FUEL RESEARCH INSTITUTE OF SOUTH AFRICA.

TECHNICAL MEMORANDUM NO. 27 OF 1966.

PART II.

BACTERIAL OXIDATION OF PYRITES IN SOUTH AFRICAN COAL MINES.

by:

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and

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FUEL RESEARCH INSTITUTE OF SOUTH AFRICA.

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BACTERIAL OXIDATION OF PYRITES IN SOUTH AFRICAN COAL MINES.

II. Manometric studies on four chemosynthetic autotrophic bacteria: Thiobacillus thio-oxidans, Thiobacillus ferro-oxidans, Ferrobacillus ferro-oxidans, F. sulfo-oxidans.

INTRODUCTION:

Four chemosynthetic autotrophic bacteria were isolated from acid water in South-African coal mines. These bacteria were identified by a qualitative determination of their energy requirements and by the appearance of their colonies growing on thiosulphate agar plates[1].

A quantitative determination of the physiological characteristics of these bacteria is necessary for further differensiation between them, and for a proper understanding of the role of each of them in the formation of acid drainage in coal mines.

This study concerns the use of a manometric method for the quantitative determination of the oxidation of ferrous sulphate, sulphur, thiosulphate and a sample of pyrites by Thiobacillus thio-oxidans, Thiobacillus ferro-oxidans, Ferrobacillus ferro-oxidans and Ferrobacillus sulfo-oxidans.

Manometric studies were used by Silverman and Lundgren[2] for studies on Ferrobicillus ferro-oxidans. Silverman, Rogoff and Wender[3] outlined the advantage of using the standard Warburg manometric method, instead of flask culture or perculation methods, to determine the bacterial oxidation of pyritic materials in coal. The manometric technique was also used by Beck[4] to study the

physiological ...../

physiological characteristics of a ferrous-ion-oxidising bacterium which was isolated from acidic leaching water at Bingham Canyon, U.S.A.

EXPERIMENTAL:

The bacteria, used for manometric studies, were the four species, previously isolated from acid drainage of a South African coal mine.

For growth of the iron oxidizing bacteria, the medium 9K developed by Silverman and Lundgren[5] was used. The medium contained the following concentration of salts per litre: $(NH_4)_2SO_4$, 3.0 g; KCl, 0.1 g; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5 g; $Ca(NO_3)_2$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 44.2 g. The medium was prepared with distilled water and 0.1 ml of 10 N sulphuric acid was added to adjust the pH of the medium to between 3.0 and 3.6. For growth of sulphur oxidizing bacteria the ferrous sulphate in medium 9K was substituted with precipitated sulphur (10 g per litre). The iron medium was sterilized in the autoclave at 121°C for 15 minutes and the sulphur medium was sterilized in flowing steam for 20 minutes on three consecutive days.

T. thio-oxidans was maintained in the sulphur medium and F. ferro-oxidans was maintained in the iron medium. Separate cultures of both T. ferro-oxidans and F. sulfo-oxidans, were maintained in the iron and in the sulphur medium. In each case the cultures were grown for at least 6 months in these media, before the manometric studies were made.

For routine culture of the bacteria, 250 conical flasks containing 100 ml of media were used. For large scale growth, 24 x 1 litre conical flasks containing 300 ml of medium were used. A 1.0 per cent inocculum was used and the flasks were incubated at 27°C for 10 days. The harvesting procedure for both sulphur and iron cultured cells was as follows. The contents of the flasks were shaken up, combined, and set aside for approximately 30 minutes to allow any particulate matter to settle to the bottom. The supernatant fluid containing the bacteria was then carefully decanted and the bacteria were concentrated in a centrifuge at an R.C.F. value of approximately 10,000 x "g".

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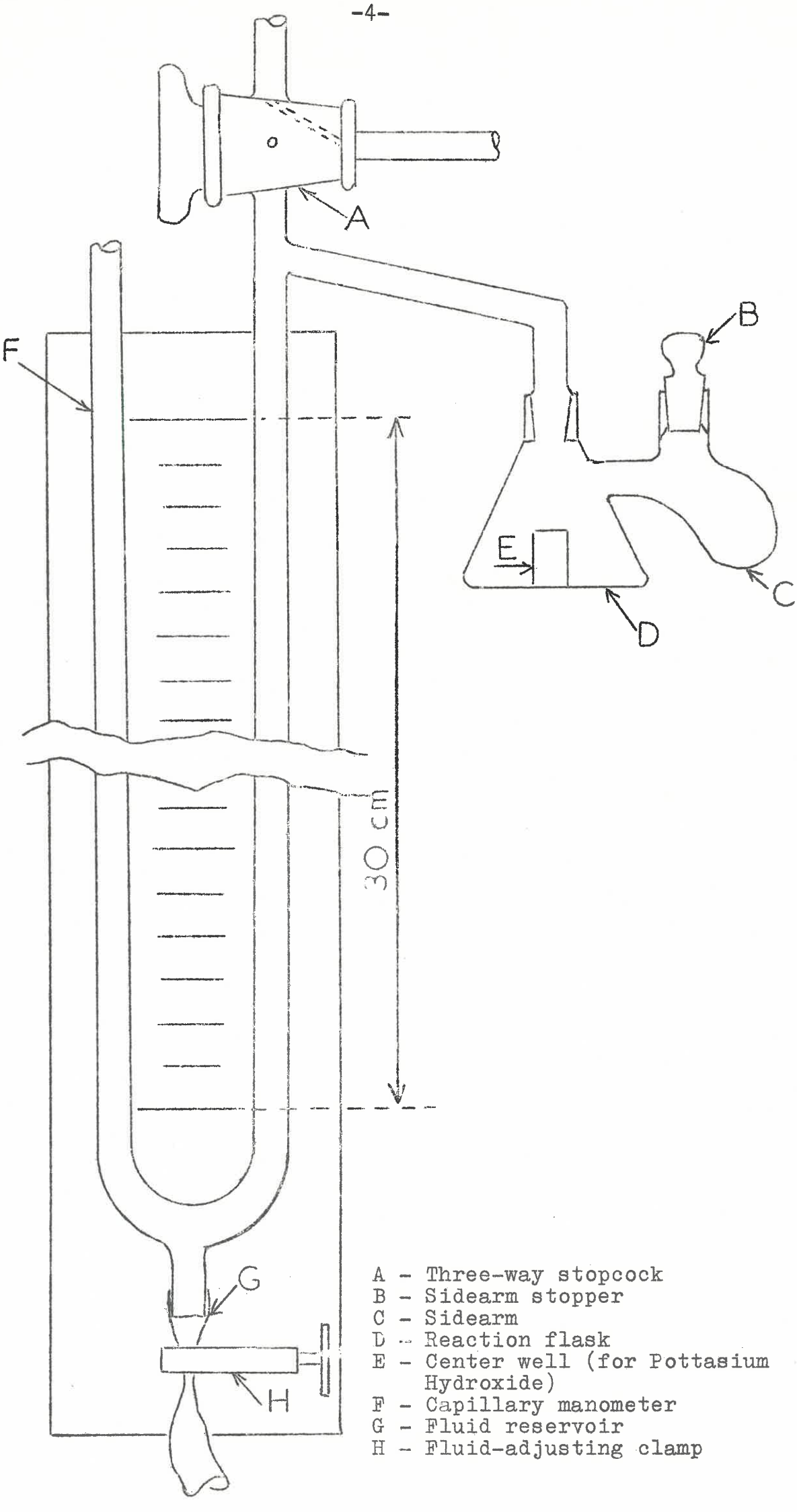
The fluid was poured off and the cell paste was suspended in distilled water acidified to a pH of 3.0 with sulphuric acid. Any solid iron oxides or sulphur particles still present in the cell suspension were removed by swinging out at an R.C.F. value of approximately 50 x "g" in the centrifuge. The supernatant cell suspension was again poured off and concentrated in the centrifuge at 10,000 x "g". This washing procedure was then repeated twice more. The final clean cell concentrate was then suspended in 20 to 50 ml of distilled water which was acidified to a pH of 3.0 with sulphuric acid, and stored in the refrigerator for further use.

Oxygen absorbtion was measured in a standard Warburg apparatus. The manometer and reaction flask (Figure 1) consist of an open-end manometer (F) graduated in millimetres, a detachable reaction flask (D) equiped with a sidearm (C) and a centre well (E). The manometer and reaction flask is attached to a shaker so that only the reaction flask is immersed in a constant-temperature water bath.

The substrate to be oxidized is placed in the main compartment and the bacteria are tipped in from the sidearm. A solution of potasium hydroxide is placed in the centre well for the absorbtion of CO_2. The manometer (previously calibrated) is used to measure the oxygen consumed during the reaction. The reaction flask is shaken at approximately 140 oscillations per minute to ensure a maximum rate of diffusion of oxygen into the solution. Small changes in pressure caused by fluctuations in the room temperature, are corrected by using a manometer without any reactants as a thermobarometer.

In this investigation, each reaction flask contained. a strip of filter paper and 0.2 ml of a 20 per cent potasium hydroxide solution in the centre well. The substrates to be oxidized i.e. ferrous sulphate ($FeSO_4 \cdot 7H_2O$), sulphur (precipitated), sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$) or pyrites were weighed directly into the main compartment. The cell suspension was tipped in from the sidearm of the flask. When less than 2.0 ml of the bacterial suspension was used, distilled water acidified to a pH of 3.0 with sulphuric acid, was added to bring the total volume of fluid in each reaction flask to 2.0 ml. Occasionally, to avoid the chance of some of the cells clinging to the wall of the sidearm, the cell suspension was placed directly in the main

compartment ...../



A - Three-way stopcock
B - Sidearm stopper
C - Sidearm
D - Reaction flask
E - Center well (for Pottasium Hydroxide)
F - Capillary manometer
G - Fluid reservoir
H - Fluid-adjusting clamp

FIGURE 1. - Manometric (Warburg) Apparatus.

compartment. The temperature of the bath was 28°C. The gas phase was air.

The chemicals used as oxidizable substrate in the manometric experiments were: $FeSO_4 \cdot 7H_2O$ merck, $Na_2S_2O_3 \cdot 5H_2O$ c.p. B.D.H., precipitated sulphur pharmaceutical grade and a sample of concentrated coal pyrites. The pyrites was not analysed as it was only used to determine the relative ability of each organism to oxidize pyrites.

RESULTS:

The oxygen uptake by cell suspensions in the presence of various quantities of ferrous sulphate, sulphur, sodium thiosulphate or pyrites, was measured to determine the optimum concentration of each substance to be used. The results are shown in figures 2 - 7.

Of the four substances only sodium thiosulphate showed a definite optimum concentration. In figure 7 the rates of oxygen uptake are plotted against the amounts of sodium thiosulphate used.

Ferrous sulphate at low concentrations seem to have little effect on the rate of oxygen uptake. The optimum amount per flask seem to be at or a little above 250 µ moles. This corresponds approximately with the amount of 44.2 gram per litre present in the medium 9K of Silverman and Landgren[5].

With sulphur a maximum rate of oxygen uptake was not obtained, even in the presence of 4,000 µ moles. Larger amounts are rather voluminous and impractical to use.

For pyrites the optimum concentration was determined with cell suspensions of two different species and is approximately between 1,000 and 2,000 µ moles.

From these results, it was decided to use the amounts shown in Table 1 in all the manometric experiments.

Table 1 ...../

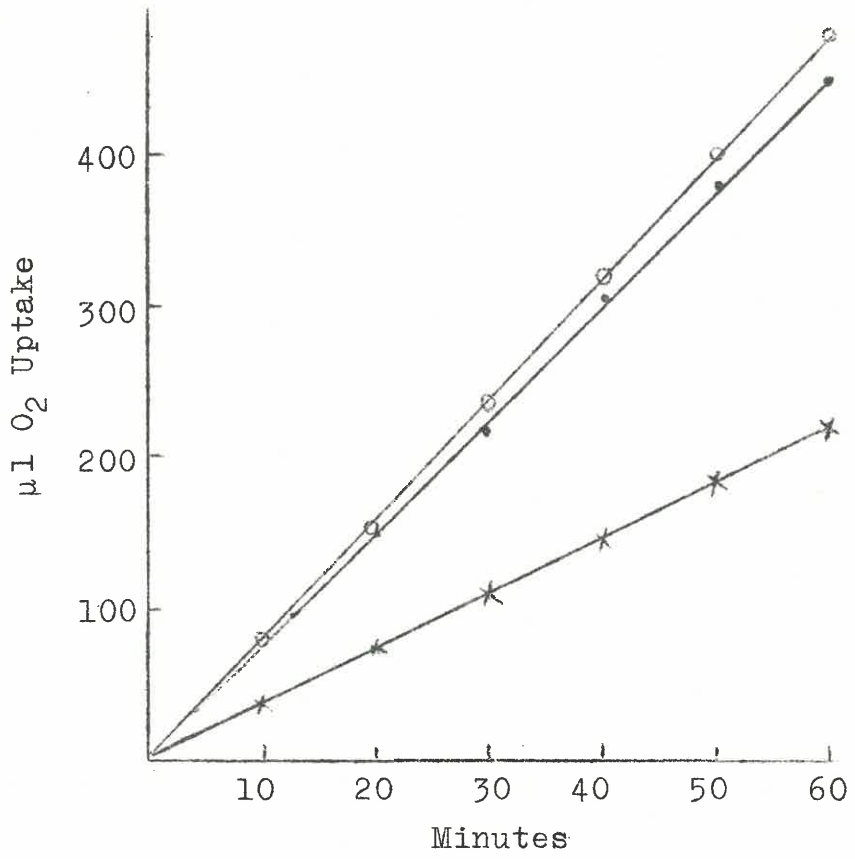


Figure 2. - Oxygen uptake by resting cells of T. ferro-oxidans in the presence of various amounts of $FeSO_4 \cdot 7H_2O$

... 125 µ moles $FeSO_4 \cdot 7H_2O$

ooo 250 µ moles $FeSO_4 \cdot 7H_2O$

xxx1000 µ moles $FeSO_4 \cdot 7H_2O$

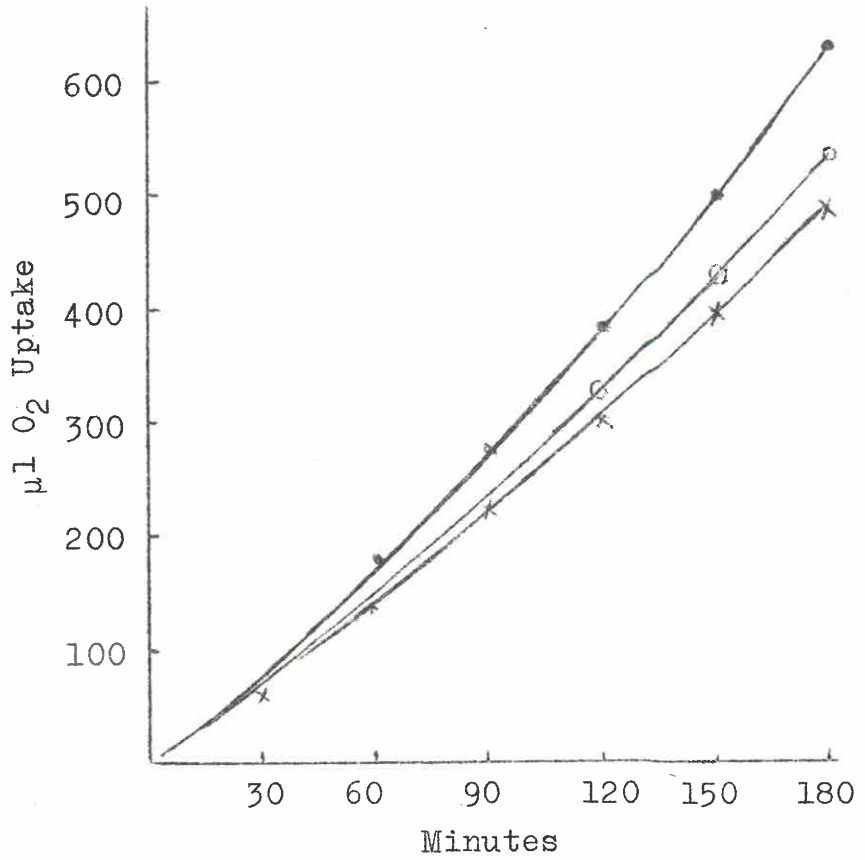


Figure 3. - Oxygen uptake by resting cells of T. thio-oxidans in the presence of various amounts of precipitated sulphur

... 4,000 µ moles sulphur

ooo 3,000 µ moles sulphur

xxx 2,000 µ moles sulphur

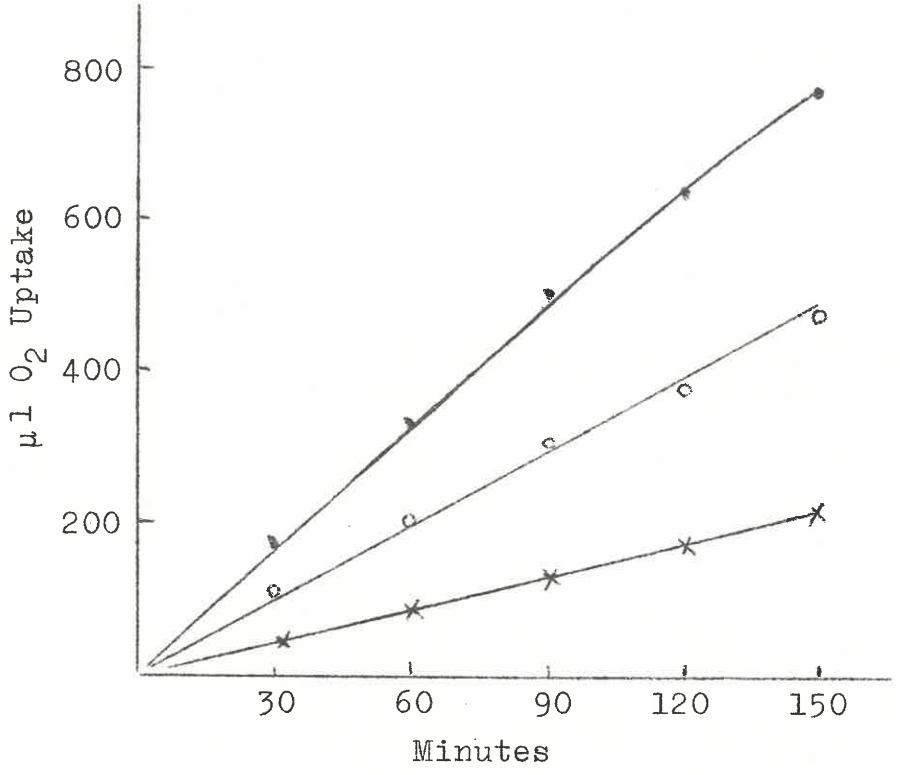


Figure 4. - Oxygen uptake by resting cells of T. ferro-oxidans in the presence of various amounts of pyrites.

... 1000 µ moles pyrites
ooo 500 µ moles pyrites
xxx 160 µ moles pyrites

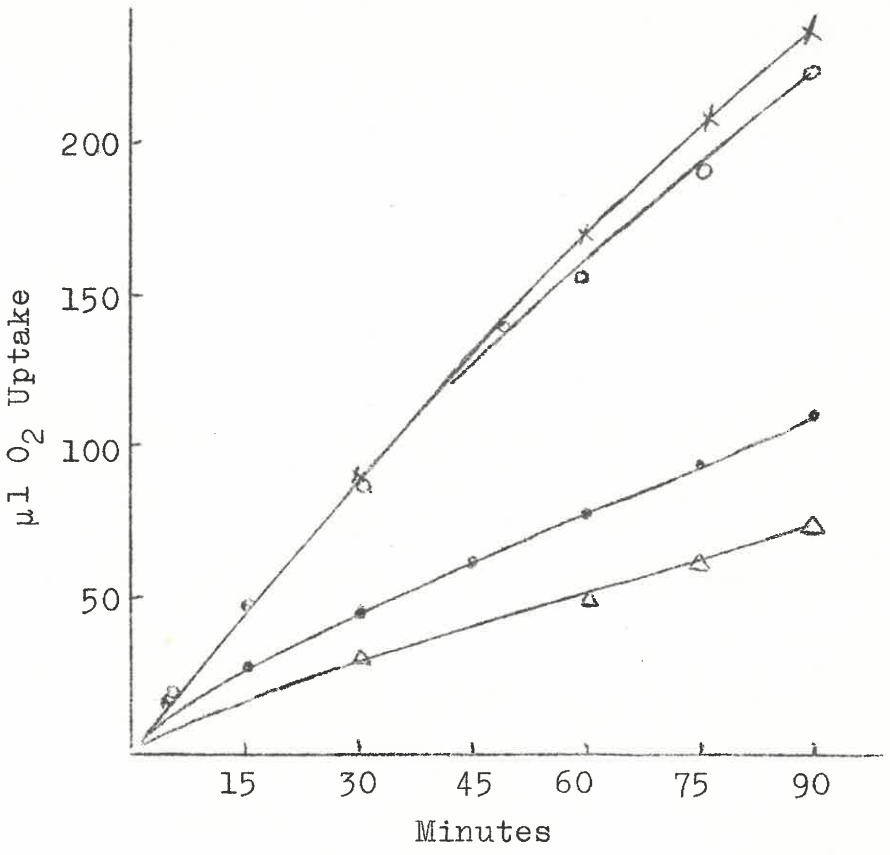


Figure 5. - Oxygen uptake by resting cells of F. sulfo-oxidans in the presence of various amounts of pyrites.

xxx 1,000 µ moles pyrites
ooo 2,000 µ moles pyrites
... 4,000 µ moles pyrites
ΔΔΔ 6,000 µ moles pyrites

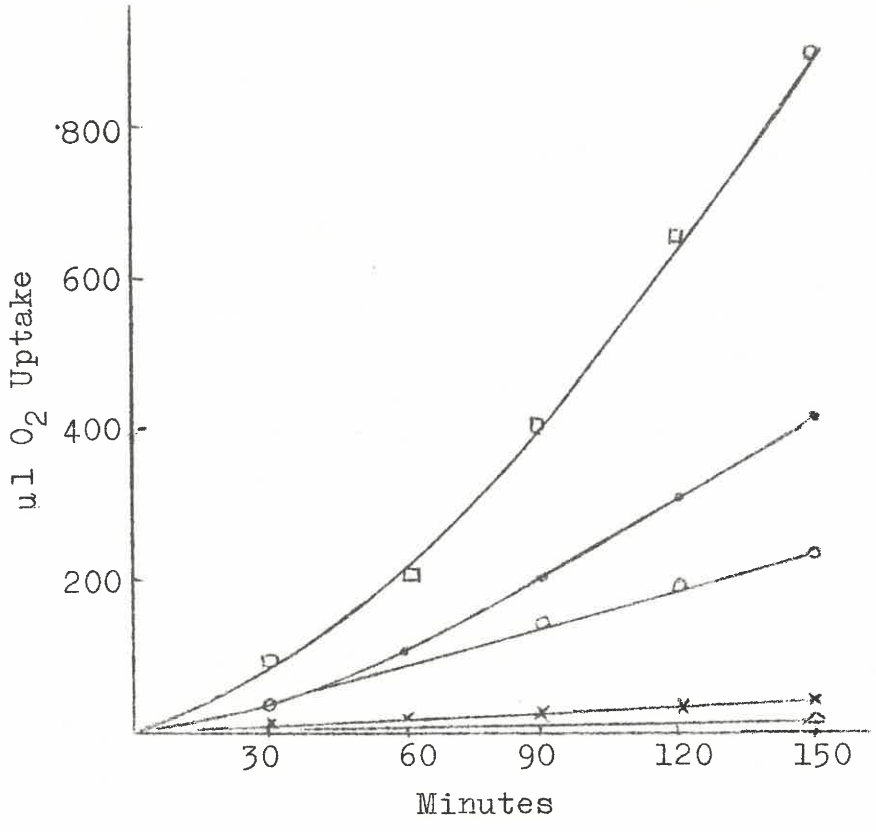


Figure 6. - Oxygen uptake by resting cells of T. thio-oxidans in the presence of various amounts of sodium thiosulphate.

... 250 µ moles sodium thiosulphate
□□□ 500 µ moles sodium thiosulphate
ooo 1,000 µ moles sodium thiosulphate
xxx 2,000 µ moles sodium thiosulphate
△△△ 4,000 µ moles sodium thiosulphate

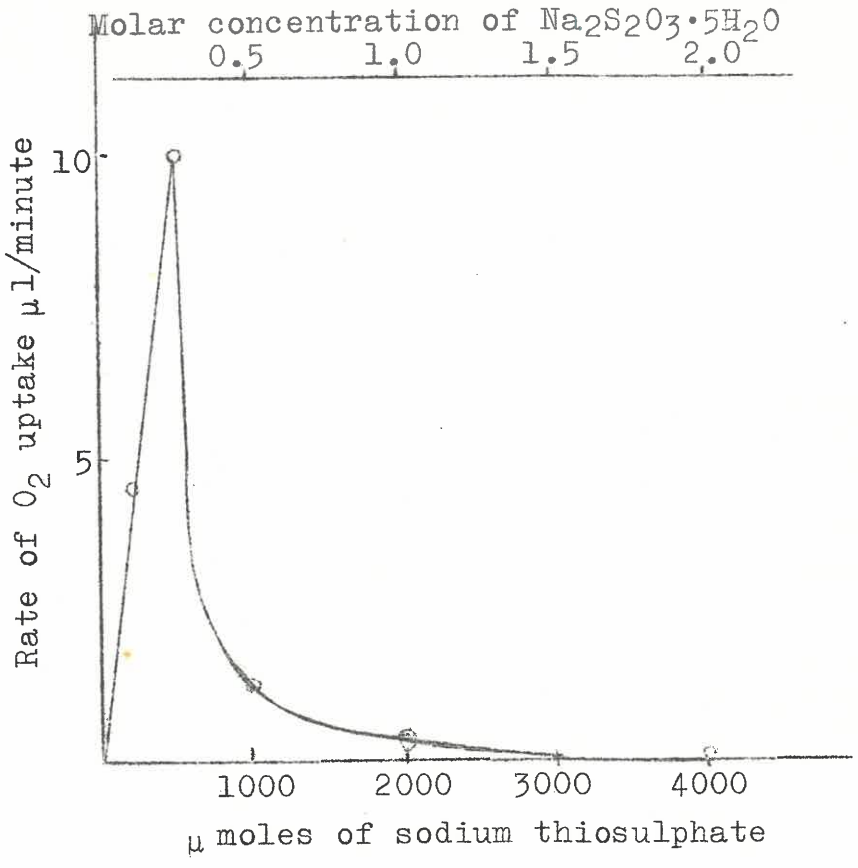


Figure 7. - Rate of oxygen uptake by resting cells of T. thio-oxidans in the presence of various amounts of sodium thiosulphate.

TABLE I.

Substrate	Amount per Warburg flask		Concentration g/litre
	mgm	µ moles	
$FeSO_4 \cdot 7H_2O$	69.5	250	34.75
Sulphur	128	4000	64.00
$Na_2S_2O_3 \cdot 5H_2O$	124.1	500	62.05
Pyrites	120	c.a. 1000	60.00

The concentrations in column 4 were calculated from the weights and the total volume of fluid i.e. 2.0 ml used in each flask. The weight of 120 mgm of pyrites correspond to only approximately 1000 µ moles and depend on the purity of the sample.

Oxygen uptake by iron oxidising bacteria in the presence of ferrous sulphate is very high, and with high cell concentrations equilibrium between the fluid and the gas phase may not be obtained. To prevent this, the activity of each cell suspension was determined by measuring the oxygen uptake in the presence of ferrous sulphate, with various volumes of the cell suspension. The results of a typical determination is illustrated in figure 8. In figure 9 the rates of oxygen uptake, obtained with various concentrations of cell suspension of different species, were plotted against the bacterial nitrogen content of each suspension. From these results it can be seen that a rate of oxygen uptake of 12 µl/minute is still low enough for equilibrium to be maintained.

The oxidation of ferrous sulphate, sulphur, sodium thiosulphate and pyrites, by the four species of bacteria were determined separately. These results are shown in Figures 10-15 and are again summarized in table II.

DISCUSSION OF RESULTS:

The results of the manometric experiments can best be discussed by comparing the $QO_2(N)$ values tabulated in Table II. With low rates of oxygen uptake the experimental error is relatively high. In experiments where the oxygen uptake was less than 5.0 µl per hour the $QO_2(N)$ values were reported as nil; which does not necessarily indicate a complete inability of all these organisms to

Table II ...../
oxidize (page 7) ...../

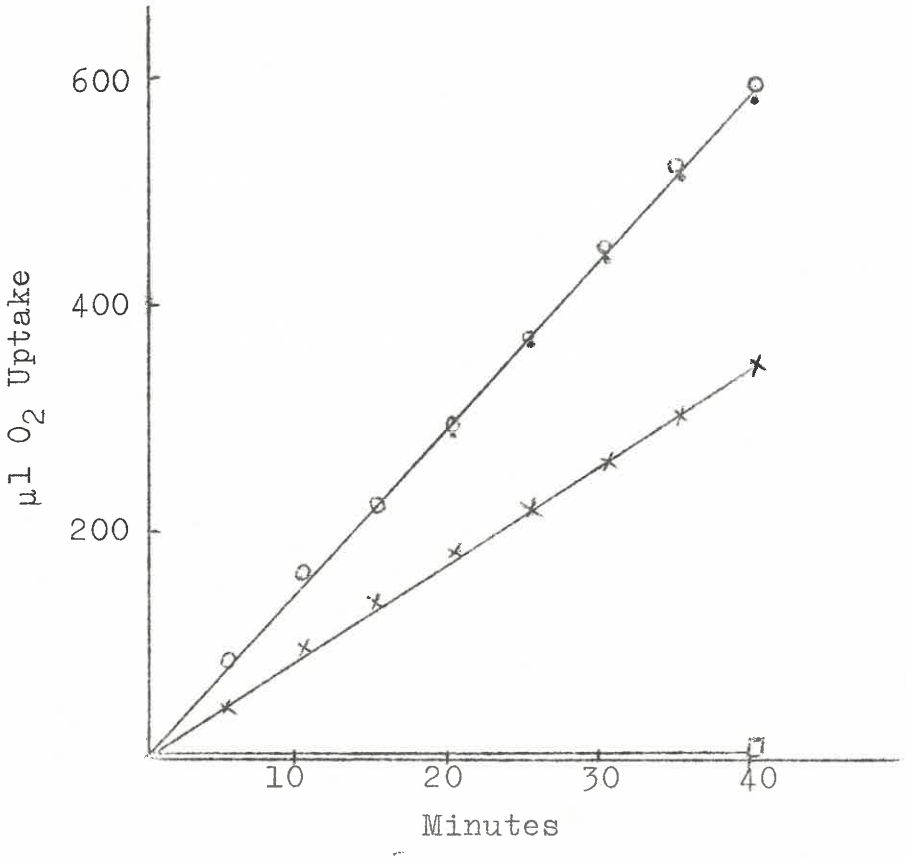


Figure 8. - Oxygen uptake by different volumes of a cell suspension of F. sulfo-oxidans in the presence of 250 µ moles of $FeSO_4 \cdot 7H_2O$.

... 2.0 ml cell suspension
ooo 1.0 ml cell suspension
xxx 0.5 ml cell suspension
□□□ No bacteria present

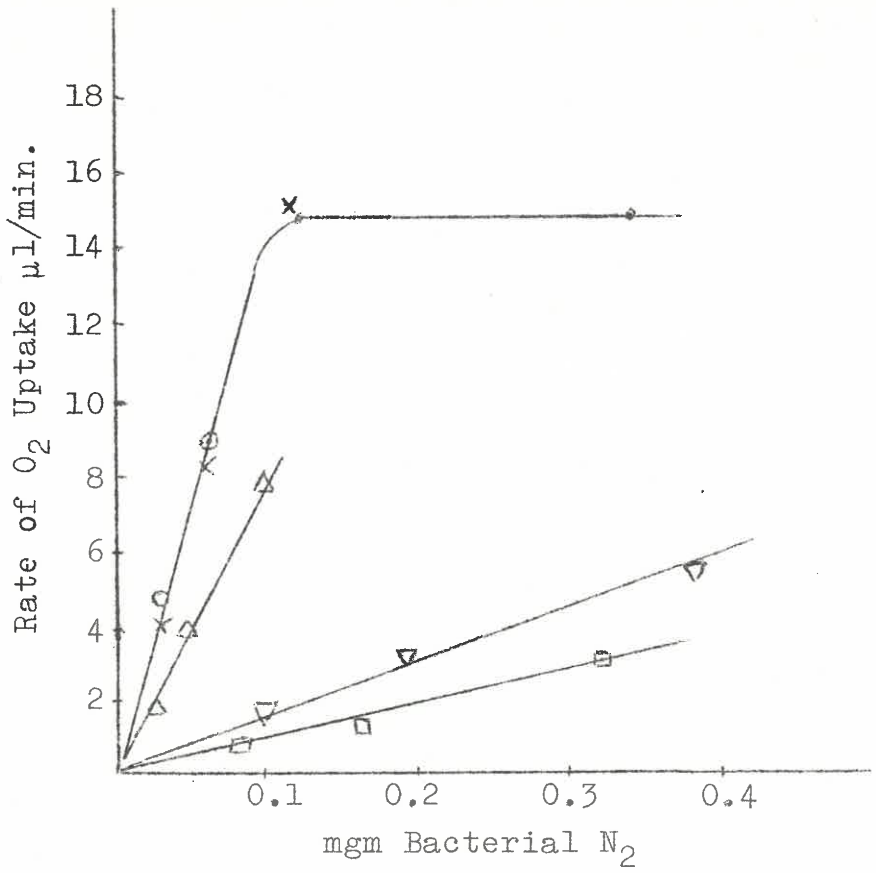


Figure 9. - Rate of oxygen uptake by various cell concentrations of different bacterial species in the presence of 250µmoles of $FeSO_4 \cdot 7H_2O$.

... Iron cultured cells of F. sulfo-oxidans
xxx Iron cultured cells of T. ferro-oxidans
ooo Iron cultured cells of T. ferro-oxidans
△△△ Iron cultured cells of F. ferro-oxidans
▽▽▽ Sulphur cultured cells of T. ferro-oxidans
□□□ Sulphur cultured cells of F. sulfo-oxidans

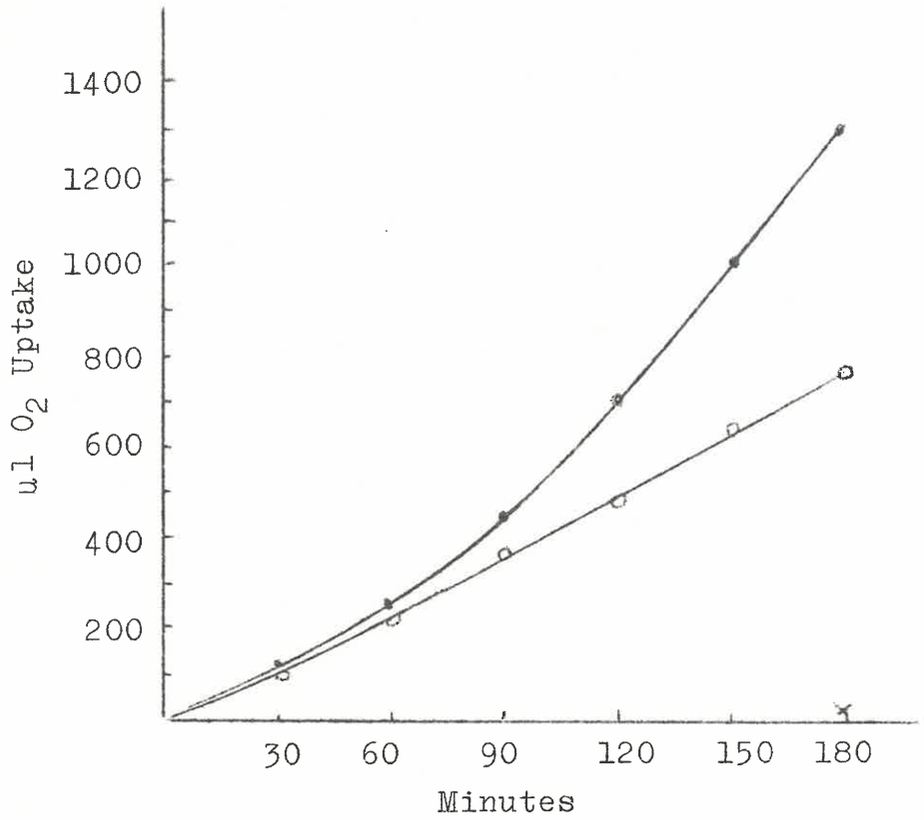


Figure 10. - Oxidation of thiosulphate 500 µ moles ..., sulphur 4,000 µ moles ooo, pyrites 1,000 µ moles xxx, Ferrous sulphate 250 µ moles xxx by resting cells of T. thio-oxidans cultured in a sulphur medium. Each flask contained 0.745 mg of bacterial nitrogen in a total solution of 2.0 ml. Temp. 28°C.

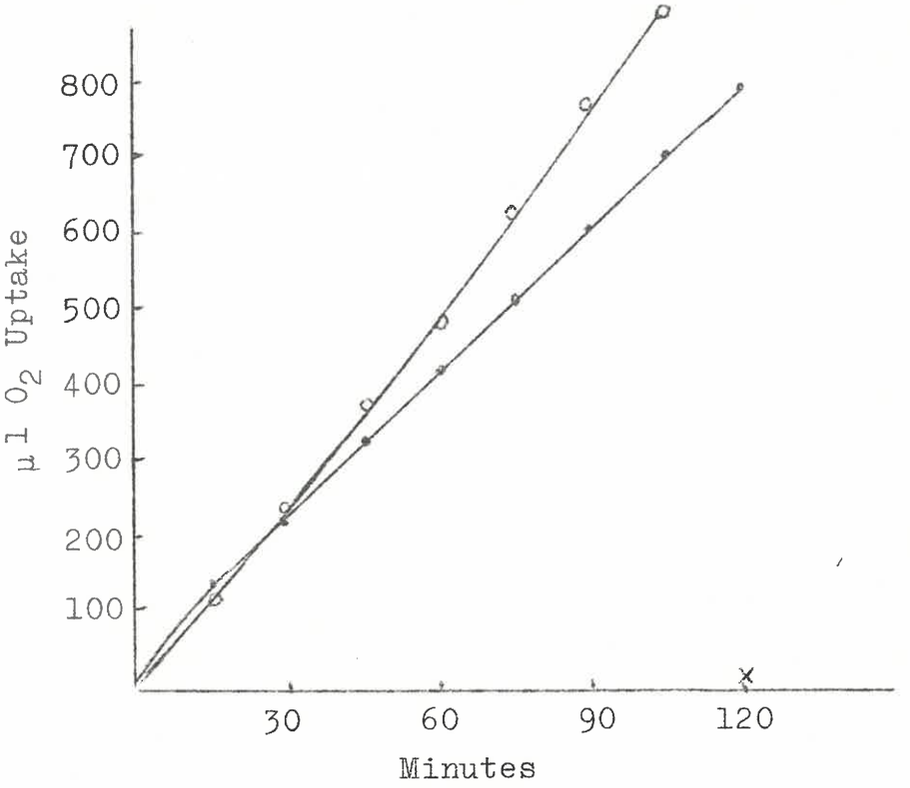


Figure 11. - Oxidation of ferrous sulphate 250 µ moles ooo, pyrites 1,000 µ moles ..., Thiosulphate 500 µ moles xxx and sulphur 4,000 µ moles xxx by resting cells of Ferrobacillus ferro-oxidans cultured in a ferrous iron medium. Each flask contained a total of 0.105mg bacterial N_2 in a total solution of 2.0 ml. Temp. 28°C.

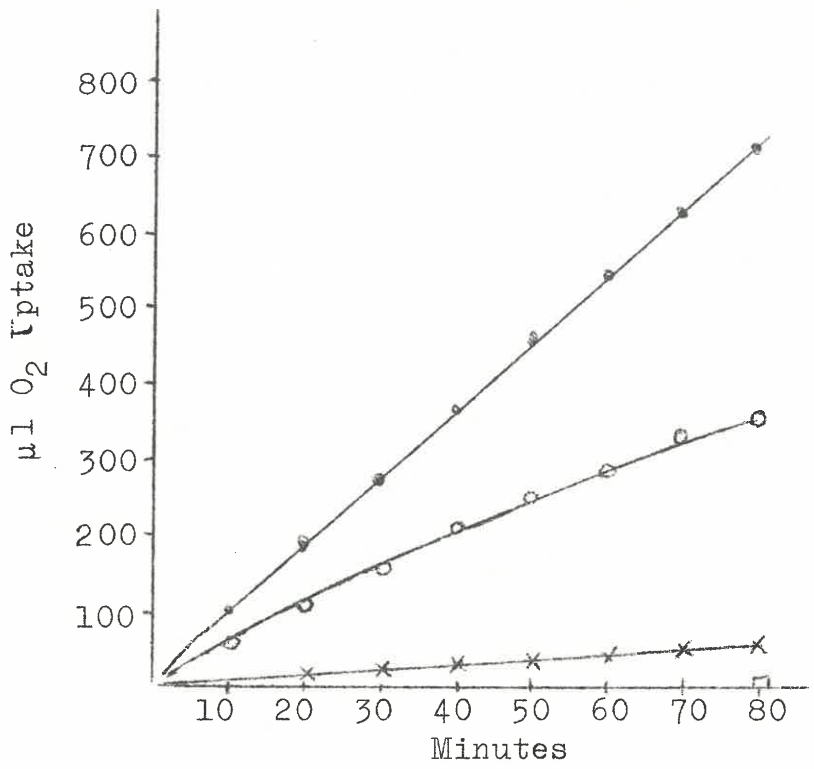


Figure 12. - Oxidation of ferrous sulphate 250µ moles ..., Pyrites 1,000 µmoles ooo, Sulphur 4,000µ moles xxx and thiosulphate 500µ moles □□□ by resting cells of Ferrobacillus sulfo-oxidans cultured in a ferrous iron medium. All flasks contained 0.060 mgm bacterial N_2 in a total solution of 2.0 ml. Temp. 28°C.

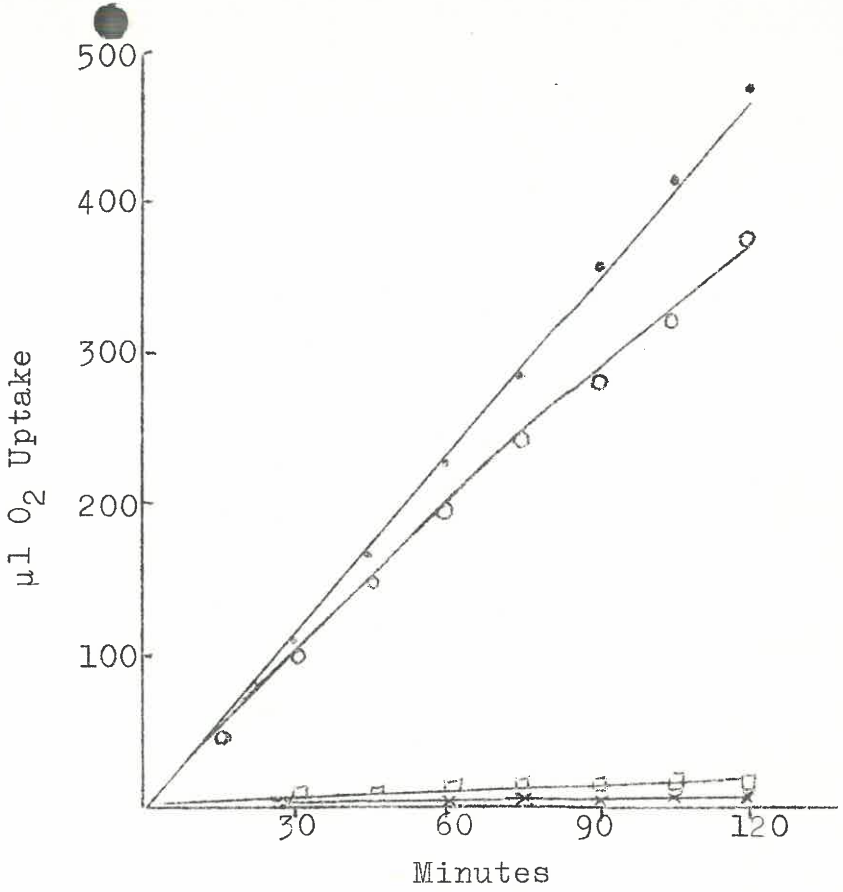


Figure 13. - Oxidation of ferrous sulphate 250µ moles ..., Pyrites 1,000µ moles ooo, sulphur 4,000µ moles □□□ and Thiosulphate 500µ moles xxx by resting cells of Thiobacillus ferro-oxidans cultured in a ferrous sulphate medium. All flasks contain 0.03 mgm bacterial Nitrogen in a total solution of 2.0 ml. Temp. 28°C.

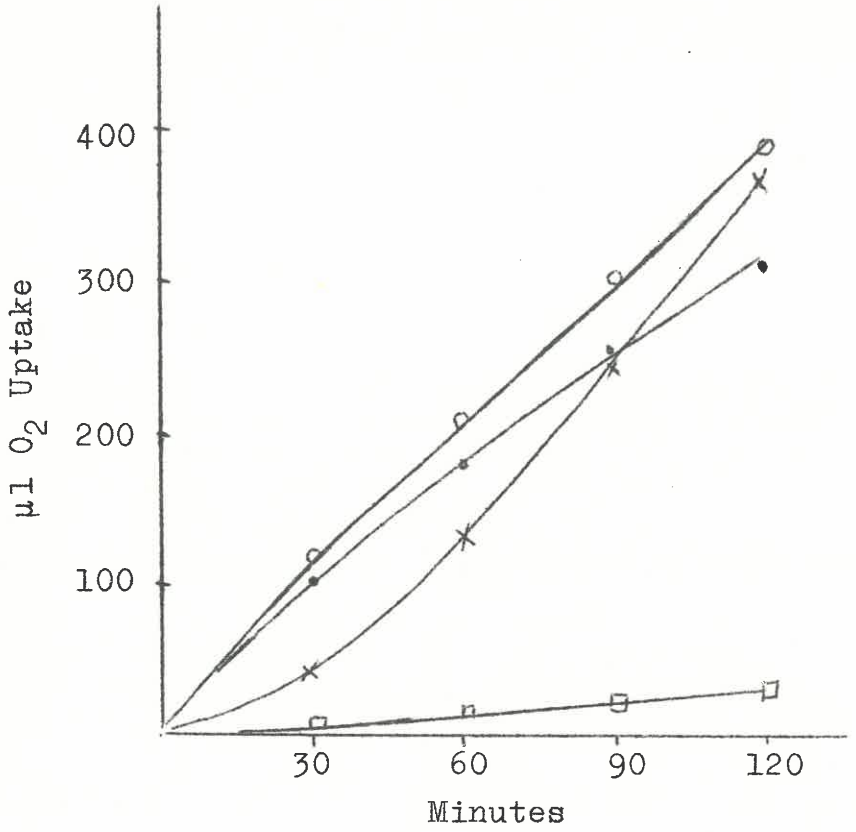


Figure 14. - Oxidation of ferrous sulphate 250 µmoles ..., Pyrites 1,000µ moles ooo, Sulphur 4,000µ moles xxx and Thiosulphate □□□ by resting cells of Ferrobacillus sulfo-oxidans cultured in a sulphur medium. All the flasks contained 0.32 mgm of bacterial N_2 in a total solution of 2.0 ml. Temp. 28°C.

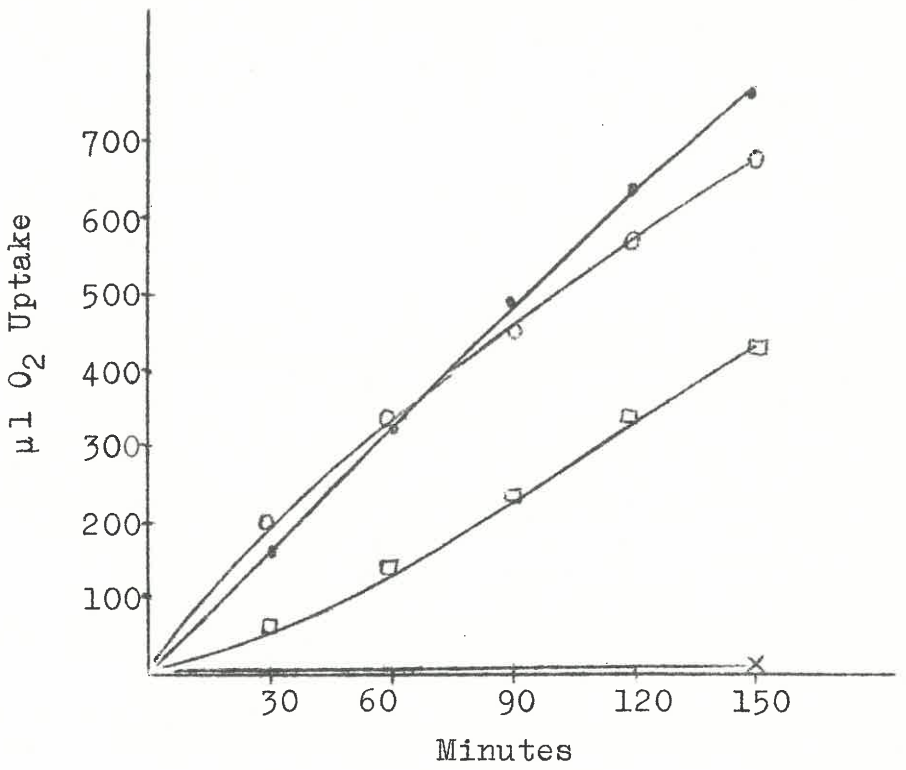


Figure 15. - Oxidation of ferrous sulphate 250 µmoles ..., Pyrites 1,000 µmoles ooo, Sulphur 4,000 µmoles □□□ and Thiosulphate 500 µmoles xxx by resting cells of Thiobacillus ferro-oxidans cultured in a sulphur medium. All flask contained 0.378 mgm bacterial N_2 in 2.0 ml total solution. Temp. 28°C.

TABLE II

RATE OF OXIDATION OF FERROUS SULPHATE, SODIUM THIOSULPHATE, SULPHUR AND PYRITES BY CELL SUSPENSIONS OF DIFFERENT BACTERIAL CULTURES.

Exp.* No.	Bacterial Species	Culture Medium	Bact. N content/flask mgm	QO_2 (N) values**			
				Fe^{++} 250 µmoles	S 4,000 µmoles	$S_2O_3^{=}$ 500 µmoles	Pyrites 1,000 µmoles
10	T. thio-oxidans	S	0.745	0	392	805	0
11	F. ferro-oxidans	Fe^{++}	0.105	4840	0	0	3520
12	F. sulfo-oxidans	Fe^{++}	0.060	8820	702	0	4420
13	T. ferro-oxidans	Fe^{++}	0.030	8940	360	0	6180
14	F. sulfo-oxidans	S	0.320	543	720	47	650
15	T. ferro-oxidans	S	0.378	860	528	0	600

* The results of these experiments are those shown graphically in the figures with corresponding numbers.

** QO_2 (N) - Micro-litres of oxygen uptake per mgm total bacterial nitrogen per hour.

oxidize the substrates in question.

The values obtained for the oxidation of ferrous sulphate indicate that T.thio-oxidans can not oxidize ferrous to ferric iron. Although F. ferro-oxidans oxidized iron at a high rate the oxygen uptake was relatively slow when compared with those of F. sulfo-oxidans and T. ferro-oxidans. The comparitive slow rate of iron oxidation by F. ferro-oxidans was also observed with the large scale growth of these bacteria in iron media. Although the same amount of medium was always used under exactly the same conditions, the cell crops of F. ferro-oxidans was always much smaller than those of F. sulfo-oxidans or T. ferro-oxidans. Even the use of a larger inoculum did not increase the cell crops of F. ferro-oxidans very much. Sulphur cultured cells of F. sulfo-oxidans and T. ferro-oxidans oxidized iron at a much slower rate than iron cultured cells of the same bacteria. (Experiments 12, 13, 14 and 15). This seem to indicate that the electron transport system for iron and sulphur oxidation by these bacteria is not exactly the same.

The $QO_2(N)$ values for sulphur oxidation indicate that F. ferro-oxidans cannot oxidize sulphur while the other species can oxidize sulphur at a reasonable high rate. The value of 392 obtained for T. thio-oxidans lie between the values of 270 and 557 reported in the literature for the same bacterium[3), 5)]. Cells of F. sulfo-oxidans and T. ferro-oxidans, cultured in iron, did not lose any of their ability to oxidize sulphur. This is rather interesting as it is opposite to that found for the oxidation of iron by sulphur cultured cells of these bacteria.

For thiosulphate oxidation the high $QO_2(N)$ value of 805 obtained with T. thio-oxidans is rather striking. Beck[5)] obtained with T. thio-oxidans a $QO_2(N)$ value of only 14 for thiosulphate oxidation when a concentration of .005M was used. The results obtained in this investigation, however, show that a maximum rate of oxygen uptake with thiosulphate was obtained at a concentration of 0.25M; and that at 0.005M, the oxygen uptake will be practically nil. (Refer to Figures 6 and 7.) The fact that oxygen uptake in the presence of low concentrations of thiosulphate, is highly dependant on the thiosulphate concentration, seem to indicate that it is not the ionic substance $S_2O_3^=$ that is oxidized, but rather the elemental sulphur that is formed in acid media according to the reaction:

$$Na_2S_2O_3 + H_2SO_4 = Na_2SO_4 + H_2SO_3 + S$$

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The steadily increasing rate of oxygen uptake with time (Figure 6) indicate that sulphur is continually being formed as more acid is produced by the bacterial oxidation of the sulphur.

With sulphur cultured cells of F. sulfo-oxidans a slow but steady rate of oxygen uptake was obtained in the presence of thiosulphate. With iron cultured cells of F. sulfo-oxidans and with cells of T. ferro-oxidans cultured in iron or sulphur, no positive indication of oxygen uptake was, however, obtained. This is rather surprising as it was found in earlier studies by the author that colonies of both F. sulfo-oxidans and T. ferro-oxidans grew readily when they were plated out on thiosulphate agar. The thiosulphate concentration in the solid medium was, however, only 0.02M. The difference in thiosulphate oxidation by these two bacteria can possibly be the result of a difference in the degree of inhibition of these bacteria in the presence of high concentrations of thiosulphate.

The values in the last column of table II indicate that the oxidation of pyrites by these organisms, parallels their ability to oxidize ferrous iron. T. thio-oxidans cannot oxidize iron and no indication of pyrite oxidation was observed. The reduced ability of sulphur grown cells of F. sulfo-oxidans (Experiment 14) and T. ferro-oxidans (Experiment 15) to oxidize iron, is also reflected in the rates obtained for pyrite oxidation. Oxidation of ferrous iron and pyrites by F. ferro-oxidans were both relatively slow when compared with the rate obtained with T. ferro-oxidans (Experiments 11 and 13). The only deviation from the general trend described above was obtained in experiment 12. The reason for this is not known at this stage.

The possibility that the oxidation of sulphur in experiments 12 to 15 was caused by the presence of T. thio-oxidans, is ruled out on the following grounds:

(i) T. thio-oxidans colonies never developed when the cultures used for inoculating the media for large scale growth, were plated out on thiosulphate agar.

(ii) T. thio-oxidans cannot grow in iron media. Thus T. thio-oxidans cells could not have been present

in ...../

in large enough numbers to result in the $QO_2(N)$ values of 702 and 360 obtained in experiments 12 and 13.

(iii) T. thio-oxidans could have grown in the medium used for culturing the cells that were used in experiments 14 and 15. If T. thio-oxidans was, however, responsible for the oxidation of sulphur in these experiments, the $QO_2(N)$ values for thiosulphate should have been just as high or even higher than the values for sulphur oxidation. The fact that thiosulphate was not oxidized by these cell suspensions prove that T. thio-oxidans could not have been present.

Very little difference seems to exist between T. ferro-oxidans and F. sulfo-oxidans when the $QO_2(N)$ values obtained for iron and sulphur oxidation in experiments 12 to 15 are compared. The values for sulphur oxidation obtained with F. sulfo-oxidans (experiments 12 and 14) are somewhat higher than the values obtained with T. ferro-oxidans (experiments 13 and 15). It is difficult to determine whether this difference actually imply a slower rate of sulphur oxidation by the latter organism or whether it was the result of an experimental error.

Although the bacterial nitrogen content of the cell suspensions are proportional to the total number of cells, it gives no indication of the number of viable cells present. The method of plating out on solid media, to obtain a count of the viable cells in a culture, can possibly be used in the case of T. ferro-oxidans, but for F. sulfo-oxidans it was found to be impossible on account of the very small size of the colonies developing on thiosulphate agar. It was also found that growth of the latter bacteria, on plates containing less than approximately 500 cells was very irregular and inconsistent.

It can thus be argued that, if a relative large number of dead cells were present in the cell suspension used in experiment 13 for instance, the $QO_2(N)$ value for sulphur could very well have been in the region of 700 instead of 360. Thus the difference in the rates of sulphur oxidation obtained in this investigation can hardly be used to differentiate between F. sulfo-oxidans and T. ferro-oxidans. However, an increase in the $QO_2(N)$ value for sulphur oxidation on the grounds of the arguement set out above, should also be accompanied by an increase in the

$QO_2(N)$ ...../

$QO_2(N)$ value for iron oxidation. By comparing the values for iron and sulphur oxidation, obtained in experiments 12, 13, 14 and 15, it can be seen that in experiment 15 this actually happened. A $QO_2(N)$ value of approximately 360 for sulphur was rather also expected in the case of experiment 15. It can be seen that the relative high value of 528 for sulphur was also accompanied by a higher value for iron oxidation than that obtained in experiment 14.

Thus there seems to be a definite difference in the physiological characteristics of F. sulfo-oxidans and T. ferro-oxidans. It is, however, difficult to say whether the difference is due to an increased ability of the one to oxidize sulphur or to an increased ability of the other to oxidize iron.

The most illustrative way to express the difference between F. sulfo-oxidans and T. ferro-oxidans is by comparing the ratio of the $QO_2(N)$ values for iron and sulphur oxidation. These ratios are tabulated in table III.

Expt. No.	Bacterial Species	$QO_2(N)$		$QO_2(N)Fe/QO_2(N)S$
		Fe^{++}	S	
12	F. sulfo-oxidans	8820	702	12.6
13	T. ferro-oxidans	8940	360	24.8
14	F. sulfo-oxidans	540	720	0.75
15	T. ferro-oxidans	860	528	1.62

The ratio $QO_2(N)Fe^{++}$: $QO_2(N)S$ obtained with F. sulfo-oxidans cells cultured in iron or sulphur is only half of the ratio obtained with T. ferro-oxidans cells cultured in the same media respectively.

SUMMARY:

The physiological characteristics of four chemosynthetic autotrophic bacteria isolated from a South African coal mine, were determined manometrically.

The results of this investigation indicate that:

(a) Thiobacillus thio-oxidans can oxidize sulphur and thiosulphate but cannot oxidize ferrous iron.

(b) Ferrobacillus ferro-oxidans can oxidize ferrous iron but cannot oxidize sulphur or thiosulphate.

(c) ...../

(c) Thiobacillus ferro-oxidans and Ferrobacillus sulfo-oxidans can both oxidize ferrous iron and sulphur at approximately comparable rates. These two bacteria are very similar physiologically, the only difference were found to be in the ratio of the rates at which each of them can oxidize iron and sulphur. Cells cultured in ferrous iron retained all their ability to oxidize sulphur while cells cultured in sulphur lost approcimately 90% of their ability to oxidize iron. This indicates that an extra step is necessary in the electron transport system for the oxidation of iron, by these bacteria, and should prove an interesting subject for further study.

(d) The ability to oxidize pyrites was found to be proportional to the ability of each of the organisms to oxidize ferrous iron.

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15th September, 1966.

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