washed away (medium dilution rate -  $0.012 \text{ hr}^{-1}$ ) and was not in contact with the coupons long enough to have any significant corrosive effect in terms of weight loss over the entire surface area. Weight losses do not indicate the degree of penetration. Intense pitting can still lead to pipeline failure (due to perforation) even though the total weight loss is negligible or even non-detectable.

In real life situations, failure of the pipeline system carrying

Plate 14. Surface of mild steel coupon exposed for 9 weeks in  
uninoculated (control) Butlin's +  $S_2O_3^{2-}$  medium.



TABLE 19. Corrosion of mild steel exposed to continuous cultures of isolate # 200 for 9 weeks.

Experimental condition	Corrosion rate	
	mdd <sup>1</sup>	mpy <sup>2</sup>
<i>B<sub>10</sub> medium</i>		
Inoculated	1.82±0.08	0.30±0.01
Uninoculated	4.57±0.04	0.75±0.02
<i>B<sub>10</sub> medium + S<sub>2</sub>O<sub>3</sub><sup>2-</sup></i>		
Inoculated	1.19±0.37	0.20±0.06
Uninoculated	4.91±1.17	0.81±0.19
<i>Butlin's medium</i>		
Inoculated	1.39±0.09	0.23±0.02
Uninoculated	1.96±0.06	0.32±0.01
<i>Butlin's medium + S<sub>2</sub>O<sub>3</sub><sup>2-</sup></i>		
Inoculated	1.73±0.14	0.28±0.02
Uninoculated	4.58±0.39	0.75±0.07

<sup>1</sup>mg/dm<sup>2</sup>/day

<sup>2</sup>mil-penetration/year

the Pembina crude oil normally resulted from pit formation. In the experimental situation, it has been demonstrated that pit formation was associated with  $S^{2-}$  production by Isolate #200. Since Isolate #200 has the capacity to produce  $S^{2-}$  from  $SO_3^{2-}$  and  $S_2O_3^{2-}$  reduction, and since  $SO_3^{2-}$  and  $S_2O_3^{2-}$  can be generated as intermediates of  $SO_4^{2-}$  reduction in the mixed flora in the Pembina crude oil system, it can be concluded that the frequent failure of the Pembina crude oil pipe system due to pit formation must be attributed to, at least in part, the activities of Isolate #200 or bacteria with similar physiological capability.

Although the corrosion rates reported in this work for the mild steel coupons in the static, semi-continuous and continuous cultures of Isolate #200 are generally low, some values compare favourably with those reported in the literature in cultures of *Desulfovibrio* and *Desulfotomaculum* spp. King, Miller and Wakerley (1973) reported a weight loss of 35 mg after 5 weeks in semi-continuous cultures of *D. desulfuricans* strain Teddington R (NCIB 8312). This figure is comparable to 38 mg loss obtained with mild steel coupon of similar surface area exposed for 2 weeks in culture of Isolate #200 in  $B_{10}$  medium containing  $S_2O_3^{2-}$ . Very similar weight losses were reported by the same workers with *D. vulgaris* strain Hildenborough (NCIB 8303) and *Desulfotomaculum orientis* strain Singapore I (NCIB 8382).

In cultures of Isolate #200 where  $S^{2-}$  was produced, pitting of the mild steel coupons occurred (Plate 13). Since pitting is a very localized attack, its destructiveness cannot be adequately quantitated by weight loss measurements. This is because corrosion rate determinations, like mdd, assume uniform weight loss.

Cyclic exposure of mild steel coupons to culture of Isolate #200 and the atmosphere

In crude oil handling systems like the Free Water Knockout Tank (FWKT) there would be periods when the tank would be empty or partially filled. Under this condition, the internal surface of the tank is exposed to the atmosphere during which oxidation of the surface material from Fe(II) to Fe(III) could occur. In the Pembina crude oil system, iron-reducing bacteria (like Isolate #200) are normally found in the produced water/oil emulsion being handled by the FWKT. As the FWKT is filled and emptied there is a cyclic exposure of the internal surface of the tank to cultures of the bacteria and the atmosphere (oxidative). The investigation reported herein was aimed at delineating the effect of such cyclic exposure on the corrosion of iron/steel.

*B<sub>10</sub> medium:* Table 20 shows the corrosion of mild steel coupons when cyclically exposed to cultures of Isolate #200 in B<sub>10</sub> and Butlin's media and the atmosphere. The rates were calculated on the basis of the actual period of exposure (12 days) to cultures of the organism. Included (in brackets) are the corrosion rates calculated on the basis of the total experimental period (28 days).

In this medium, the calculated corrosion rate in uninoculated medium was again higher than in the inoculated medium. This observation had been explained earlier as due to corrosive effect of dissolved O<sub>2</sub> in the uninoculated flasks. The calculated corrosion rates in B<sub>10</sub> medium were higher than those obtained in the same medium under the conditions of static or semi-continuous cultivation of

TABLE 20. Corrosion<sup>1</sup> of mild steel during cyclic exposure to cultures of isolate #200 and the atmosphere.

Experimental condition	Corrosion rate	
	mdd <sup>2</sup>	mpy <sup>3</sup>
<i>B<sub>10</sub> medium</i>		
Inoculated	30.92±1.71 (13.25)	5.06±0.28 (2.17)
Uninoculated	65.50±1.89 (28.07)	10.71±0.32 (4.59)
<i>Butlin's medium</i>		
Inoculated	104.95±5.68 (44.98)	17.17±0.93 (7.36)
Uninoculated	68.65±14.69 (29.42)	11.22±2.40 (4.81)

<sup>1</sup>Corrosion rate calculated on the basis of the actual immersion time in the culture. In brackets are shown (for comparison) the corrosion rates calculated on the basis of the total experimental period.

<sup>2</sup>mg/dm<sup>2</sup>/day

<sup>3</sup>mil-penetration/year

Isolate #200.

*Butlin's medium:* The corrosion rate of 105 mdd was very high; significantly higher than was obtained under any condition in static and semi-continuous cultures of the organism.

During the atmospheric exposure of the previously immersed coupons, they became covered by a layer of yellowish-brown crust which could not be easily scraped off. After each submersion in the culture of Isolate #200 this crust of corrosion was removed in the culture but was reformed during atmospheric exposure of the coupons. It was thought that the yellowish-brown crust formed on the coupon exposed to the atmosphere was an Fe(III), probably the oxide. When immersed in culture of Isolate #200 this Fe(III) covering was reduced to the more soluble Fe(II). The removal of a protective Fe(III) crust by Isolate #200 would expose fresh metal for further atmospheric oxidation.

Therefore, by the constant reoxidation of coupon to Fe(III) and removal of this crust, there was a continuous eating away (loss of material) of the coupon which resulted in the appreciable weight loss. Since the corrosion rate calculated for this process for the experimental period was significantly higher than those obtained with  $S^{2-}$  generation, it must be concluded that the ability of Isolate #200 to reduce insoluble, protective Fe(III) forms to the soluble, non-protective Fe(II) forms is a very important factor in the corrosive effect of this organism.

Formation of Fe(III) coatings under natural conditions can be expected to occur both under aerobic and anaerobic conditions, provided the environment is oxidizing enough. Protective Fe(III) forms can easily form in oxygenated environments due to oxidative



effect of  $O_2$ . This situation was reported by Booth *et al.* (1963, 1965) in iron/steel buried in tidal waters of the Thames estuary. On the other hand, under anaerobic conditions and in environments containing  $NO_3^-$ , the  $NO_2^-$  formed by the reduction of  $NO_3^-$  (during anaerobic respiration) by microorganisms would oxidize Fe(II) to the less soluble, protective Fe(III) forms. When iron-reducing bacteria are present, the protectiveness of Fe(III) would be lost because of the formation of the soluble Fe(II) forms provided adequate electron donor was available. In oilfield operations fresh water injections containing as much as 6 ppm of dissolved  $O_2$  may be injected to producing wells to aid the recovery of oil. However, produced water for such wells may have dissolved  $O_2$  content of only about 10 ppb. Presumably, the initial  $O_2$  content of 6 ppm in the fresh water injection had been part utilized to oxide iron of the pipe system to Fe(III). Since iron-reducing bacteria have been found in such systems the situation similar to the cyclic oxidation of the coupon and subsequent reduction by iron-reducing bacteria can be said to exist. Therefore, corrosion of pipeline systems due to reduction of any protective Fe(III) to Fe(II) can be expected to occur in oilfields that employ fresh water injection for enhanced oil recovery.

## CONCLUSIONS

The ferric iron-reducing bacterium, a *Pseudomonas* sp. designated Isolate #200, caused the anodic depolarization of mild steel. The intense anodic depolarization of mild steel passivated by the oxidizing inhibitor,  $\text{NO}_2^-$ , by activity of Isolate #200, indicated that this organism caused the anodic depolarization by the removal of the protective ferric film.

Corrosion rate, as measured in terms of weight loss, due to the activities of Isolate #200 was low (when there was no sustained oxidizing condition) and may not singularly be responsible for the high corrosion rate observed in Pembina crude oil pipeline system. A very high corrosion rate of  $105 \text{ mg dm}^{-2} \text{ day}^{-1}$  of mild steel was obtained during cyclic exposure of the steel specimen to cultures of Isolate #200 and the atmosphere. This result further indicated that the corrosive activity of this organism was due to the removal of protective ferric film/coat. Isolate #200 also caused the transformation of mild steel from the passive to the active states. Consequent on these observations, pipeline systems, or any other iron/steel structures, contaminated by Isolate #200 (or similar iron-reducing bacteria) cannot be protected by oxidizing inhibitors, like  $\text{NO}_2^-$ , which function by the formation of protective ferric film. Also, anodic protection methods, which passivate by the formation of protective ferric film, cannot be expected to be an effective protection against corrosion, in systems contaminated by Isolate #200 or similar ferric iron-reducing bacteria.

Pitting corrosion of mild steel specimens in cultures of Isolate #200 producing  $\text{S}^{2-}$  indicated that this and like organisms are very active contributors to the failure of the Pembina pipeline system.

In the situation as it exists in Pembina crude oil pipeline system, anodic depolarization as caused by Isolate #200 would be expected to complement cathodic depolarization due to sulphate-reducing bacteria known to be present. Synergistic effects on corrosion by the activities of these two bacterial types (Isolate #200-like bacteria and  $\text{SO}_4^{2-}$  reducers) cannot be ruled out. Interactions between different groups of bacteria (as indicated by 'cascade system of  $\text{S}^{2-}$  generation') exist in the Pembina crude oil system. The total corrosion picture in the Pembina pipeline system is complex, involving the interaction of different bacteria, aerobic and anaerobic. Therefore, the corrosion of mild steel due to iron-reducing bacteria, as determined in this work, must be considered as being additional to that which may be independently caused by sulphate-reducing bacteria (or any other group) known to be present in the system.

A relationship between coloration of the cells, cytochrome content and the ability to reduce  $\text{Fe(III)}$  to  $\text{Fe(II)}$  by Isolate #200 has been demonstrated. Cells which possessed little or no demonstrable cytochrome content had no colour and reduced negligible amounts of  $\text{Fe(III)}$ . The inhibition of  $\text{Fe(III)}$  reduction in cells of Isolate #200 by specific cytochrome inhibitors suggested that  $\text{Fe(III)}$  might be used as a terminal electron acceptor in this organism. The perpetuation of the lag phase during  $\text{Fe(III)}$  reduction by the addition of chloramphenicol (which prevents the initiation of protein synthesis) and the decline of  $\text{Fe(III)}$  reduction by osmotic shock have shown the involvement of an Fe-inducible protein whose addition to washed shocked cells increased slightly the  $\text{Fe(III)}$ -reducing activity of the previously osmotically shocked cells. This latter characteristic is reminiscent of transport protein factor

as was observed in other organisms (Pardee *et al.*, 1966). No significant Fe(III)-reducing activity was present, under the experimental conditions, in the cell components. Only the whole cells or lysozyme-treated cells (plasmids) reduced Fe(III) unequivocally.

Isolate #200 is widely distributed, having been found in all Pembina crude oil pipelines flowing from North Central Alberta to Montreal, Quebec. It has been demonstrated that this organism (Isolate #200) produced exopolysaccharide with which it attached to surfaces of coupons, thus enabling it to maintain a successful hold in the natural environment. Moreover, Isolate #200 has a wide range of physiological activities, for example nutritional versatility which would enable it to scavenge and grow on a wide range of substrates and modify the environment sufficiently to affect the integrity of any iron/steel structure. Thus, the ability of Isolate #200 to transform ferric iron [Fe(III)] to ferrous iron [Fe(II)] would change the redox potential,  $E_h$ , to more negative values; solubilize iron (by the formation of the more soluble Fe(II) forms); and decrease the resistivity of any environment: conditions known to engender corrosion of iron/steel in soils. Furthermore, the ability of Isolate #200 to reduce  $\text{SO}_3^{2-}$  and  $\text{S}_2\text{O}_3^{2-}$  (known intermediates of  $\text{SO}_4^{2-}$  reduction by  $\text{SO}_4^{2-}$ -reducing bacteria) would contribute to the total  $\text{S}^{2-}$  pool and, therefore, the corrosivity of such an environment.

Thus, Isolate #200 has the capacity to cause and enhance the corrosion of iron/steel. It is, therefore, recommended that in the assay for corrosion bacteria in any environment, iron-reducing bacteria or bacteria showing physiological activities similar to Isolate #200 should be considered in addition to the traditional sulphate-reducing bacteria.

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APPENDIX 1aB<sub>10</sub> Medium

K <sub>2</sub> HPO <sub>4</sub>	0.8 g	
KH <sub>2</sub> PO <sub>4</sub>	0.2 g	
MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.2	
<sup>1</sup> NaCl	0.2 g	
MnSO <sub>4</sub>	0.001 g	
NaMoO <sub>4</sub>	0.001 g	1 ml of 0.1% solution
CaSO <sub>4</sub> (sat. soln)	10 ml	
Yeast extract (Difco)	5.0 g	
Peptone (Difco)	5.0 g	
FePO <sub>4</sub>	4.7 g	
Distilled water	990 ml	(to make up to 1000 ml)
pH	7.2	

<sup>1</sup>May be replaced with 0.4 g Na<sub>2</sub>SO<sub>4</sub>



APPENDIX 1bModified Butlin's medium (Jobson, 1975)

$K_2HPO_4$	0.5 g
$NH_4Cl$	1.0 g
<sup>1</sup> $Na_2SO_4$	2.0 g
<sup>2</sup> $MgSO_4 \cdot 7H_2O$	0.1 g
<sup>3</sup> $FeSO_4 \cdot 7H_2O$	0.1 g
Sodium lactate (60%)	1.5 ml
Yeast extract (Difco)	1.5 g
Distilled water	1000 ml
pH adjusted to	7.2

<sup>1</sup>May be replaced with 1.52 g NaCl

<sup>2</sup>Or - 0.1 g  $MgCl_2 \cdot 6H_2O$

<sup>3</sup>Or - 0.07 g  $FeCl_2 \cdot 4H_2O$

APPENDIX 1cC<sub>4</sub>F<sub>1</sub> Medium

$\beta$ -glycerophosphate	3.46 g
Tris	14.5 g
NaCl	4.67 g
NH <sub>4</sub> Cl	1.07 g
KCl	1.49 g
MgCl <sub>2</sub>	0.20 g
KH <sub>2</sub> PO <sub>4</sub>	0.09 g
Na <sub>2</sub> SO <sub>4</sub>	0.07 g
CaCl <sub>2</sub>	0.03 g
FeCl <sub>3</sub>	0.005 g (trace)
Distilled water	1000 ml
pH adjusted with HCl to	7.4

APPENDIX 1dModified Brewer's Medium (Jobson, 1975)

Beef extract (Difco)	1.0 g
Yeast extract (Difco)	2.0 g
Peptone (Difco)	5.0 g
Sodium lactate (60%)	2.5 ml
$\text{Na}_2\text{SO}_4$	2.0 g
Sodium thioglycollate	1.0 g (50 ml of 2% solution)
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.10 g
Distilled water (enough to bring total volume to 1000 ml)	
pH adjusted to	7.2

APPENDIX 1eSynthetic (defined) Medium)

$K_2HPO_4$	0.5 g
$Na_2SO_4$	2.0 g
$NH_4Cl$	1.0 g
$CaCl_2 \cdot 2H_2O$	0.15 g
$MgSO_4 \cdot 7H_2O$	0.1 g
<sup>1</sup> $FeSO_4 \cdot 7H_2O$	0.1 g
<sup>2</sup> Sodium lactate (60%)	1.5 - 3 ml
Distilled water	1000 ml
pH adjusted to	7.2

<sup>1</sup> $FeSO_4$  reduced to 0.02 g where necessary to avoid precipitation.

<sup>2</sup>Or - any appropriate carbon source.

APPENDIX 2Fixation and embedding procedures for electron microscopy

- Reagents:*
- (i) 70% glutaraldehyde
  - (ii) 0.1 M cacodylate buffer, with or without ruthenium red (0.025 %, w/v)
  - (iii) Propylene oxide
  - (iv) Resin mixture: vinyl cyclohexene dioxide (10 g), Diglycidyl ether of polypropylene glycol (6 g), Nonenyl succinic anhydride (26 g) and Dimethyl amino ethanol (0.4 g).

*Procedure:*

## (a) Fixation

- (i) Completely submerge specimen in glutaraldehyde-cacodylate buffer mixture (5% glutaraldehyde in 0.1 M cacodylate buffer), with or without ruthenium red, and incubate for 2 hr at room temperature.

For liquid culture, prefix cells in 1:10 mixture of fixative and culture; then fix in 5% glutaraldehyde-cacodylate system.

- (ii) Centrifuge where necessary, wash 5 to 10 minutes in cacodylate buffer, with or without ruthenium red.

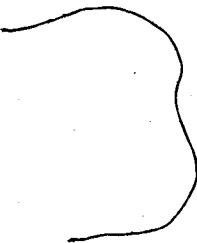
## (b) Post Fixation

- (i) Form agar core by resuspending pellet in equal volume of 4% Bacto agar (molten and cooled to 50-60°C). Suck up mixture in Pasteur pipette and extrude the core as it solidifies. Slice core into short cylinders and place in vials.
- (ii) Cover core with 2% osmic acid in 0.1 M cacodylate buffer, with or without ruthenium red, and incubate in fume hood at room temperature for 2 hr.

## (c) Dehydration

Dehydrate sample according to the protocol below:

30% acetone (30 minutes)



50% acetone	(30 minutes)
70% acetone	(30 minutes)
90% acetone	(30 minutes)
100% acetone	(10 minutes)
100% acetone	(10 minutes)
Propylene oxide	(10 minutes)
Propylene oxide	(10 minutes)

(d) Infiltration

Fill vials containing the dehydrated samples with propylene oxide-embedding resin mixture (3:1, v/v) and incubate overnight with shaking, at room temperature.

(e) Embedding

- (i) Remove core and blot off old resin with absorbent paper (filter paper) and replace core in new resin; incubate for 3-4 hours.
- (ii) Transfer core to capsule and fill with resin and cure (at 60°C for 8 hours).

APPENDIX 3a

Typical data used in plotting polarization curves. The data shown below were used in plotting Fig. 55. Incubation time: 0 hr.

Cathodic Polarization		Anodic Polarization	
Potential (V)	Current density (mA cm <sup>-2</sup> )	Potential (V)	Current density (mA cm <sup>-2</sup> )
-0.110	0	-0.11	0
-0.135	0.005	-0.085	0.008
-0.160	0.008	-0.060	0.011
-0.185	0.012	-0.035	0.013
-0.210	0.018	-0.010	0.014
-0.235	0.025	+0.015	0.015
-0.260	0.032	+0.040	0.015
-0.285	0.036	+0.065	0.016
-0.310	0.041	+0.090	0.016
-0.335	0.043	+0.115	0.017
-0.360	0.046	+0.140	0.021
-0.385	0.046	+0.165	0.032
-0.410	0.044	+0.190	0.058
-0.435	0.040	+0.215	0.110
-0.460	0.033	+0.024	0.217
-0.485	0.028	+0.265	--
-0.510	0.027	+0.290	--

APPENDIX 3b

Incubation time: 24 hr.

Cathodic Polarization		Anodic Polarization	
Potential (V)	Current density (mA cm <sup>-2</sup> )	Potential (V)	Current density (mA cm <sup>-2</sup> )
-0.500	0	-0.500	0
-0.525	0.017	-0.475	0.018
-0.550	0.028	-0.450	0.027
-0.573	0.040	-0.425	0.029
-0.600	0.047	-0.400	0.028
-0.625	0.054	-0.375	0.025
-0.675	0.065	-0.325	0.028
-0.700	0.072	-0.300	0.033
-0.725	0.079	-0.275	0.039
-0.750	0.089	-0.250	0.046
-0.775	0.094	-0.225	0.056
-0.800	0.105	-0.200	0.068
-0.825	0.117	-0.175	0.082
-0.850	0.140	-0.150	0.097
-0.875	0.164	-0.125	0.111
-0.900	0.206	-0.100	--



APPENDIX 3c

Incubation time: 48 hr.

Cathodic Polarization		Anodic Polarization	
Potential (V)	Current density, (mA cm <sup>-2</sup> )	Potential (V)	Current density (mA cm <sup>-2</sup> )
-0.280	0	-0.280	0
-0.305	0.014	-0.255	0.023
-0.330	0.019	-0.230	0.035
-0.355	0.025	-0.205	0.047
-0.380	0.032	-0.180	0.058
-0.405	0.039	-0.155	0.070
-0.430	0.046	-0.130	0.075
-0.455	0.053	-0.105	0.082
-0.480	0.060	-0.080	0.091
-0.505	0.068	-0.055	0.096
-0.530	0.075	-0.030	0.105
-0.555	0.082	-0.005	0.112
-0.580	0.086	+0.020	0.117
-0.605	0.090	+0.055	0.124
-0.630	0.092	+0.070	0.129
-0.655	0.096	+0.095	0.136
-0.680	--	+0.120	0.143

APPENDIX 3d

Incubation time: 96 hr.

Cathodic Polarization		Anodic Polarization	
Potential (V)	Current Density (mA cm <sup>-2</sup> )	Potential (V)	Current density (mA cm <sup>-2</sup> )
-0.520	0	-0.520	0
-0.545	0.017	-0.495	0.023
-0.570	0.026	-0.470	0.035
-0.595	0.033	-0.445	0.051
-0.620	0.039	-0.420	0.070
-0.645	0.045	-0.395	0.086
-0.670	0.051	-0.370	0.105
-0.695	0.058	-0.345	0.131
-0.720	0.065	-0.320	0.154
-0.745	0.074	-0.295	0.182
-0.770	0.085	-0.270	0.215
-0.795	0.098	-0.245	0.250
-0.820	0.118	-0.220	0.292
-0.845	0.145	-0.195	0.339
-0.870	0.183	-0.170	0.386
-0.895	--	-0.145	0.437
-0.920	--	-0.120	--

APPENDIX 3e

Incubation time: 168 hr.

Cathodic Polarization		Anodic Polarization	
Potential (V)	Current density (mA cm <sup>-2</sup> )	Potential (V)	Current density (mA cm <sup>-2</sup> )
-0.390	0	-0.390	0
-0.415	0.013	-0.365	0.030
-0.440	0.019	-0.340	0.042
-0.465	0.023	-0.315	0.051
-0.490	0.030	-0.290	0.061
-0.515	0.034	-0.265	0.070
-0.540	0.039	-0.240	0.082
-0.565	0.044	-0.215	0.098
-0.590	0.049	-0.190	0.110
-0.615	0.054	-0.165	0.133
-0.640	0.058	-0.140	0.159
-0.665	0.065	-0.115	0.199
-0.690	0.068	-0.090	0.250
-0.715	0.074	-0.065	0.327
-0.740	0.080	-0.040	0.432
-0.765	0.089	-0.015	0.572
-0.790	0.098	+0.010	--

## APPENDIX 4

Miscellaneous determinations

Density of mild steel coupon (av.)  $8.8 \text{ g cm}^{-3}$

Surface area of mild steel coupons (av.)  $0.14 (0.137) \text{ dm}^2$