STUDIES ON THE REDUCTION INTENSITY (rH), IRON-REDUCING CAPACITY AND GLEY FORMATION OF SOME FACULTATIVE ANAEROBIC BACTERIA, VERIFIED ON ESKRIDGE SHALE

by

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INTRODUCTION

Iron has a very intimate relationship with life in general and with primitive and simple forms of life in particular. It is well established that the microbiological oxidation of ferrous iron serves as an energy source for a specific group of chemoautotrophic microorganisms, the iron bacteria. The reduction of iron, however, seems to be unspecific and not peculiar to any single group or family, but is an attribute of a variety of bacteria. The involvement of different bacteria with an assortment of metabolic properties in the reduction of iron, suggests a diversity of reducing mechanisms implicated with the transformation of ferric iron to the ferrous state.

Several mechanisms have been proposed which may account for the microbiological reduction of iron. Since the reduction requires the addition of electrons, it was believed that the ferric ion acted as a final electron acceptor in cell respiration. This would be in a manner analogous to the reduction of nitrate by denitrifying bacteria. Another possible mechanism for the solubilization of iron might be the direct reaction of fermentation products with ferric hydroxides and oxides. Roberts (1947) for example, found that resting cells of Bacillus polymyxa in phosphate buffer and glucose reduced ferric hydroxide rapidly and suggested that a three-carbon intermediate, formic acid or hydrogen was the hydrogen donor involved in the reduction. On the other hand, it was conjectured that iron reduction in soil might be not more than a side reaction of bacterial activity, and that at least a part of the transformation was

caused by degradation and water soluble products of organic matter. Bétrémieux (1951) suggested that the medium became reducing because bacteria utilized molecular oxygen from sources such as nitrate, sulphate or ferric oxide under anaerobic conditions. The work of Bromfield (1954b), however, indicated that the reduction of iron oxide is not, in fact, due to bacterial utilization of oxygen, but more likely associated with the dehydrogenase activity of the reducing organisms.

Nevertheless, among the many mechanisms and factors proposed, two environmental components were claimed most frequently, namely (a) an increase in acidity accompanying fermentation processes, and (b) the depletion of oxygen as a consequence of metabolic activity. The consumption of oxygen electrochemically means an increase of electrons in the environment resulting in a drop of the redox-potential, whereas the degree of acidity is an expression of proton activity. By measuring the Eh and pH, these parameters will reflect the development of the reduction intensity, rH, in the milieu. The level of this rH would then represent the limits and characteristics of the bacterium involved. It was reasoned that, if iron reduction by bacteria is unspecific, there might be a relation between rH and the iron-reducing capacity of the organism concerned.

The iron-reducing capacity of certain known and unknown, isolated facultative anaerobic bacteria was determined colorimetrically and compared using different media as growth substrate. Since the reduction of iron

$$Fe^{+++} + e \rightleftharpoons Fe^{++}$$

requires the supply of electrons, only those metabolic products could be potentially responsible for the transformation, that are in the reduced state, such as molecular hydrogen or "reductones". Thus the hypothesis was introduced that iron reducing bacteria are principally those which elaborate those substances in their surroundings. In order to support this postulate, unknown bacteria capable of producing hydrogen were isolated from soil and water and tested on their iron-reducing capacity. In addition these isolates were studied for their ability to bleach a hematite-rich shale; this process is known as gleying.

REVIEW OF LITERATURE

Mechanisms of Iron Reduction

Processes and mechanisms involved in the reduction of iron from the ferric to the ferrous state are still not completely understood. Most of the experimental results are without conformity. This may be partly explained by the relative small number of investigations performed, especially during the last decade. Further it is generally believed that iron reduction is due to a variety of bacteria and there seems to be no particular significance of it in the metabolism of the organisms. Nevertheless, this neglect is surprising, if one realizes the world-wide distribution of the process, which plays an important role in Gley- and Pseudogley soil type formation. In these soil-types the reduction process is revealed by characteristic gray- and bluish-green discolorations as a result of periodically waterlogged, anaerobic conditions.

According to the present concept, the reduction of iron oxides and hydroxides is caused principally by microbial activity. The role of microorganisms in dissolving iron compounds has long been known. In 1836, Kindler noted the solution of iron around roots of plants growing in ferruginous sands. This may be the result of carbon dioxide. Winogradski (1888) used anaerobic conditions to promote solution of ferric iron as a source of this element for studies on iron bacteria. More recently, Lieske (1912) observed the reducing effect of molds, particularly of Hyphomycetes, on ferric compounds. He further noticed that sucrose had a solvent effect on hydrated iron oxides. Wright (1922) studied the influence of bacterial dextrose fermentation on mineral solubility, and concluded that solubilization was the consequence of an increased hydrogen-ion concentration. The pH change seemed to be an important factor in iron-reduction.

Besides the reaction of the medium, several other mechanisms may account for the reducing effect. Starkey and Halvorson (1927) deduced from their experiments that microorganisms may alter two environmental conditions, which control the ratio of reduced to oxidized iron; namely (a) the reaction of the medium, and (b) the oxygen pressure in the milieu. These findings were supported by experiments of Starkey (1945). Starkey treated half of his experimental flasks with ferric hydrate and inoculated both parts with a little soil. To proportional numbers of the flasks dextrose or peptone was added and the cultures examined for ferrous iron and hydrogen-ion concentration after 3 weeks. Considerable iron was dissolved in the dextrose medium, partly due to the production of acid.

Further, it was noted that the iron exerted a buffering effect and prevented the shift to the low pH shown in the medium free of iron. The reaction of the peptone medium, however, remained nearly constant, irrespective of the presence of ferric hydrate. Though the pH was close to neutrality, appreciable amounts of iron were detected in solution. This indicated that iron was dissolved not only by an increase of acidity, but partly through other factors, such as the exhaustion of oxygen.

Mention should be made also of the changes which are caused by differences in the concentration of dissolved carbon dioxide (Halvorson, 1931). This gas is produced in such quantities by certain microorganisms that it may greatly affect the solubility of iron in waterlogged soils (Ahmad, 1963) or natural waters (Hem, 1960). The increased partial pressure of carbon dioxide will drop the pH and some ferric hydrate becomes reduced to ferrous carbonate.

Not only under anaerobic conditions, but also in the presence of air, an increased hydrogen-ion concentration may bring large amounts of iron into solution. This was reported by Harmsen (1938), dealing with changes in some soils of the Wieringermeer Polder, an area of land reclaimed from the Zuider Zee in the Netherlands. When the area was diked and the water pumped out, the black sulfide of the soil was oxidized biologically and chemically to elemental sulfur, which was then attacked by Thiobacillus thiooxidans with the production of sulphates. In locations where there was little lime, a rapid increase in the acidity took place and large amounts of iron were brought into solution.

A more specific mechanism of reduction was suggested by studies by Roberts (1947). He isolated 265 microorganisms from soil and reported that only one unidentified species of the genus Clostridium and the facultative anaerobe Bacillus polymyxa were capable of reducing ferric oxide in pure cultures. In studies with B. polymyxa, Roberts found that glucose was fermented more rapidly in the presence of ferric hydroxide than without the mineral. Also the amount of molecular hydrogen produced per mole glucose was less in the samples treated with ferric hydroxide. These findings suggested the participation of the trivalent iron ion as a final H-acceptor.

Not only iron, but also manganese may serve as a biological H-acceptor. Mann and Quastel (1946) showed that the reduction of manganese dioxide (Mn^{4+}) could be accomplished by bacterial suspensions in the presence of suitable H- donators (an excess of carbohydrates) or compounds such as thiols or polyhydroxyphenols, elaborated by bacterial metabolism or break-down products of organic matter. However, the reduction process required the intervention of a hydrogen transporter, like pyocyanin, a soluble pigment produced by Pseudomonas aeruginosa. Similar conclusions were proclaimed more recently by Pichinoty (1963a, b) for the nitrate reducing ability of Aerobacter aerogenes, an organism known for its iron reducing capacity (Bromfield, 1954a). Pichinoty observed that culture suspensions of A. aerogenes, grown under anaerobic conditions on a nitrate free medium, did not consume any molecular hydrogen in the presence of nitrate, until a little amount of benzyl-viologen was added. It seemed that some organisms require specific electrontransport systems in order to couple hydrogenase and reductase.

In the cyclic sequence of iron interconversions, we saw some evidence suggesting that the divalent ion may be regenerated through acid production or by removal of oxygen as a result of microbial metabolism. Bétrémieux (1951) reasoned that microorganisms may utilize the oxygen of ferric oxides if conditions became anaerobic. Therefore he determined the influence of various anions on the iron reducing rate of Bacillus polymyxa. When sulphate, chloride, nitrate or phosphate were added to the media--in the form of ammonium salts and based on the same amount of nitrogen--it was found that only sulphate allowed an intensive iron reduction. Reduction was still evident in the presence of chloride. The amount, however, was less than glucose alone. The phosphate ions reacted to produce insoluble iron phosphates, whereas nitrate did not produce any change at all in iron solubility. Betremieux concluded that oxygen of ferric oxide was used under anaerobic conditions in the presence of sulphate. In the presence of nitrate, however, B. polymyxa preferred the nitrate oxygen to the ferric oxide, leaving the ferric iron in the undissolved state. The same suggestion was made by Leeper (1947) for the reduction of manganese dioxide. Earlier, Quastel et al. (1925) found that some bacteria could grow under anaerobic conditions on glycerol or lactate if nitrate was used as a nitrogen source, but not if ammonium was applied. The glycerol and lactate could not be oxidized to yield energy for growth in the absence of oxygen or a substance, such as nitrate, which could replace molecular oxygen. Bromfield (1954a) proved that B. polymyxa was unable to derive oxygen for growth from ferric oxide under anaerobic conditions. He showed growth and reduction of iron only in a

medium containing glycerol, nitrate and ferric oxide, but not in a glycerol-ferric oxide combination. There was no growth and reduction under oxygen depletion in a glycerol-ammonium-sulphate medium, not even when ferric oxide was present. From further studies by Bromfield (1954b), the iron reduction by Bacillus circulans appeared to be associated with the dehydrogenase activity of the organism. Treatments that inhibited dehydrogenase activity prevented the reduction of methylene blue as well as iron.

Since dehydrogenase activity is coupled with the reduction of oxygen, this biogenesis of electrons might well partly be responsible for the drop in redox-potential, Eh, observed in soils. The decrease in Eh has been reported to parallel the rate of iron reduction and solubilization of other elements in many different waterlogged and submerged soils (Tokai et al., 1956; Clark and Resnicky, 1956; Stobe, 1956; BaasBecking et al., 1959; Oborn, 1960a, b; Alexander, 1961; Ahmad, 1963; Savant and Roscoe, 1964).

Tokai et al. (1956) found a rapid reduction of iron simultaneous with a decrease in Eh during the first week following flooding. Drop in Eh and the rapid reduction of iron were associated with denitrification and vigorous production of carbon dioxide; successively hydrogen and methane generation dominated and finally an active sulphate reduction took place. Oborn (1960), cited the work of Stobe (1956), who also observed that waterlogging and consumption of oxygen by microorganisms caused a decided decrease in redox-potential and a lower pH, whereas iron and manganese solubilization increased. In terms of oxidation-reduction

potential, ferrous iron became prominent at Eh values below 200 mV during a period of intense bacterial action. The significance of bacterial activity was apparent since treatment with organic matter enhanced the reduction. The quantity of ferrous iron released was related to the amount of fermentable substrate (Clark and Resnicky, 1956). Further, the formation of divalent iron was terminated when soils, incubated with glucose under waterlogged conditions, were treated with a little amount of a bacterial inhibitor. This showed that certain iron reducing bacteria were responsible for iron transformation under anaerobic conditions (Yamanaka and Motomura, 1959). Similar features were found in natural waters. Mortimer (1942) showed the relationship between the fall in redox-potential with increasing depth in lake water and the rise of ferrous iron. At the point of total oxygen depletion, the Eh had dropped to 150 mV corrected to a pH of 7, and ferrous iron was detected in the water.

This review of investigations on the mechanisms and factors in iron reduction leads to the tacit assumption that iron transformation is probably not related to any specific microbial metabolism. In the course of gathering the data on iron reduction it soon became clear that both the intensity of the redoxpotential and the degree of acidification, i.e., the activity of electrons and protons were essential in shifting the chemical equilibria between the oxidized and reduced form of iron.

A theoretical approach to the ferrous-ferric equilibria in solutions, representing the relationship between pH and Eh, is shown in the stability-field diagram prepared by Hem and Cropper (1959). This diagram indicates under what conditions the specific ferrous and ferric ionic species dominate under atmospheric conditions (see Fig. 1).

FERROUS-FERRIC CHEMICAL EQUILIBRIA
AND REDOX-POTENTIALS

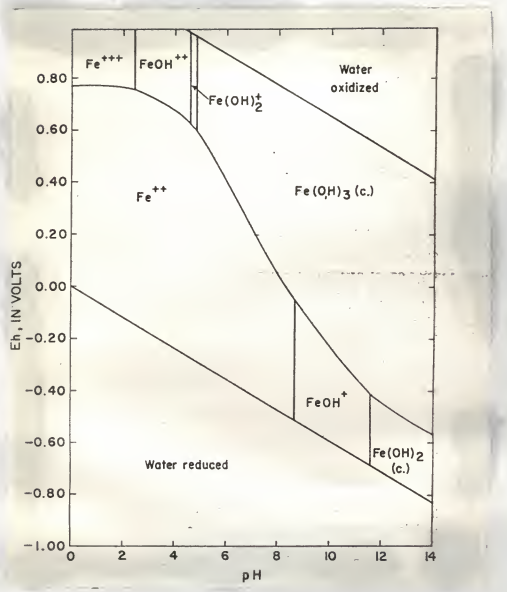


Fig. 1. Stability-field diagram for aqueous ferric-ferrous systems (after Hem and Cropper, 1959).

Iron Reduction and Organic Ligands

Though iron reduction may be essentially of microbiological origin, Bloomfield (1951, 1953a, b) has put forward the view that the transformation of iron and its solubilization is at least partly caused by plant degradation and extractable products. He proved that sterile solutions of fermented grass and aqueous extracts of Kauri pine leaves and bark (Agathis australis) dissolved and reduced ferric oxides. It is well known that iron combines readily with many organic ligands. Delong and Schnitzer (1955) reported that leaf extracts and solutions from forest canopies were "capable of mobilization and transportation of iron", and they believed an "acidic polysaccharide" to be the active material of the extracts. Moreover, not only polyhydroxy-alcohols, but also solutions of tannic acid were found to reduce dissolved ferric iron to the ferrous state (Hem, 1960). Dilutions of this acid from 5 to 50 ppm reduced iron extensively when the pH was less than 4. Tannic acid, a monoprotonic acid, is very common not only among plants, but is also present in certain green algae and representative of Pore fungi (Polyporales). Recently, Muir et al. (1964) isolated an iron dissolving fraction of organic acids from dried pine needles. The active part of the fraction consisted of alfa-hydroxycarboxylic acids. This is interesting, since many holofermentic- and mixed-acid bacteria generate alfa-hydroxyacids, such as lactic- and citric-acid, among their waste products.

One would expect complexed ferrous iron to be stable only in reducing environments. In fact, the complexing ligands did not alter the general

stability relationship between Eh and pH; however, both factors might be changed and the rate of reduction could be delayed compared to conditions without complexing substances (Hem and Cropper, 1959).

Iron Reduction and Gleying

When Yamanaka and Motomura (1959) incubated soil with the addition of glucose under waterlogged conditions, a gray discolorization was noticed while pH and Eh decreased. Although it has long been known that microorganisms play an important part in pedological processes of many kinds, it is not certain when it was first realized that gleying is caused by microbiological reduction of trivalent iron compounds. Albrecht (1941) may be the first who explained gleying by microbiological activity. He regarded the process as necessarily involving the production of hydrogen sulfide. The reduction of sulphates to hydrogen sulfide by Vibrio desulphuricans resulted in the formation of ferrous sulfide, imparting a dark blue or black colour, characteristic among "gley-spots". However, this process may be regarded as a special example, different from the general pattern of gley formation. Allison and Scarseth (1942), in developing a biological method for iron determination in soils, discovered that the anaerobic incubation of soil with sucrose resulted in a gray bleaching of the material. These discolorizations were verified by Bloomfield (1950), and were similar to those found in Gley and Pseudogley soil types. When Bloomfield fermented a mixture of clay and sugar under microaerophilic conditions, he found that the rate of gleying followed about the same sigmoid growth curve as the disappearing of the energy

source, indicating the activity of microorganisms. However, no pure cultures responsible for the discolorization could be isolated. Bromfield (1954a) collected samples of gleyed material aseptically from different zones in a soil profile. Iron reducing bacteria could only be detected in the surface layers of gleyed soils, but not in samples taken at a depth of about 3 m. when the isolated bacteria were tested for iron reducing ability, 5 different species gave positive results.

Iron-Reducing Bacteria

Among the bacteria converting ferric iron to the ferrous state, two groups may be recognized: (1) species of the obligate anaerobic Clostridia (Starkey and Halvorson, 1927; Roberts, 1947), and (2) a heterogenous group of facultative anaerobic organisms, belonging to the families Bacillaceae and Enterobacteriaceae. The latter group includes the species Escherichia coli (Starkey and Halvorson, 1927), Bacillus polymyxa (Roberts, 1947; Bromfield, 1954a), B. circulans, Escherichia freundii, Aerobacter aerogenes and Paracolobactrum sp. (Bromfield, 1954a, b). All the facultative anaerobes were reported to transform iron from the tri- into the divalent state when grown in pure cultures, whereas among the strict anaerobes only Clostridium sporogenes was found to do so (Starkey and Halvorson, 1927).

Studies on the dissolution of iron by specific fungi have not been reported yet. In completion, it should be mentioned that Oborn and Hem (1961) isolated Verticillium sp. and Cladosporium herbarum from anaerobic incubations of a mixture of soil and organic matter. Though considerable amounts of iron were dissolved, it is not evident whether these

spore-forming fungi were responsible. Heavy gas production was noted which could have been bacterial activity. Reduction by bacteria is likely since both fungi are known as organic matter decomposers.

Characterization of the Reduction Intensity by the rH

Redox conditions of the medium in which microorganisms develop may be ascertained by determining the oxidation-reduction potential of the system. It provides a means of judging the intensity with which the system is capable of reducing or oxidizing the material occurring within it. The Eh alone, however, does not characterize the redox-conditions completely. When the hydrogen ion concentration varies, the ionization phenomena should be taken into account, because it affects oxidation-reduction systems by altering the ionic equilibria. Hewitt (1950) applied Nernst's electrode equation to a bacterial system and derived:

$$Eh = Eo + \frac{RT}{F} \ln \frac{(Ox)}{(Red)} - \frac{RT}{F} Ln \frac{Kd}{(H^+) + Kd}$$

in which Kd represents the dissociation constant of the reduced form. It will be seen that the Eh depends on the hydrogen-ion concentration and the dissociation constant of the reduced form. Therefore it is necessary to quote results in terms of Eh and pH.

Derivation of the Reduction Intensity, rH

A redox system can also be regarded as being in equilibrium with hydrogen gas of 1 atm., the hydrogen electrode. The Nernst equation for the hydrogen electrode

$$Eh = Eo + \frac{RT}{2F} \ln \frac{(H^+)^2}{(H_2)}$$

will apply to this hypothetical pressure of hydrogen gas, since the following equilibrium may establish at the electrode

$$H_2 \rightleftharpoons 2H^+ + 2e$$

The Eo for a hydrogen electrode is zero and thus the equation will be

$$Eh = -\frac{RT}{2F} \ln (H_2) + \frac{RT}{F} \ln (H^+)$$

After rearranging to common logs and introducing the term rH, defined as the logarithm of the reciprocal of the hydrogen pressure, $rH = -\text{Log}(H_2)$, we derive

$$Eh = 0.03\ (-2pH + rH) \text{ because } \frac{2.3 \times RT}{2F} = 0.03 \quad (30\ C)$$

$$rH = \frac{Eh}{0.03} + 2pH$$

The range of the rH values extends from 0 to 42.6; i.e., from totally reduced (rH = 0) until completely oxidized (rH = 42.6) conditions. At totally reduced conditions at 1 atm. hydrogen pressure and equilibrium between atmosphere and the hydrogen in solution, the rH becomes -log 1 = 0. In an aqueous system, at completely oxidized situation, the rH value reaches 42.6, since at 1 atm. of oxygen pressure the hydrogen pressure will be decreased to $10^{-42.6}$ atm., because at these conditions water may be regarded as dissociated into hydrogen and oxygen:

$$K = (H_2)^2 \times (O_2) = 10^{-85.2}$$

$$2 \times rH + rO = 85.2$$

$$rH\ 3\ 42.6$$

The reduction intensity expresses the degree of saturation with hydrogen and represents a quantitative index of the redox-situation in the medium. In contrast to the Eh, it reflects the redox-balance regardless of the magnitude of the pH (Tendeloo, 1957; Kuznetsov et al., 1963; Rabotnowa, 1963; Stephenson, 1966).

Characterization of Facultative Anaerobic Bacteria by the rH

The study of changes in oxidation-reduction potentials in cultures has been pursued by several investigators, since Potter (1911) first noted that electrode potentials of inoculated media were more negative than a sterile control. After numerous publications, the interest in this topic ceased again in the early forties. The question, however, whether bacteria can be characterized by the redox-potential or reduction intensity they create, has been studied repeatedly.

Aubel and Aubertin (1927), for example, cultivated anaerobic- and aerobic bacteria in an agar medium and defined the rH-range using different rH- indicators. From their studies they concluded that obligate anaerobes, like different Clostridium species, develop well in a rH-range from 0-12, whereas facultative anaerobes such as Escherichia coli, Proteus vulgaris and Pseudomonas fluorescens could grow easily in a wider rH-range from 0-20.

The concept that obligate anaerobic bacteria prefer low redox-potentials has been strengthened by other experiments. Plotz and Geloso (1930) detected the growth range of Clostridium tetani colorimetrically and electrometrically in a meat-extract broth culture and found an optimal

proliferation between rH 4 and 15, whereas Reed and Orr (1943) reported an rH of 8 for most Clostridium species. Aubel, Rosenberg and Grunberg (1946) further recorded the establishment of an rH range of 11-12 for Cl. saccharobutyricum and Cl. sporogenes. It seems that the most favorable rH-range for obligate anaerobes is established around a rH value of 10.

Escherichia coli may be mentioned as a typical representative of facultative anaerobic bacteria. This organism was able to grow in completely anaerobic conditions, in which the Eh decreased to the level of the hydrogen electrode: rH = 0 (Clifton and Cleary, 1934). Plotz and Geloso (1930) noted that E. coli dropped the rH to 5.5 within 48 hrs. This value was reached independent of the potential at the time of inoculation. A lower minimum was reported by Clifton, Cleary and Beard (1934). In a sugar containing medium the rH decreased during the log phase of E. coli from 27 to 1, after which it increased again because of unfavorable conditions (pH about 4). If they maintained the rH at certain levels, it was found that E. coli was able to grow in the wide rH range from 30 to 1; i.e., from rather oxidizing to strong reducing environmental conditions. Thus, opposite to the obligate anaerobes, the facultative anaerobic bacteria may be characterized by a less specific and broader rH-range. Organisms of both groups, however, seem to establish redox-conditions during the stationary phase that are typical for each species (Gillespie and Rettger, 1938a, b; Gillespie and Porter, 1938).

METHODS AND MATERIALS

In order to elucidate the significance of the rH of a bacterial culture on the reduction of iron, a hematite ($Fe_2O_3.3H_2O$) rich shale was chosen as test material. The development of the hydrogen ion concentration and the redox-potential during the reduction process in the system were followed electrochemically by a special designed experimental set-up.

An Erlenmeyer flask of 250 ml contents, having a wide path of about three cm, was filled with a 1.5 cm layer of sieved (100 mesh) experimental clay. Each flask was equipped with a platinum electrode, specially constructed for the measurement of R/O-potentials. The tip of the electrode, a "non-attackable" platinum spiral, had been soldered to a copper wire which was sealed in a glass conduit in order to prevent polarization. This electrode was permanently inserted in the growth medium and reached into the sieved shale, nearly in the center of the flask.

Before inoculation, the shale in the test flask was soaked with a 2% sucrose solution, filled half with more sucrose solution and closed with an aluminum foil cap. The set-up was autoclaved for thirty minutes at 121 C. After cooling down, the flask was completed up to the neck with separately sterilized 2% sucrose solution.

Origin of the Test Material

The experimental substrate, an iron rich, red colored shale, was collected on the northern slope of the Tuttle Creek Dam overflow. This sediment belonged to an Eskridge formation which is defined as overlying the Neva Limestone member of the Grenola Limestone and underlying the

Cottonwood member of the Beattie Limestone. The formation, typically red-colored by oxidized iron, is one unit of a series of vari-colored shales assigned to the Lower Permian System which crop out in many places in Kansas (Moore et al., 1944). The Eskridge formation is a littoral sediment, which was deposited in a subaerial plain of deltioc origin adjoining a sea fluctuating in level. The tropical conditions prevented the accumulation of organic matter, whereas the periodical dry environment produced the large amount of hematite, accumulatee on the surface of the clay mineral illite (Waterman, 1951). Waterman showed the presence of both ferrous and ferric iron in samples of the Eskridge shale. From a total amount of 0.32-0.34% Fe_2O_3, only 0.08-0.10% occurred in the ferrous state, the remainder being trivalent oxidized iron.

Electrometric Determination of pH and Eh of the Iron-reducing System

After inoculation of the medium with three loops of a 24-48 hr old pure culture, the flasks were incubated at 30 C in a jar in which a lighted candle was placed. This oxygen depleted, carbon dioxide enriched environment simulated anaerobic conditions.

Changes in the pH and Eh were measured using a Beckman pH-meter, model H-2, as the recording instrument. The pH was detected by employing a glass electrode as the experimental half-cell with reference to the calomel electrode. The Eh was obtained by recording the differences in e.m.f. between the immersed Pt-electrode of the flask and the glass electrode. The e.m.f. with reference to the hydrogen electrode was calculated from the equation:

$$Eh = E_{measured} + 0.191\ V \qquad (at\ 30\ C)$$

The potential + 0.191 V represents the difference in e.m.f. between the glass electrode and the normal hydrogen half-cell. It was derived as follows: The recommended standard half-cell potential of the saturated calomel electrode at 30 C is 0.241 V with respect to the hydrogen half-cell. The difference in potential between the glass electrode and the saturated calomel half-cell was measured with the Beckman pH-meter, model H-2, in a standard Beckman pH-buffer solution of pH = 7.0. The potential of the glass electrode was found to be 0.050 V lower than the e.m.f. of the calomel electrode (Ives and Janz, 1961; Clark, 1960).

The reduction intensity was calculated from:

$$rH = \frac{Eh}{0.03} + 2pH \qquad (at\ 30\ C)$$

Before and after each determination the electrodes were thoroughly cleaned by washing them with sterilized distilled water. To ascertain sterility, the half-cells were disinfected carefully with cotton soaked in 10% phenol and subsequently immersed two times for a few minutes in 10% phenol and 95% ethanol. The inoculated flasks were checked for contamination by streaking nutrient agar plates at regular intervals.

Methods for the Determination of Total Dissolved Iron ($Fe^{3+} + Fe^{2+}$) and Ferrous Iron

Determination of Total Iron. After the electrometric determinations, the total amount of iron brought into solution from the shale by bacterial activity was determined colorimetrically after Jackson (1958). An 1 ml aliquot of the solution was pipetted in a volumetric tube graduated at 15 ml and acidified with 1 ml of a 0.6 N HCl solution. Next, 2 ml of a 1%

hydroxylamine hydrochloride preparation and 1 ml of a 1.5% orthophenanthroline solution were added, resulting in formulation of an orange iron complex. The sample was made up to volume with distilled water, centrifuged for 5 minutes at 5,000 rpm and the transmission read in a Bausch and Lomb Spectronic 20 spectrophotometer at 490 mu wavelength. The concentration of the amount of total iron released was calculated from a calibration curve, prepared from a standard iron solution.

Determination of Ferrous Iron. The reducing capacity of bacteria was tested as the amount of ferrous iron dissolved from a ferric salt after a certain time of incubation. Therefore, 1 ml samples of the culture broth were pipetted into a 15 ml graded tube. To this 1 ml of a 0.2% solution of alpha alpha'-dipyridyl in 10% acetic acid was added and then the volume made up to the mark with distilled water. The solution was centrifuged for 5 minutes at 5,000 rpm in a Serval table centrifuge to clear. The intensity of the red colored ferrous complex was measured in the same Bausch and Lomb spectrophotometer mentioned above, at a maximum absorption of 525 mu. From a standard calibration curve, the readings are converted to concentrations of ferrous iron and expressed in mg Fe^{2+}/l (modified after Bromfield, 1954; Asami and Kumada, 1959).

The calibration curve for the total- and ferrous-iron determination was prepared as follows. Precisely 0.7023 g ferrous ammonium sulphate ($Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$) was weighed out and dissolved in distilled water acidified with 20 ml 0.6 N HCl/l. The solution was then transferred to a liter volumetric flask and completed with distilled water to the mark. This solution contained 100 ppm Fe. From this stock solution, 10 ml were diluted to 100 ml and from this dilution, aliquots (0.1; 0.2; 0.3; 0.5;

1.0; 2.0; 3.0; 4.0 and 5.0 ml) were taken and either reacted with 1 ml of 0.2% alpha alpha'-dipyridyl solution or a 1.5% orthophenantroline reagent, according to the described procedures, for the outline of the Fe^{2+} and total iron calibration curves. An average of five determinations was used in plotting against the iron concentration.

Determination of the total- and ferrous iron calibration curves

Fe in ppm	Reading in % transmission	
	490 mu	525 mu
0.1	98	98
0.2	95	96
0.3	91	89
0.5	85	81
1.0	76	64
2.0	55	48
3.0	39	37
4.0	29	24
5.0	20	18

Method for the Determination of the Iron-reducing Capacity of Facultative Anaerobic Bacteria

The various media were dispersed in 25 ml portions into 6 in. x 1 in. boiling tubes and sterilized at 120°C for 20 minutes. After cooling, 5 ml of a sterile ferric hydroxide suspension (1%) of 5 ml of a ferric chloride

solution (0.2%) was pipetted to each tube and afterwards inoculated with 2 drops of a suspension of a 24-48 hour slope culture, grown on nutrient agar. The test media were either incubated aerobically or anaerobically in a jar, in which a lighted candle was placed, for 5 days at 30°C. Ferrous iron was determined in 1 ml aliquots taken from the broth as described earlier. At the same time the final pH was recorded.

Bacteria and Media used in Studies on the Iron-reducing Capacity

The following cultures of bacteria used during the experiments were collected from various sources:

Bacillus circulans	Prof. Harris, Foltz
B. polymyxa	" "
B. cereus	" "
B. subtilis	" "
B. megaterium	" Foltz
Escherichia freundii	" Harris
E. coli B	" "
Serratia marcescens	" "
Pseudomonas aeruginosa	" McMahon
Ps. vulgaris	" Harris
Micrococcus luteus	" Foltz
Erwinia carotovora	" "
Unidentified coccus TF	" Harris
Aerobacter aerogenes	Isolated by author from soil
A. cloacae	" " " " "

These cultures were maintained on nutrient agar throughout this work. To insure viability the organisms were transferred every two to three weeks.

The iron-reducing capacity of bacteria was studied in different growth substrates in order to detect the effect of the media on the amount of reduced iron.

The following media were selected (in g/100 ml aqua dist.):

I. Halvorson and Starkey's medium (1927):

$(NH_4)_2SO_4$, 0.01 g; K_2HPO_4, 0.01 g; $MgSO_4 \cdot 7H_2O$, 0.001 g; $CaCl_2$, 0.001 g; glucose, 1.0 g.

II. Roberts' medium (1947):

K_2HPO_4, 0.31 g; KH_2PO_4, 0.08 g; KCl, 0.02 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; asparagine, 0.5 g; glucose 2.4 g.

III. Bromfield's medium (1954a):

KH_2PO_4, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; $(NH_4)_2SO_4$, 0.1 g; sucrose, 0.5 g; $CaCO_3$, 0.5 g; $Fe(OH)_3$, 0.05 g; Difco yeast extract, 0.015 g.

IV. Pichinoty's medium (1963):

Na_2HPO_4, 0.36 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.003 g; $FeSO_4$ and $CaCl_2$ each 0.001 g; KNO_3, 0.05 g; Difco yeast extract 0.05 g; glucose 0.4 g.

The media were adjusted to pH 7.

Procedure of Isolating Unknown Iron-reducing Bacteria

Water, soil and fecal samples were collected from the Blue River Pond area near Tuttle Creek Dam. The soil (about 5 g) was suspended in 100 ml sterile water and after shaking for a few minutes allowed to settle. From the natural water samples and from the soil and fecal extracts, 0.1 ml and 1.0 ml aliquots were transferred to lactose-broth fermentation tubes and observed for the production of acid and gas after 24 hours incubation at 30 C. Those tubes which showed acid and gas production, EMB-agar plates were streaked and after 24 hours, 18 different colonies selected for further studies. These organisms were isolated in pure culture and tested for the production of hydrogen. In order to determine the generation

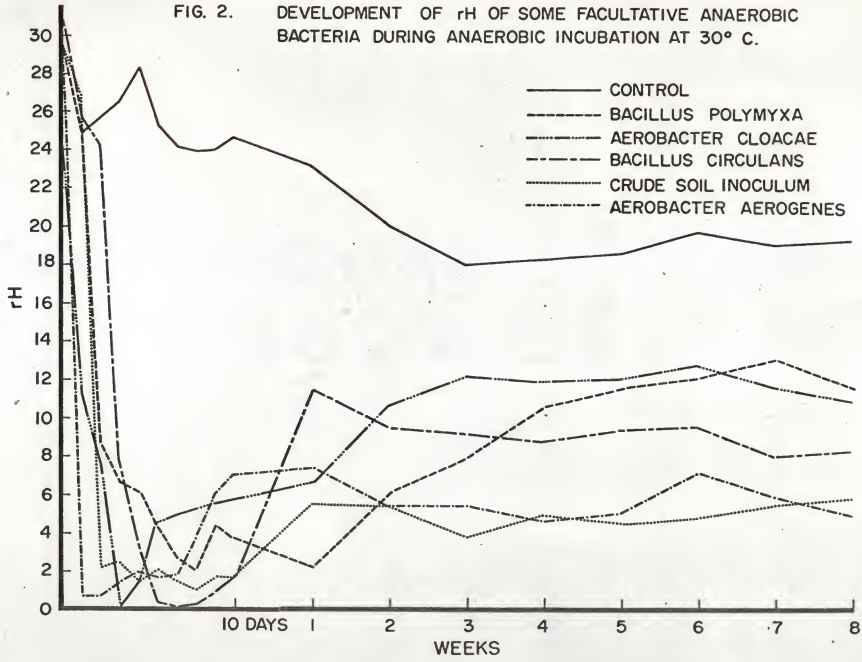
of molecular hydrogen, 18 strains were inoculated in Smith tubes containing a 5% glucose broth. After 48 hours, the tubes were completely filled with 20% potassium hydroxide and shaken for 5 minutes to absorb carbon dioxide. The difference in gas volume before and after the treatment with alkali was measured and the carbon dioxide/hydrogen ratio estimated.

EXPERIMENTAL RESULTS

I. Characterization of the Facultative Anaerobes *Aerobacter aerogenes*, *A. cloacae*, *Bacillus polymyxa* and *B. circulans* by the rH

In the present investigation the 4 tested facultative anaerobic bacteria could be characterized by the degree and level of the rH. When a clay fraction of the Eskridge shale was inoculated with either *Aerobacter aerogenes*, *Bacillus polymyxa*, *B. circulans* or strain 17 (an iron-reducing bacteria later identified as *A. cloacae*), a marked drop in the reduction intensity during the log phase was observed. These results are shown in Fig. 2. *Bacillus circulans* developed the lowest reduction intensity (rH = 0), whereas *B. polymyxa* reached its minimum at a value of 2.1, the highest minimal rH recorded. With respect to the time needed to establish these minimal values, *A. aerogenes* and *A. cloacae* were most active; the latter required three days, the former just 24 hours. In contrast to these species, neither species of Bacillus established maximal reducing conditions until 5-6 days. The flasks inoculated with a crude soil inoculum exhibited a less pronounced minimum. The control, indeed, developed some reducing properties. However, a minimal reduction intensity of 18 was only recorded after 31 days of incubation.

FIG. 2. DEVELOPMENT OF rH OF SOME FACULTATIVE ANAEROBIC BACTERIA DURING ANAEROBIC INCUBATION AT 30° C.



In general, changes in reduction intensity in media with different bacteria show principally the same features; the potential decreases more or less rapidly, reaches a minimum at which it remains for some time, and increases than to a certain level (Rabotnowa, 1963). These characteristics are revealed clearly by the bacteria tested (see Fig. 2). After 30 days of incubation, certain levels of reduction intensity were established. From the 30th day until the end of the determinations after 66 days, the bacteria exhibited rather marked and constant differences in rH. The reduction intensity appeared to be specific for the organism in question.

Table 1. Average pH and Eh determinations and derived rH from the 30th until the 66th day of anaerobic incubation in a jar.

Organism	rH	Eh(mV)	pH
Crude soil inoculum	4.8	-215	6.0
A. aerogenes	5.5	-220	6.4
B. circulans	8.9	-75	5.7
B. polymyxa	10.7	-31	6.0
A. cloacae	11.8	+19	5.6

Characterization of the Facultative Anaerobes A. aerogenes, A. cloacae, Bacillus polymyxa and B. circulans by Gleying and Iron-reducing Capacity

Besides the test organisms Bacillus polymyxa and B. circulans, obtained in pure culture from stock cultures, two other actively iron-reducing bacteria, Aerobacter aerogenes and Aerobacter cloacae, were used.

A. aerogenes was isolated in pure culture by streaking plates (Bromfield's medium solidified with 2% agar) from a two-week-old flask, which had been inoculated with a crude inoculum. The presence of an iron-reducing organism was detected by the bleaching of the top layer of the red shale in the experimental flask. A crude inoculum was prepared by incubating 5 g garden soil with 200 ml of a 0.5% sucrose solution and 0.1 g ferric oxide at 30°C. After 4 days the active crude culture (heavy gas formation) was shaken, allowed to settle for a few minutes and 3 ml of the supernatant added to a fresh autoclaved experimental set-up. The only organism isolated was tested biochemically and was easily classified as Aerobacter aerogenes (see biochemical tests, strain 19).

The isolation of a second unknown iron-reducing bacterium (strain 17) has been described under Methods and Materials. The identification by biochemical tests will be discussed on page 49.

Iron Reduction and Gleying. The flasks inoculated with B. polymyxa, A. aerogenes, A. cloacae (str. 17) or other crude inoculum, showed heavy gas production after 48 hours. Bacillus circulans, however, did not produce any gas. Growth was recognized by turbidity and was ascertained by the addition of 2 ml of a 0.1 sterile yeast extract, since it is known that B. circulans requires certain vitamins for growth.

In the flasks showing gas production, the smooth surface of the shale was disturbed by the formation of many "craters" from which the gas escaped. These "funnels" reached deeper the more the incubation proceeded, indicating the movement of the bacteria to the bottom of the flasks (see Plate I).

Iron reduction was noticed visually by bleaching of the shale. Both Bacillus circulans and Str. 17 showed first graying after 7 days, whereas

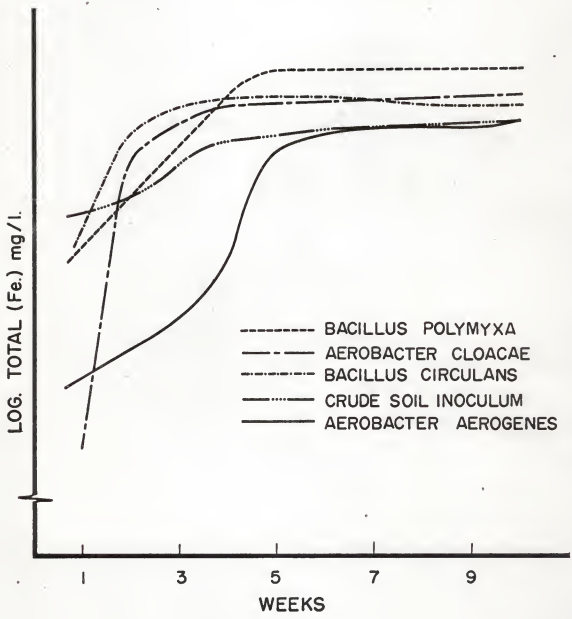
EXPLANATION OF PLATE I

Iron reduction and patterns of gleying.

B. polymyxa and A. aerogenes did not show any discolorization until 14 days. However, it should be mentioned that one of the duplicates of A. aerogenes did not display any bleaching throughout the whole period of incubation, though iron reduction was detected colorimetrically. The crude inoculum produced its first gleying after 13 days. No gray layer was formed, but little, isolated gray-blue spots as well as more grayish-diffuse zones were observed, scattered through the shale. These findings suggest the presence of at least two different organisms, since type of discolorization and intensity seemed to be specific properties. B. circulans, for example, created a thin green-gray layer on the surface of the clay only, whereas A. aerogenes could be distinguished by an ash-gray zone, intensively bleached in the upper part but decreasing with depth. Like A. aerogenes, B. polymyxa was characterized by a zone of bleaching. However, a sharp margin separated the reduced and oxidized parts. Further, a typically blue-gray color was assigned to the reduced zone. Strain 17, on the other hand, may be recognized by a more gray-brown type of reduction, of which the intensity decreased with depth. These features may be regarded as characteristic for each organism, since they were also noted in the duplicate set-up.

Reduction Intensity and Iron-reducing Capacity. The development of the iron solubilization and the maximal quantity of iron brought into solution by B. polymyxa, B. circulans, A. aerogenes and A. cloacae are presented in Fig. 3. These graphs indicate the differences in iron reducing capacity of the organisms tested, revealed on Eskridge shale. About 4 to 5 weeks after the incubation period was started, the maximal amount of iron transformed was reached and remained at a constant level.

FIG. 3. DEVELOPMENT AND QUANTITY OF IRON REDUCED FROM A HEMATITE-RICH SHALE BY SOME FACULTATIVE ANAEROBIC BACTERIA UNDER ANAEROBIC CONDITIONS.



This period corresponds well with the time at which the organisms had established their characteristic rH levels. However, no relationship seemed to exist between this rH level and the amount of iron dissolved. These conclusions are derived from the results presented in Table 2.

Table 2. Average values of rH and the quantity of dissolved iron from the 30th day until the end of the incubation period of 66 days.

Organisms	rH	Fe^{2+} (mg/l)
B. polymyxa	10.7	875
A. cloacae	11.8	342
B. circulans	8.9	318
A. aerogenes	5.5	154
Crude soil inoculum	4.8	175

From Table 2 it is further evident that the iron-reducing capacity of the tested organisms differs greatly. B. polymyxa elicited a reducing ability about 5 times stronger than A. aerogenes.

Even during the first 10 days of the incubation period, no relation between the decreasing rH and the intensification of the reduction of iron could be found. The development of the reduction intensity and the quantity of iron transformed by the tested bacteria during the initial growth is shown in Table 3.

From Table 3 it may be concluded that only in the case of B. circulans a decrease in rH caused a rapid increase of the amount of iron reduced during the first 10 days of incubation. Since an increase in reduction

Table 3. Development of rH and amount of iron dissolved from Eskridge shale during the initial 10 days of anaerobic incubation.

Time in days	2		4		6		8		10	
Culture	rH	Fe^{2+} (a)	rH	Fe^{2+}	rH	Fe^{2+}	rH	Fe^{2+}	rH	Fe^{2+}
Crude inoculum	2.2	0.0	1.5	22.0	1.5	30.0	1.7	38.0	1.7	40.0
A. aerogenes	0.8	0.0	2.0	2.5	1.9	5.0	6.3	6.0	10.0	8.0
B. polymyxa	8.7	1.5	6.3	4.0	4.2	10.5	2.1	35.0	6.4	50.0
B. circulans	24.3	1.0	4.0	11.0	0.0	31.0	1.0	105.0	6.6	185.0
A. cloacae	7.4	0.0	1.5	2.0	5.0	3.0	5.8	22.0	7.0	120.0

(a) Fe^{2+} (reduced iron) in ppm.

intensity is caused by lowering pH and Eh of the environment by metabolic activity, we may ask what metabolic products could be responsible for this feature. All tested bacteria ferment glucose with a variety of end products, among which organic acids are the major components (Wood, 1961). The striking difference between B. circulans and the other organisms lies in the absence of the gases CO_2 and H_2. Instead, the available hydrogen is utilized by B. circulans to form glycerol, a neutral product. If it is assumed that molecular hydrogen is the electron donor in the reduction process of A. aerogenes, A. cloacae and B. polymyxa then no reduced end products seem available to explain iron reduction and drop in rH by B. circulans. When, however, the formation of some unknown reducing substances, "reductones" (Euler and Hasselquist, 1955; Rabotnowa, 1963; Rabotnowa et al., 1963) is supposed, which accumulate in the medium, then the findings found with B. circulans could be elucidated. The slow increase in the amount of iron brought into solution by A. aerogenes, A. cloacae and B. polymyxa could be ascribed to the escape of hydrogen from the shale in opposite to the accumulation of other reducing substances in the case of B. circulans.

II. Determination and Comparison of the Iron-reducing Capacity

The iron-reducing capacity of microorganisms may be determined by the addition of ferric compounds, such as $Fe_2(SO_4)_3$, $Fe(NO_3)_3$, $Fe(Cl)_3$ or $Fe(OH)_3$ to the medium, followed by the measurement of the amount of iron reduced after a certain period of incubation. The advantage of the first 3 substances mentioned compared to ferric hydroxide is their greater

solubility. This means a better distribution through the medium and thus an increased reactivity.

In order to compare the effect of the different ferric salts on the reducing capacity of an organism, tubes containing 25 ml Bromfield Medium (pH = 7.0) received either 5 ml ferric sulfate, ferric nitrate or ferric hydroxide solution, in concentrations of 1.0, 0.5 and 1.0%, respectively. After an anaerobic incubation period of 5 days, pH and the amount of iron reduced were determined.

Independent of the pH of the medium before the inoculation, a slightly acid environment was established by microbial activity, as is seen in Table 4. In the presence of ferric hydroxide, the acidity became more pronounced than when ferric sulfate or ferric nitrate were added. This indicates that the organisms in the acid sulfate medium have produced neutral rather than acid waste products.

Table 4. The reduction of ferric iron from ferric sulfate, nitrate and hydroxide after a 5-day anaerobic incubation period. Average values of two determinations.

Organism	I[a]		II[a]		III[a]	
	Fe^{2+} (mg/l)	pH	Fe^{2+} (mg/l)	pH	Fe^{2+} (mg/l)	pH
Aerobacter aerogenes	25	5.9	4.5	5.9	1.5	5.9
Bacillus polymyxa	4.5	6.0	0	6.4	0.5	5.8
Escherichia freundii	8.0	6.1	0	6.4	0	5.4
E. coli B	4.5	5.8	0	6.6	0.5	5.3
Serratia marcescens	3.5	6.0	0	6.2	3.0	5.2
Unidentified coccus TF	4.0	6.5	0	7.0	0	5.5
Control	1.5	5.2	0	5.1	0	6.7
pH before inoculation		5.0		4.9		8.3

(a) I = $Fe_2(SO_4)_3$; II = $Fe(NO_3)_3$; III = $Fe(OH)_3$.

The influence of the pH on metabolism is well established for certain mixed-acid fermentators. The dependence of 2,3-butanediol fermentation of Aerobacter indologenes from the acidity was studied by Michelson and Werkman (1938). The critical pH was located at 6.3; above this value, acetic and formic acid accumulated and the production of hydrogen, carbon dioxide, acetoin and the neutral products acetylmetylcarbinol and 2,3-butanediol was prevented. Below this critical value large amounts of CO_2 and H_2 were generated from glucose. Further, under acid conditions the main acid produced was lactic acid. Similar results were reported by Orlowa (1950, cited after Rabotnowa, 1963), who followed the fermentation products of A. aerogenes at different pH values. The critical pH for this organism was reported at 6.0; only below this limit the production of neutral waste products could be detected. Further, at pH 8.1 about 64.5% of the total amount of acids was acetic acid; at pH 4.9 acetic acid only could be found. In general, for both A. aerogenes and B. polymyxa, most studies agreed that the critical pH was about 6.3-6.5. A higher pH value excluded the formation of neutral products and organic acids accumulated (Long and Patric, 1963). A similar regulation mechanism was observed with E. coli by Stokes (1949). In the alkaline range, mainly volatile acids were produced (acetic, formic and little lactic acid), whereas CO_2 and H_2 were generated in the acid range.

This marked effect of pH on metabolic behaviour may explain the differences in iron reducing capacity in the presence of ferric sulphate or ferric hydrate. The generation of reducing molecular hydrogen by

A. aerogenes, B. polymyxa, E. freundii, E. coli and Serratia marcescens may account for the iron reduced at the lower pH levels.

The inability to reduce iron in a ferric nitrate containing medium may be interpreted by some recent findings for E. coli and Aerobacter aerogenes. Under anaerobic conditions, E. coli converts glucose predominantly to acetate, CO_2, H_2, ethyl alcohol, lactate and succinate, with minor amounts of 2,3-butanediol, acetoin and glycerol (Wood, 1961). Anaerobic controlled cultures of A. aerogenes produced primarily ethyl alcohol, acetate and formate, with smaller amounts of CO_2, H_2, succinate and lactate. Forget and Pichinoty (1964) and Dobrogosz (1966) discovered, however, that nitrate reduction coupled with anaerobic glucose metabolism altered the pattern of fermentation end products in A. aerogenes and E. coli, respectively. Both investigators found that with nitrate included in the medium, formation of ethyl alcohol, hydrogen, succinate and lactate was virtually eliminated, while pyruvate, acetate, formate and CO_2 accumulated as major end products. Nitrate seemed effective in competing for electrons that would otherwise have been directed to other waste products.

The results found and interpreted suggest the participation of molecular hydrogen in the reduction of ferric iron to the divalent state under certain conditions.

Comparative Studies Between Molecular Hydrogen and Non-hydrogen Producing Bacteria. In further studies, iron-reducing capacities were compared using various media containing ferric hydroxide or ferric chloride. The latter has been used by Bromfield (1954a). Two groups

of organisms were chosen and tested for their reducing capacities. The first group contained bacteria known for the production of hydrogen, while the second group included fermenters which failed to do so. The iron reducing capacity was tested upon different media used by other investigators. These culture media differed greatly in quality and quantity (see Fig. 2). Pichinoty's culture medium was chosen, since nitrogen was present as nitrate. The results, from an average of three determinations, are presented in Tables 4 and 5.

From these tables we may draw the following over-all conclusions. The organisms of group I, capable of producing hydrogen (except B. circulans), exhibited varying iron-reducing capacities, whereas those listed under group II and unable to produce hydrogen during fermentation processes, can not (or only to a minor extent) transform ferric iron to the ferrous state. Further, it is clear that the amount of iron reduced under partially aerobic conditions is greater than in the absence of oxygen. Only when the organismsof group I were incubated in Bromfield's medium, with the addition of ferric chloride, more iron had been reduced under anaerobic conditions.

The influence of the medium is unequivocal. All organisms tested manifested maximal iron-reducing capacities when incubated in Roberts' medium, the richest substrate with respect to both carbohydrate (2.4 g glucose/100 ml) and nitrogen (0.5 g asparagin/100 ml). Again the significance of nitrate in the medium on the reduction of iron under anaerobic conditions is revealed. Iron reduction in Pichinoty's medium is only shown in the presence of oxygen. Bacillus circulans was unable to

Table 5. Amounts of ferrous iron (mg/l) in solution and final pH values in various media which received $Fe(OH)_3$ (Group I) or $Fe(Cl)_3$ (Group II) before inoculation. Incubation aerobically and anaerobically for 5 days at 30°C.

Organism	Halvorson and Starkey's				Roberts'				Bromfield's				Pichinoty's			
	aerobic		anaerobic		aerobic		anaerobic		aerobic		anaerobic		aerobic		anaerobic	
	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}
Group I - 5 ml 1% $Fe(OH)_3$																
Aerobacter aerogenes	4.4	6.5	4.3	1.0	5.4	15.5	5.6	3.5	5.7	7.0	5.8	1.0	6.4	6.5	5.6	0
Bacillus polymyxa	4.5	2.5	5.0	0	5.9	3.5	5.9	1.0	5.3	2.5	5.6	0	6.2	2.0	5.1	0
Escherichia freundii	5.8	4.0	3.7	0	5.5	5.0	4.2	1.5	5.5	2.0	5.3	0	5.9	4.0	4.9	0
Escherichia coli B	5.6	2.5	3.7	0	4.4	9.0	4.4	1.5	4.8	2.5	5.1	0.5	5.2	2.5	4.9	0.5
Serratia marcescens	4.7	2.0	3.3	0	4.8	11.5	5.4	0.5	4.0	4.5	5.0	4.0	5.8	3.5	5.0	0
Bacillus circulans[a]	6.0	0	5.7	0	6.4	1.0	6.0	0	6.0	4.0	5.8	4.0	5.7	0	5.6	0
Control	5.7	0	5.5	0	6.8	1.0	6.7	0	6.0	1.0	6.5	0	5.6	1.0	4.7	0
Group II - 5 ml 0.2% $Fe(Cl)_3$																
A. aerogenes	2.8	0	-	-	4.9	19.5	4.8	2.0	5.6	1.0	5.9	1.0	5.3	0	-	-
B. polymyxa	2.9	0	-	-	5.8	3.0	5.9	2.0	5.5	0	5.6	3.0	4.6	0	-	-
Escherichia freundii	2.9	0	-	-	4.2	11.5	4.2	12.0	4.4	0	6.0	1.0	4.4	0	-	-
E. coli B	2.8	0	-	-	4.2	6.0	4.1	2.0	4.5	0	5.7	0.5	4.3	0	-	-
Serratia marcescens	2.7	0	-	-	4.4	10.0	5.3	1.5	4.6	0	5.0	1.5	5.3	0	-	-
B. circulans	2.6	0	-	-	6.0	3.5	6.3	8.0	4.8	2.0	5.5	5.0	4.7	0	-	-
Uninoculated control	2.6	0	-	-	6.0	1.0	6.6	1.0	5.2	0	5.2	0	5.2	0	-	-

(a) Does not produce hydrogen.

reduce iron at all, though the medium contained yeast extract, required for optimal growth by this organism. This may explain the highest yield of ferrous iron by Bacillus circulans in Bromfield's medium.

Predominating in the ability to reduce ferric iron were the organisms Aerobacter aerogenes, Serratia marcescens and Escherichia coli, dissolving more than 5 mg Fe^{2+}/l from $Fe(OH)_3$. However, in the presence of ferric chloride, this quantity was surpassed by A. aerogenes, Serratia marcescens, Escherichia freundii, Bacillus circulans and E. coli. The highest amount of ferrous iron scored under anaerobic conditions was 12.0 mg Fe^{2+}/l by E. coli, closely followed by Bacillus circulans with 8.0 mg Fe^{2+}/l, in Roberts' and Bromfield's media, respectively. Bromfield (1954a), however, using his medium reported a reduction capacity for B. circulans of 40 mg Fe^{2+}/l and for two B. polymyxa strains 20 and 25 mg Fe^{2+}/l. In the present investigation, not more than 3 mg Fe^{2+}/l could be calculated for B. polymyxa under anaerobic conditions.

Further, there was obviously no relation between the acidity and the dissolving ability of the organism tested. Moreover, the opposite may be derived: the greater the iron reducing capacity, the higher the final pH of the medium, especially under anaerobic conditions. This associates metabolic activity with the amount of iron transformed to the divalent state. Such a relation is supported by the fact that maximal iron reduction took place in Roberts' medium.

Reduction of Iron by Bacterial Culture Fluids. Bacillus circulans reduced iron to a maximal extent in Bromfield's medium under anaerobic conditions, as is shown in Table 4. Since no gases are produced by the

Table 6. Amounts of ferrous iron (mg/l) in solution and final pH values in various media which received $Fe(OH)_3$ or $Fe(Cl)_3$ before inoculation. Incubation aerobically and anaerobically for 5 days at 30°C.

	$Fe(OH)_3$								$Fe(Cl)_3$							
	Roberts'				Bromfield's				Roberts'				Bromfield's			
	aerobic		anaerobic		aerobic		anaerobic		aerobic		anaerobic		aerobic		anaerobic	
	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}
Bacillus cereus	6.8	1.0	6.3	0	5.5	1.0	6.4	0	6.1	2.0	6.4	1.0	5.6	0	6.2	0
B. megaterium	6.5	0	6.7	0	6.6	0	6.8	0	6.6	1.0	6.4	3.5	6.6	0	6.3	0
B. subtilis	6.8	0	6.7	0	6.5	0	6.7	0	6.6	0	6.4	1.0	6.7	0	6.1	0
Pseudomonas vulgaris	5.8	0	5.7	0	6.6	0	7.0	0	5.9	1.5	5.7	1.5	6.7	0	6.3	0
Ps. aeruginosa	6.3	0	6.1	0	6.4	0	6.9	0	6.5	2.5	5.9	1.5	6.2	0	6.1	0
Micrococcus luteus	6.5	0	6.5	0	6.8	0	7.1	0	6.3	0.5	6.3	1.5	7.0	0	6.4	0
Erwinia carotovora	5.7	0	5.7	0	6.2	1.0	6.5	0	5.8	9.0	5.9	1.5	5.7	0	5.6	0
Control	6.8	0	6.7	0	7.3	0	6.5	0	6.6	0.5	6.5	1.0	6.5	0	6.2	0

organism, other reducing substances must have been excreted into the medium.

To determine whether B. circulans, B. polymyxa, A. aerogenes and an unknown, but strong reducing organism (strain 9) reduced iron by the formation of accumulating reducing substances, the following experiment was carriet out. Boiling tubes, containing 25 ml of Roberts' medium, enriched with yeast extract (0.015 g/100 ml) were inoculated and incubated aerobically and anaerobically at 30 C for 5 days. The cultures were then either autoclaved or centrifuged during 15 minutes at 5,000 rpm and filtered through a 0.2 mu millipore filter. To these preparations 5 ml of a 1% sterile ferric hydroxide suspension was added and the culture fluids incubated anaerobically for 48 hrs at 30 C. Determinations of ferrous iron in solution and final pH values were made on the fluids. The results are presented in Table 7.

Table 7. Reduction of ferric hydroxide by 5-day culture fluids of various facultative anaerobic bacteria. pH and Fe^{2+} (mg/l), recorded in duplicate, after 48 hours.

Treatment	Autoclaved				Filtered (0.3 mu)			
	aerobic		anaerobic		aerobic		anaerobic	
	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}
A. aerogenes	5.9	0	6.1	0	6.0	0	6.0	0
B. polymyxa	5.5	0	5.4	0	5.2	0	5.5	0
B. circulans	6.2	0	6.2	0	5.9	1.0	5.7	0
Strain 9	6.0	0	6.0	0	5.7	0	6.1	0
Control	6.6	0	6.5	0	6.8	0	6.7	0

Apparently, the constituents of the culture fluids possessed virtually no capacity for iron reduction. Only the culture filtrate of B. circulans exhibited a slight reducing ability, if the organism had been grown under aerobic conditions. This favors the idea that iron reduction by bacteria other than B. circulans is caused by hydrogen, since no accumulation of reducing substances could be detected.

III. Determination of Iron-reducing Capacity of Some Unknown Iron-reducing Bacteria Isolated from Soil and Water

From different natural environments 18 unknown bacteria were isolated as described earlier in this paper. Origin of the strains are listed in Table 8.

Table 8. Origin of 18 selected strains producing acid and gas from lactose after 24 hours.

Origin of sample	Acid	Gas	Strain No. isolated
Catfish Pond	+	+	10, 11
Farmer's stock tank	+	+	12
Cedar Creek	+	+	13, 14, 15, 16, 17
Barn drainage	+	+	6, 7, 8, 9
Road side ditch	+	+	18
Cattle feces	+	+	1, 2
Fermenting grass	+	+	3, 4, 5

The amount of molecular hydrogen generated from a glucose broth by each of these 18 strains is shown on Table 9.

Table 9. The CO_2/H_2-ratio from fermented glucose by 18 isolated bacteria.

Strain number	Acid	Gas	CO_2:H_2
1	+	-	
2	+	+	1 : 1
3	-	-	
4	-	-	
5	+	-	
6	+	+	3 : 1
7	+	+	1.2: 1
8	+	+	2 : 1
9	+	+	5 : 1
10	-	-	
11	+	+	2 : 1
12	-	-	
13	+	+	1.5: 1
14	+	-	
15	+	+	1.1: 1
16	+	+	2.5: 1
17	+	+	4.1: 1
18	+	+	1.7: 1

Qualitative Determination of the Iron-reducing Capacity. The iron-reducing ability of all isolated strains was tested with alpha alpha'-dipyridyl, which turns red in the presence of ferrous iron. The bacteria were plated on Bromfield's medium, to which 0.05 g $Fe(OH)_3$/100 ml had been added. After 48 hours the cultures were tested for ferrous iron. Reduction was considered to have taken place when strips of filter paper, soaked with a 0.1% solution of alpha alpha'-dipyridyl in acetic acid (10%), turned red when placed over the cultures.

In another test for iron reduction, fermentation tubes containing Bromfield's broth with ferric hydroxide were inoculated and incubated for 5 days. After the incubation, 1 ml of a 0.2% alpha alpha'-dipyridyl reagent was added and the formation of a pink-red color regarded as a positive test (see Table 10).

According to this table, 11 bacteria could be regarded as positive. Coordinating results were recorded between reduction in the broth and on solidified Bromfield's agar. An exception was strain 15, which did not manifest the formation of ferrous iron in the broth.

Quantitative Determination of the Iron-reducing Capacity. From the qualitative positive strains, 9 were selected and tested for their reducing ability. The cultures chosen were inoculated both in Bromfield's and Roberts' medium and the amount of iron reduced from ferric hydroxide determined. Determinations were made from aerobic and anaerobic incubations and results calculated from an average of three determinations (see Table 10).

Table 10. Pigmentation and iron-reducing capacity of 18 unknown isolated bacteria.

Strain No.	Pigmentation	Pink-red colorization with alpha alpha'-dipyridyl in: Bromfield's broth after 5 days aerobic	Bromfield's broth after 5 days anaerobic	Bromfield's agar after 48 hours aerobic
1	white-gray	-	-	-
2	white	+	+	+
3	yellow	-	-	-
4	yellow	-	-	-
5	white	-	-	-
6	white	++	-	+
7	white-gray	-	-	+
8	white	+	+	+
9	white	++	+	+
10	intense yellow	-	-	-
11	white	++	+	+
12	yellow	-	-	-
13	white	+	+	+
14	white	-	-	-
15	white	-	-	+
16	white	+	+	+
17	white	+	+	+
18	white	+	++	+

Determinations in average of 2.
+ = weak pink color; ++ = intense red.

Table 11. Amount of ferrous iron (mg/l) and final pH values in Bromfield's and Roberts' media after a 5-day incubation period at 30°C.

Medium condition	Bromfield's				Roberts'			
	aerobic		anaerobic		aerobic		anaerobic	
Strain No.	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}	pH	Fe^{2+}
6	5.8	5.0	5.3	14	5.8	6.0	5.6	1.0
7	5.7	1.0	5.2	4.5	4.6	2.0	4.5	5.5
8	5.7	2.5	5.6	5.0	5.9	6.5	5.5	1.0
9	5.6	13.0	5.6	1.0	6.1	25.0	5.8	17.0
11	5.8	6.5	6.0	1.5	5.4	17.5	5.5	5.5
13	5.5	6.5	5.5	1.5	4.5	5.0	4.4	4.0
16	5.6	1.0	5.2	0	4.7	3.5	4.6	1.5
17	5.9	4.5	5.5	4.5	5.5	1.0	5.2	0
18	5.8	2.0	5.6	5.5	5.5	6.5	5.0	6.5
Control	5.9	0.5	5.7	0	6.6	0.5	6.3	0

In general, iron reduction was more intensive under aerobic conditions, i.e., in those tubes which had not been incubated in the jar. However, it should be emphasized that these "aerobic" conditions were in fact mainly anaerobic throughout the incubation period. Only the initial proliferation of the inoculum might have found sufficient oxygen as final electron acceptor. The increasing population developed an anaerobic environment and forced the metabolism to switch to alternative pathways with the production of molecular hydrogen. This might explain the larger

amounts of reduced iron under "aerobic" rather than anaerobic conditions, because of the greater number of bacteria developed during the initial presence of oxygen.

The experimental findings thus strengthen the theory postulated during this work.

Identification of the Iron-reducing Strains. Biochemical tests were applied on the strains 6, 7, 8, 9, 11, 13, 16, 17 and 18 following the procedure of Lord (1959) for the identification of Enterobacteriaceae. The bacteria were classified as to species using Bergey's Manual of Determinative Bacteriology, 7th edition (1957). The differentiation of species among the coliform group was based on the IMViC-reactions, motility, the ability to liquefy gelatin, the utilization of glycerol and differences in the ability to ferment glucose, sucrose, dulcitol, Inositol and salicin. For complete survey of test performed, see Table 12.

Strain 6: Aerobacter aerogenes. (1)

Properties of str. 6 correlated best with the description of A. aerogenes, except for the inability to produce gas from glycerol. On the other hand, the IMViC-reactions, motility and gelatin liquefication were significant.

Strain 7: Escherichia coli.

This identification was clear cut. All biochemical tests performed agreed with the properties listed for E. coli.

Strain 8: Aerobacter cloacae. (1)

Aerobacter cloacae differs from A. aerogenes in the ability to liquefy gelatin and the inability to ferment glycerol with the production

Table 12. Survey of biochemical tests.

Strain No.		Gram stain	lactose		glucose		Voges-Proskauer	Tryptone broth (indol)	Kosser's citrate	Methyl red test	Motility	Frazier's gelatin	15% gelatin	glycerol		cellobiose		Dulcitol		Inositol		maltose		mannitol		Rhamnose		salicin	
			acid	gas	acid	gas								acid	gas	acid	gas	acid	gas	acid	gas	acid	gas	acid	gas	acid	gas	acid	gas
6.	short rod	-	+	+	+	+	+	-	+	-	-	+	+	+	-	+	+	-	-	-	-	+	+	+	+	+	+	+	+
7.	short rod	-	+	+	+	+	-	+	-	+	-	+	+	+	+	-	+	-	-	-	-	+	+	+	+	+	+	+	+
8.	short rod	-	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	-	-	-	-	+	+	+	-	-	-	+	+
9.	short rod	-	+	+	+	+	+	-	+	+	-	+	+	+	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+
11.	short rod	-	+	+	+	+	+	-	+	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+	+	+	+	-	+
13.	coccus	-	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16.	short rod	-	+	-	+	+	+	-	+	-	+	+	+	+	-	-	+	-	-	-	-	+	+	+	-	-	-	+	+
17.	short rod	-	+	+	+	+	+	-	+	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+	+	-	-	-	+
18.	short rod	-	+	-	+	+	+	-	+	-	+	+	+	+	-	+	+	-	-	+	-	+	+	+	+	+	+	+	+
19.	short rod	-	+	+	+	+	+	-	+	-	-	+	+	+	-	+	+	-	-	+	-	+	+	+	+	-	-	+	+

Table 12 (cont'd.).

Strain No.		sorbitol		sucrose		arabinose		galactose		levulose		xylose		inulin		Malonate	Uschinsky's		Starch	$(NH_4)_2PO_4$-glucose	urea	nitrite +		catalase	7% NaCl	20% NaCl	T.S.I.	
		acid	gas	acid	gas	acid	gas	acid	gas	acid	gas	acid	gas	acid	gas		turbidity	sediment					gas				acid	gas
6.	short rod	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	-	+	-
7.	short rod	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	-	+	-	+	-	-	+	-
8.	short rod	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	+	-
9.	short rod	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	+	-
11.	short rod	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	-	+	+	+	-	+	-	+	-	-	+	-
13.	coccus	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	+	-
16.	short rod	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	+	+	+	+	-	+	-	+	-	-	+	-
17.	short rod	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	+	-
18.	short rod	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	+	-
19.	short rod	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	+	-	+	-	-	+	-

Table 12 (concl.).

Strain No.	Litmus milk: acid	alkali	gas	reduction	peptonization	curd
6. short rod	+	-	+	+	+	+
7. short rod	+	-	-	-	-	-
8. short rod	+	-	+	+	-	-
9. short rod	+	-	+	+	-	-
11. short rod	+	-	+	+	-	-
13. coccus	+	-	+	+	-	-
16. short rod	+	-	+	+	+	+
17. short rod	+	-	+	+	+	-
18. short rod	+	-	+	+	-	-
19. short rod	+	-	+	+	-	-

of gas. These characters found with strain 8 classified it as a strain of A. cloacae, though the organism was incapable of fermenting maltose and hydrolized starch.

Strain 9: A. aerogenes. (3)

This isolate did not match with A. aerogenes completely, since it failed to produce gas from glycerol and to ferment inositol. However, the inability to liquefy gelatin in both Frazier's gelatin and 15% gelatin stabs, excluded the organism as a strain of A. cloacae and was therefore regarded a A. aerogenes.

Strain 11: A. aerogenes. (4)

Like strain 9, this isolated organism differed from A. cloacae in the inability to produce gelatinase and was classified best as a strain of A. aerogenes, though no gas was produced from glycerol. This would place the organism as an A. cloacae: however, the liquefication of gelatin is considered by Bergey as a more reliable characteristic than the production of gas from glycerol. Furthermore, manitol and sorbitol were not attacked, even after 10 days.

Strain 16: Paracolobactrum aerogenoides. (1)

In this case the fermentation of lactose was considerably delayed (no gas and acid after 4 days), which is characteristic for the genus Paracolobactrum. Representatives of this genus may be found abundantly in surface water, soil and the intestinal tracts of animals. P. aerogenoides exhibits the same properties of A. aerogenes and A. cloacae, except for the delayed lactose fermentation. Strain 16 showed the highly fermentive characters of A. aerogenes, its IMViC-reactions and motility. Further, the strain was gelatinase positive, an attribute of A. cloacae.

Strain 17: A. cloacae. (2)

The fermentation of glycerol without the production of visible gas and the ability to produce gelatinase, classified this bacterium as an A. cloacae. However, no motility was observed. The fermentation of carbohydrates agreed well with those listed in Bergey's Manual.

Strain 18: Paracolobacterum aerogenoides. (2).

Again a consistently delayed fermentation of lactose was observed. Furthermore, both properties of A. aerogenes and A. cloacae were present: IMViC-reactions, motility and gelatin liquefication. Starch was fermented, which is rare according to Bergey's Manual.

Strain 13.

The previously reported strains were all gram negative rods. This organism, however, was classified as a gram negative coccus. The biochemical tests showed a highly fermented character, since all the carbohydrates tested (18), with the exception of inulin, were rapidly fermented with the production of acid and gas. Further, citrate and malonate were utilized as the only carbon sources, while the catalase test revealed an extremely strong production of oxygen. A gram negative coccus with these characteristics could not be identified by Bergey's Manual of Determinative Bacteriology (1957). Whether this isolate represents a new group of unnamed soil or water bacteria requires further research.

The Formation of Gley from Eskridge Shale. When these isolates were examined for their ability to form gley, bleaching was noted with each tested strain. Discolorization in the flask with Paracolobactrum aerogenoides started about 14 days after inoculation and was characterized by brown-gray streaks diffused and scattered through the red shale. The

three-week incubation was not as intensive as that observed with Aerobacter aerogenes or A. cloacae. Strain 7, identified as Escherichia coli, differed from the Aerobacter type in that only diffuse gray-brown spots were developed. These regions were formed throughout the layer; however, no bleaching occurred in the surface layer. No test for gleying was performed with strain 13, the unidentified coccus.

DISCUSSION

The natural microbial environment, taken as a unit, contains so many variables, that it seems almost impossible to ascribe certain processes to the action of a single factor. However, since the reduction of iron requires the supply of electrons, only those parameters need to be assessed which influence the redox-potential in the milieu, i.e., the proton and electron activity. By determining Eh and pH of the system, the reduction intensity "rH" of the organism in question could be calculated. It was hoped that the level of reduction intensity could be seen as an expression of the iron-reducing capacity. Thus the processes involved may be presented as:

$$Fe_2O_3 + H_2O \rightleftharpoons 2FeO.OH$$

$$2FeO.OH + 6H^+ + 2e \rightleftharpoons 2Fe^{2+} + 4H_2O$$

When the rH of the facultative anaerobes Aerobacter aerogenes, A. cloacae, Bacillus polymyxa and B. circulans was followed during the gleying process of the red oxidized Eskridge shale, the expected relation between the rH degree and the amount of iron brought into solution could not be detected. B. polymyxa, for example, established a rH value of about 11 during

stationary growth while dissolving about 875 mg Fe^{2+}/l, whereas Aerobacter aerogenes maintained a reduction intensity of 5.5, but appeared incapable of reducing more than 154 mg Fe^{2+}/l. Identical studies performed with B. circulans and Aerobacter cloacae confirmed these negative results. Even when the drop in rH during the first 10 days was related to the amount of iron reduced, no relationship was revealed. B. circulans, however, showed a more or less parallel relationship between electron and ferrous ion activity. The interpretation of these idfferences was sought in metabolism. While A. aerogenes, A. cloacae and Bacillus polymyxa elaborate hydrogen among their waste products, B. circulans must discharge its hydrogen as other products since in this organism molecular hydrogen is absent under anaerobic conditions. The drop of rH to the level of the hydrogen electrode, however, indicates the accumulation of unknown reducing substances, often designated as "reductones".

The question of the nature of these reducing compounds has been raised before. Euler and Hasselquist (1955) supposed that during the degradation of sugars by microorganisms, substances of the aldehyde-type were produced. Euler claimed triose reductone, a 2,3-dihydroxyacrylaldehyde, responsible for the reducing environments of bacteria. This compound, the enolic form of which is

can be reducing because of its tautomeric forms. The production of similar substances is also conjectured by Rabotnowa (1963). She and her co-workers found that during the development of Streptomyces aureofaciens, the amount

of reductone increased while the Eh of the culture decreased. Further, it was noted that the increase of reductone was paralleled by a proportional decrease of dioxyaceton, suggesting this glycolytic intermediate as a pre-stage of reductone. The accumulation of these reducing substances in the case of B. circulans could account for the stronger increase in the amount of reduced iron, compared to the molecular hydrogen producing bacteria. The formation of reductones by B. circulans was confirmed, since experiments showed that sterile culture fluids of this organism were able to reduce ferric hydroxide (1.0 mg/l).

The fermentation of Aerobacter sp. and Bacillus polymyxa differs from B. circulans in the formation of the enzyme hydrogenlyase. When the iron reducing capacity of other non-hydrogen producing bacteria was compared with hydrogen producers, only the latter were found capable of transforming ferric iron to the ferrous state. The extent of reduction, however, depended on the environmental conditions. It was found that (1) the iron-reducing capacity was greater under partially aerobic than anaerobic conditions, and that (2) all reducing organisms transformed largest quantities of iron when inoculated in Roberts' medium, the most nutritive of the substrates tested. B. circulans, however, requiring certain vitamins, yielded a maximum of ferrous iron in Bromfield's medium.

In interpreting these findings, metabolic behaviour needs to be included. There is, of course, no paucity of information on end products of glucose metabolism in the group of mixed-acid fermentors. Under aerobic conditions, acetate and CO_2 are the major products formed, whereas during anaerobic conditions glucose is converted predominantly to acetate, ethyl alcohol, lactate, succinate, carbon dioxide and hydrogen, with minor or no

amounts of 2,3-butanediol, acetoin and glycerol (Wood, 1961). Fermentation, however, is not necessarily restricted to completely anaerobic conditions. Heavy growth in fermentation tubes, containing a carbohydrate rich medium, will consume the available oxygen soon, forcing the organisms to change to anoxidative metabolism. Much less growth occurs under anaerobic conditions. Thus, early stimulation of growth with greater number of organisms in the initially aerobic incubations and the excess of fermentable substrate will lead later to a stronger production of hydrogen than cultures maintained oxygen free after inoculation. This speculation is supported, since it was found that in a poor medium (Bromfield's) and in the presence of ferric hydrate, more iron had been transformed under anaerobic than aerobic conditions. The depletion of oxygen as inducer for the enzyme formic acid hydrogenlyase was not required, as was proved by Waring and Werkman (1944). Their enzymatic studies with Aerobacter indologenes revealed that formic acid hydrogenlyase was formed as freely in strongly aerated cultures as anaerobically, provided that sufficient iron was present in the medium.

The involvement of hydrogen in creating reducing conditions has been suspected before. Aubel et al. (1946) assumed that facultative and obligate anaerobic bacteria were surrounded by a zone saturated with hydrogen. This prevented the inactivation of enzymes by oxygen. Also the reduction of various inorganic compounds by molecular hydrogen has been reported. Stephenson and Stickland (1931) presented evidence that the reduction of sulphate to sulfide was caused by the microbiological generation of hydrogen. Recently, the reduction of nitrate to nitrite could be achieved in a reconstituted enzymatic system with molecular hydrogen (DelCampo et al.,

1965). Even the reduction of nitrite to ammonia by molecular hydrogen, generated by Anabaena cylindrica, was possible, provided that light was present (Hattori, 1963).

Iron reduction by hydrogen producing bacteria could be substantiated through the isolation of unknown bacteria from soil and water. When these unknown hydrogen producers were tested for the ability to reduce iron, all were found capable of doing so, both under partially aerobic and anaerobic conditions. When these organisms were classified, representatives of Aerobacter aerogenes, A. cloacae, Escherichia coli and Paracolobactrum aerogenoides were found. A gram negative coccus, with high fermentive properties, capable of producing hydrogen and reducing iron, could not be identified, using Bergey's Manual of Determinative Bacteriology (1957). The identified bacteria, according to Bergey, all produce gas from carbohydrate with a CO_2/H_2-ratio of 1:1 or higher.

It might be pointed out in conclusion that iron reduction is probably a widespread property among bacteria, and may be an attribute of most Clostridia, some Micrococcaceae and other groups capable of producing molecular hydrogen. Manometric determinations of hydrogen production and uptake in the presence of soluble iron, both under aerobic and anaerobic conditions, might yield useful information. Nevertheless, besides hydrogen as reducing power, other possible substances may function similarly. Further, certain electron transport systems may occur between hydrogenase and final reduction of iron.

SUMMARY

The bacteriological reduction of iron by hydrogen producing bacteria was studied under controlled conditions in the laboratory. Since the reduction of ferric iron requires a supply of electrons, a relation was sought between the reducing intensity, rH, and the iron dissolving capacity. No relationship could be detected. However, iron reduction and gleying were found to be associated processes. The discolorization and bleaching intensity, revealed on hematite-rich Eskridge shale, were found to be specific properties of Aerobacter aerogenes, A. cloacae, Bacillus polymyxa and B. circulans.

A comparative study of the reducing capacity of hydrogen-producing and non-hydrogen producing bacteria showed that only the former were capable of transforming ferric iron to the divalent state. This hypothesis was tested for validity by the isolation of hydrogen elaborating bacteria. When the iron reducing ability was determined, positive results were obtained, supporting the theory put forward. The isolates were identified as strains of A. aerogenes, A. cloacae, Escherichia coli and Paracolobactrum aerogenoides.

A gram negative coccus, with highly fermentative properties and capable of producing hydrogen as well as reducing iron, could not be classified according to the Bergey scheme.

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LITERATURE CITED

Ahmad, N. 1963. The effect of evolution of gases and reducing conditions in a submerged soil on its subsequent physical status. Trop. Agric., 40:205-209.

Alexander, M. 1961. Introduction to soil microbiology. Wiley & Sons, Inc. New York, p. 396-401.

Albrecht, W. A. 1941. Calcium saturation and anaerobic bacteria as possible factors in gleyzation. Soil Sci., 51:213-220.

Allison, L. E. and G. D. Scarseth. 1942. A biological reduction method for removing free iron oxide from soil and colloidal clays. J. Amer. Soc. Agron., 34:616-630.

Asami, T. and K. Kumada. 1959. A new method for determining free iron in paddy soils. Soil and Plant Food, 5:141-146.

Aubel, E. and E. Aubertin. 1927. Sur le potentiel d'oxydo-réduction des milieux ou croissent les anaérobies strictes et les anaérobies fakultatifs. C. R. Soc. Biol. 97, 543-551.

Aubel, E., A. J. Rosenberg and M. Grünberg. 1946. Étude sur le potentiel d'oxydo-réduction. Limites de croissance des bactéries anaérobies. Helv. Chim. Acta, 29:1267-1278.

Baas Becking, L. G. M., I. R. Kaplan and D. Moore. 1960. Limits of the natural environment in terms of pH and oxidation-reduction potentials. J. Geol., 68:243-284.

Betremieux, R. 1951. Etudes experimentale de l'evolution de fer et du manganese dans les sols. Ann. Agron. A 2:194-297.

Bloomfield, C. 1950. Some observations on gleying. J. Soil Sci., 1:203-213.

Bloomfield, C. 1951. Experiments on the mechanism of gley formation. J. Soil Sci., 2:196-211.

Bloomfield, C. 1953a. A study on Podzolization. I. The mobilization of iron and aluminum by scots pine needles. J. Soil Sci., 4:5-16.

Bloomfield, C. 1953b. A study on podzolization. II. The mobilization of iron and aluminum by the leaves and bark of Agathis australis (Kauri). J. Soil Sci., 4:17-23.

Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's Manual of Determinative Bacteriology. Williams & Wilkins, Baltimore. 7th ed.

Bromfield, S. M. 1954a. The reduction of iron by bacteria. J. Soil Sci. 5:129-139.

Bromfield, S. M. 1954b. Reduction of ferric compounds by soil bacteria. J. Gen. Microbiol., 11:1-6.

Clark, W. M. 1960. Oxidation-reduction potentials of organic systems. Williams & Wilkins Co., Baltimore, p. 113-115, 291-295.

Clark, F. E. and J. W. Resnicky. 1956. Some mineral element levels in the soil solution of a submerged soil in relation to rate of organic matter addition and length of flooding. 6th Intern. Congr. Soil Sci., Paris, Vol. C:545-548.

Clifton, C. E. and J. P. Cleary. 1934. Oxidation-reduction potentials and ferri-cyanide reducing activities in glucose-peptone cultures and suspensions of Escherichia coli. J. Bact., 28:561-569.

Clifton, C. E., J. P. Cleary and P. J. Beard. 1934. Oxidation-reduction potentials and ferri-cyanide reducing activities in peptone cultures and suspensions of Escherichia coli. J. Bact. 28:541-559.

DelCampo, F. F., A. Paneque, J. M. Ramirez and M. Losada. 1965. Nitrate reduction with molecular hydrogen in a reconstituted enzymatic system. Nature, 205:387-388.

Delong, W. A. and M. Schnitzer. 1955. The mobilization and transport of iron in forested soils. Soil Sci. Soc. Amer. Proc., 19:360-368.

Dobrogosz, W. J. 1966. Altered end-product patterns and catabolic repression in Escherichia coli. J. Bact., 91:2263-2269.

Forget, P., and F. Pichinoty. 1964. Influence de la respiration anaérobique du nitrate et du fumarate sur le metabolisme fermentaire d'Aerobacter aerogenes. Biochem. Biophys. Acta. 82:441-444.

Gillespie, R. W. H. and L. F. Rettger. 1938. Bacterial oxidation-reduction studies. I. Differentiation of species of spore-forming anaerobes by potentiometric technique. J. Bact., 36:605-620.

Gillespie, R. W. H. and L. F. Rettger. 1938. Bacterial oxidation-reduction studies. II. Differentiation of Lactobacilli of diverse origin. J. Bact., 36:621-631.

Euler, H. von and H. Hasselquist. 1955. Uber Galactose als Ausgangsmaterial für Reduktone and über die biologische Bildung der Ascorbinsäure. Arch. Kemi, 8:197-204.

Halvorson, H. O. 1931. Studies on the transformation of iron in nature. II. The effect of CO_2 on the equilibrium in iron solutions. Soil Sci., 32, 141-165.

Harmsen, G. W. 1938. Biologische ijzeromzettingen in de bodem. Chem. Weekblad. 35:495-500.

Hattori, A. 1965. Effect of hydrogen on nitrite reduction by Anabaena cylindrica. In: Japanese Society of Plant Physiologists. University of Tokyo Press, Tokyo, p. 485-492. Ref. Biol. Abstr. 46, 7779.

Hem, J. D. and W. H. Cropper. 1959. Chemistry of iron in natural water. A survey of ferrous-ferric chemical equilibria and redox-potentials. U. S. Geol. Survey Water-Supply Paper, 1459-A, 1-31.

Hem, J. D. 1960. Restraints on dissolved ferrous iron imposed by bicarbonate, Redox-potential and pH. U. S. Geol. Surv. Water-supply Paper, 1459-B, 33-50.

Hem, J. D. 1960. Complexes of ferrous iron with tannic acid. U. S. Geol. Surv. Water-Supply Paper, 1459-D, 75-94.

Hewitt, L. F. 1950. Oxidation-reduction potentials in bacteriology and biochemistry. 6th ed. The Williams & Wilkins Co., Baltimore. p. 3-10, 29.

Ives, D. J. G. and G. J. Janz. 1961. Reference electrodes. Theory and practice. Academic Press, New York. p. 26, 161, 240, 261-263.

Jackson, M. L. 1958. Soil chemical analysis. Prentice Hall, Inc. Englewood Cliffs, N. J. p. 390.

Kindler, A. 1942. Bemerkungen über die Bildung einiger Eisenerze. Poggendorff's Ann. Phys. Chem., 37:203-206. (1836). Cited after Allison and Scarseth.

Kuznetsov, S. I., M. V. Ivanov and N. N. Lyalikova. 1963. Introduction to geological microbiology. McGraw Hill, New York, p. 20-22.

Lieske, R. 1942. Untersuchungen über die Physiologie eisenspeichernder Hypomyceten. Jahrb. Wiss. Bot., 50:328-354 (1912). Cited after Allison and Scarseth.

Leeper, G. W. 1947. The form and reaction of manganes in soil. Soil Sci., 63:79-88.

Long, S. K. and R. Patrick. 1963. The present status of 2,3-butylen glycol fermentation. Adv. Appl. Microbiol., 5:135-155.

Lord, T. H. 1959. Determinative bacteriology. (Laboratory manual). Burgess Publ. Co., Minneapolis.

Mann, P. J. G. and J. H. Quastel. 1946. Manganese metabolism in soils. Nature, 158:154-156.

Moore, R. C., J. C. Freye, and J. H. Jewitt. 1944. Tabular description of outcropping rocks in Kansas. Kansas Geol. Surv. Bul., 52.

Mortimer, C. H. 1942. The exchange of dissolved substances between mud and water in lakes. J. Ecology, 30:147-201.

Muir, J. W., J. Logan, and C. J. Bown. 1964. The mobilization of iron by aqueous extracts of plants. II. Capacities on the amino-acid and organic-acid fraction of pine-needle extract to maintain iron in solution. J. Soil Sci., 15:226-237.

Oborn, E. T. 1960a. Chemistry of iron in natural water. A survey of pertinent biochemical literature. U. S. Geol. Surv. Water-Supply Paper, 1459-F, 111-189.

Oborn, E. T. 1960b. Chemistry of iron in natural water. Iron content of selected water and land plants. U. S. Geol. Surv. Water-Supply Paper, 1459-G, 191-205.

Oborn, E. T. and J. D. Hem. 1961. Chemistry of iron in natural water. Microbiological factors in the solution and transport of iron. Geol. Surv. Water-Supply Paper, 1459-H, 213-220.

Orlowa, N. W. 1963. Die Bildung von Aceton und 2,3-butylenglycol durch eine Kultur von Aerobacter aerogenes. (russ.) Mikrobiologija, 19, 4-20. (1950). Cited after Rabotnowa.

Pichinoty, F. 1963a. Étude et mise au point de deux méthodes permettant de mesurer l'activité des nitrate-reductases bactériennes. Ann. Inst. Pasteur, 104:219-237.

Pichinoty, F. 1963b. Recherches sur la nitrate-reductase d'Aerobacter aerogenes. Ann. Inst. Pasteur, 104:394-418.

Plotz, H. and J. Geloso. 1930. Relation entre la croissance des microorganisms anaérobies et le potentiel du milieu de culture. Ann. Inst. Pasteur, 45:623-640.

Potter, M. C. 1963. Electrical effects accompanying the decomposition of organic compounds. Proc. Roy. Soc. B., 84:260-272 (1911). Cited after Rabotnowa.

Quastel, J. H., M. Stephenson and M. D. Whetham. 1925. Some reactions of resting bacteria in relation to anaerobic growth. Biochem. J., 19:304-316.

Rabotnowa, I. L. 1963. Die Bedeutung physikalisch-chemischer Faktoren (pH und rH_2) für die Lebenstätigkeit der Mikroorganismen. Fischer Verlag, Jena, East-Germany. (Transl. from russ.), p. 20-22, 89-112, 163.

Rabotnowa, I. L., V. G. Plakunova, M, A. Paluva and L. V. Shenderova. 1963. Reasons for the decrease in the redox-potential in cultures of microorganisms. Mikrobiologija, 32:954-961. (Russ. with Engl. summ.)

Reed, G. B. and J. H. Orr. 1943. Cultivation of anaerobes and oxidation-reduction potentials. J. Bact., 45:309-320.

Roberts, J. L. 1947. Reduction of ferric hydroxide by strains of Bacillus polymyxa. Soil Sci., 63:135-141.

Savant, N. K. and E. Roscoe. 1964. Changes in redox-potential and phosphorus availability in submerged soil. Soil Sci., 98:388-394.

Starkey, R. L. and H. O. Halvorson. 1927. Studies on the transformation of iron in nature. II. Concerning the importance of microorganisms in the solution and precipitation of iron. Soil Sci., 14:381-402.

Starkey, R. L. 1945. Transformation of iron by bacteria in water. J. Amer. Water Works Assoc. 37:963-984.

Stephenson, M. 1966. Bacterial metabolism. M. I. T. Press, Cambridge, p. 35-40.

Stephenson, M. and L. H. Stickland. 1931. Hydrogenase. II. The reduction of sulphate to sulphide by molecular hydrogen. Biochem. J., 36:712-720.

Stobe, G. 1956. Oxidation-reduction potentials of soils. Latvijas SSR zinatnu Akad. Vestis, 6:41-47. Cited after Oborn (1960).

Stokes, J. 1949. Fermentation of glucose by suspensions of Escherichia coli. J. Bact. 57, 147-158.

Tendeloo, H. J. C. 1957. Anorganische analyse, physische chemie en kolloid chemie. Kniphorst Press, Wageningen, Netherlands, p. 61-65.

Tokai, Y., T. Koyama and T. Kamura. 1956. Microbiological studies and the reduction process of paddy soils. 6th Intern. Congr. Soil Sci. Paris. Vol. C:527-532.

Waring, W. S. and C. H. Werkman. 1944. Iron deficiency in bacterial metabolism. Arch. Biochem., 4:75-81.

Waterman, W. D. 1951. Factors determining the colors of red and green shales. Master's Thesis, Dept. Geol. and Geogr., Kansas State University.

Winogradsky, S. 1888. Über Eisenbakterien. Bot. Zeitg., 46, 262-270. Cited after Allison and Scarseth (1942).

Wright, D. 1922. Equilibrium studies with certain acids and minerals and their probable relation to decomposition of minerals by bacteria. Calif. Univ. Pub. Agr. Sci., 4:245-337.

Yamanaka, K. and S. Motomura. 1959. Studies on the gley formation of soils. I. On the mechanism of formation of active ferrous iron in soils. Soil and Plant Food, 5, 134-140.

STUDIES ON THE REDUCTION INTENSITY (rH), IRON-REDUCING CAPACITY AND GLEY FORMATION OF SOME FACULTATIVE ANAEROBIC BACTERIA, VERIFIED ON ESKRIDGE SHALE

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The main problem under study was the mechanism involved in the reduction of iron in nature. Iron reduction and the associated phenomena of gleying baffled microbiologists and soil scientists for several decades. The survey of literature, informative though it may be, did not reveal uniform conclusions. This is not surprising if one realizes that iron reduction is due to unrelated bacteria, the metabolism of which have no particular relation to iron.

Since the reduction of the ferric ion to the divalent state requires a supply of electrons, it was reasoned that the proton and electron activity in the environment of the organism in question, might be an expression of its reducing capacity. The significance of the assumption may be presented by:

$$Fe_2O_3 + H_2O \rightleftharpoons 2FeO.OH$$

$$2FeO.OH + 6H^+ + 2e \rightleftharpoons 2Fe^{2+} + 4H_2O$$

By measuring Eh and pH of the medium, the "reduction intensity", rH, of some facultative anaerobic bacteria could be calculated. Though the level of the rH established during the stationary phase of Aerobacter aerogenes, A. cloacae, Bacillus polymyxa and B. circulans characterized the organism under study, no relation could be detected between this degree of rH and the amount of iron transformed. In these experiments an hematite ($Fe_2O_3 \cdot 3H_2O$) rich test substrate was used. This originated from the Lower Permian Eskridge shale formation, cropping out in many places in Kansas.

During the process of reduction, the tested organisms bleached the shale, which is characteristic for the process of gleying. Discoloration and intensity of gleying appeared to be characteristic for each organism.

The ability to reduce iron was restricted to bacteria or other organisms capable of producing molecular hydrogen. Among the bacteria without hydrogenlyase activity, only B. circulans brought about reduction of iron. This supported the theory of molecular hydrogen as iron reducing power. However, an exception was Bacillus circulans. It was assumed that this organism excreted the excess of reducing power under anaerobic conditions as dihydroxyacetone. Dihydroxyacetone converts spontaneously in 2,3-dihydroxyacrylaldehyde, a substance known as "reductone" because of its tautomeric properties.

The hypothesis considered was then tested for validity by the isolation of hydrogen producing bacteria from water and soil. All 10 strains were positive for iron-reducing capacity. An earlier suggestion that iron reduction is not always accompanied by the formation of gley could not be confirmed. Using the Eskridge shale as test material, the isolated bacteria, identified as Aerobacter aerogenes, A. cloacae, Escherichia coli and Paracolobactrum aerogenoides, all developed characteristic patterns of gleying.

It may, indeed, be concluded that iron reduction and gleying are both characteristic properties of those organisms, capable of producing hydrogen during fermentation processes. Among the facultative anaerobic Enterobacteriaceae, the species Aerobacter aerogenes, A. cloacae, Paracolobactrum aerogenoides, Escherichia freundii, E. coli, Serratia

marcescens as well as the spore formers *Bacillus polymyxa* and *B. circulans* may be regarded as potentially responsible for the reduction of iron in general, and for discoloration in gley and Pseudogley soil types in particular.