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ELECTROCHEMICAL OBSERVATIONS IN MICROBIOLOGICAL PROCESSES. I.

GROWTH OF THIOBACILLUS THIOOXIDANS

SUMMARY

The growth of Thiobacillus thiooxidans utilizing sulfur in three media was studied by observing changes in half-cell emf, bacterial cell count and production of acid as a function of time. A comparison of the biological half-cell emf with comparable control half cells reveals that T. thiooxidans makes an electrochemical contribution to half-cell voltage. A change from the more complex medium of Skerman's mineral salts to A.T.C.C. allowed a clearer delineation of T. thiooxidans' ability to make an electrochemical contribution.

Reproducible biological half-cell emf's were obtained when the ferrous sulfate was removed from the A.T.C.C. medium. One half cell comprising T. thiooxidans utilizing sulfur in A.T.C.C. was observed over a 111-day period. During this time the initial half cell voltage of -0.35 volts, decreased to a negative value of -0.64 volts (hydrogen emf series). T. thiooxidans in utilizing sulfur produces only sulfate ion, thereby simplifying the identification of an electrochemical contribution during growth.

I. INTRODUCTION

The concept of converting chemical energy from natural occurring fuels into electrical energy by biochemical reaction has intrigued man for many years. Potter¹ in 1911 was the first to conduct experiments with biochemical galvanic cells. He observed that "the disintegration of organic compounds by microorganisms is accompanied by the liberation of electrical energy." His experiments were conducted primarily with the yeast-glucose system which gave open circuit voltages between 0.3 and 0.5 V. These exploratory experiments led to investigations in 1931 by Cohen,² who studied several bacterial cultures as electrical half cells. More recently, Bean,^a Canfield,^b Bitterley^c and their co-workers have been working on various aspects of bioelectricity for the National Aeronautics and Space Administration. Emphasis in their investigations was placed on the utilization of organic foodstuffs as an energy source.

In order to gain a better understanding of voltages developed in biological oxidations, a decision was made by the authors to investigate some of the autotrophic bacteria. Autotrophic bacteria, because of their ability to utilize inorganic substrates as an energy source

^a Philco Corporation, Newport Beach, California.

^b Magnō Corporation, Anaheim, California.

^c The Marquardt Corporation, Van Nuys, California.

and carbon dioxide for their carbon requirements, offered a different and perhaps a simpler approach to associating electrochemical potentials with metabolic activity of bacteria. The sulfur oxidizing bacteria, Thiobacillus, were chosen since they were among the most metabolically active autotrophs. In these studies, primary emphasis was placed on T. thiooxidans.

The electrochemical investigations reported in this paper assume that sulfate ion is the only metabolic product associated with the oxidation of sulfur by T. thiooxidans. This assumption has the support of earlier workers such as Starkey,^{3,4} Starkey, Jones and Fredrick,⁵ Vogler and Umbreit,⁶ and Parker and Prisk.⁷

II. ELECTROCHEMICAL ACCESSORIES

Carbon (UP-62-R) from the United Carbon Company, Bay City, Michigan, was cut into electrodes. The ends of the electrodes were plated with copper from a CuSO_4 solution. Copper leads were soldered to the plated surfaces. The leads and their contact with the carbon were treated with paraffin to eliminate wetting and direct contact between the copper and nutrient.

Platinum electrodes were prepared from platinum gauze (45 mesh, 0.0078 in diameter) obtained from J. Bishop and Company. This gauze was cut into 2-in lengths, approximately 5/16 in wide. Copper leads were soldered to one end of the gauze. To prevent possible

oxidation of the copper, the lead wires were covered with plastic tubing. This tubing was then anchored to the copper-platinum solder joint by coating the end of the tubing and the junction with an epoxy resin. To further prevent any possible diffusion of water to and through the plastic tubing and the epoxy-covered junction, the lead wires were kept above the biological half-cell liquid level. The thermocouple effect for these copper-platinum electrodes was found to be negligible ($5 \mu\text{V}/^\circ\text{C}$).

These biological half-cell investigations involved maintaining an air atmosphere above the media in the cells. The electrochemical effect of supplying fresh air above versus bubbling it directly into the stirred media was negligible as long as gaseous concentration gradients did not exist within the media (Table I).

TABLE I
DYNAMIC AND STATIC AEROBIC CONDITIONS FOR STIRRED
ELECTROCHEMICAL HALF CELLS

<u>Medium</u>	<u>Aerobic Atmosphere</u>	<u>Potential, V</u>		
		<u>Static</u>	<u>Dynamic (Bubbling)</u>	<u>δ</u>
Sterile distilled water	Air	-0.323	-0.329	0.006
Skerman's	Air	-0.365	-0.365	0.000
A.T.C.C.	Air	-0.390	-0.390	0.000
A.T.C.C. (minus FeSO_4)	Air	-0.420	-0.420	0.000

Since composition gradients were known to be generated through utilization of substrate by the uneven suspensions of bacteria, it was considered necessary to uniformly stir the half cells. The electrochemical effect of turning off the stirrer was checked for the uninoculated media in which such composition gradients were absent. Table II reveals that the effect of not stirring was appreciable in the sterile distilled water. It became negligible when conducting nutrients were added to the water. In all large control and biological half cell experiments reported in this paper, the cell constituents were stirred and atmospheric air with its carbon dioxide was available to the media through sterile cotton plugs.

TABLE II
EFFECT OF NOT STIRRING LARGE ELECTROCHEMICAL HALF CELLS
(AIR BUBBLING INTO CELL)

<u>Medium</u>	<u>Potential, V</u>		
	<u>On</u>	<u>Off</u>	<u>δ</u>
Sterile distilled water	-0.329	-0.240	0.089
Skerman's	-0.365	-0.363	0.002
A.T.C.C.	-0.390	-0.388	0.002
A.T.C.C. (minus FeSO ₄)	-0.420	-0.418	0.002

During the course of experimentation three basic bicell designs were implemented. The first design involved combining a biological half cell with either a control or a reference half cell in a U-tube. An agar plug in the bend of the tube separated the two half cells. Although positive results for associating emf's with growth of T. thiooxidans were obtained with this initial design, a modification (Fig. 1), which made a separate opening to the agar bridge for connecting a reference calomel cell, was desired. Each of the original half cells, the biological and control, could now be monitored individually with the calomel half cell. Experiments with this modified U-tube were satisfactory, however it was deficient in size and allowed concentration gradients to form so a new large biological half cell was designed.

The above cell designs limited the amount of liquid to be used to about 20 ml of nutrient. Furthermore, the long narrow tubes presented little opportunity for changes in electrode design, stirring, and continuous measurement of pH. Therefore, the new design used a large three- and, later, a five-necked 1 000 ml round-bottom flask (Fig. 2). A Teflon stirrer was suspended through the center neck surrounded with a glass bearing. Its action diminished acid and mineral concentration gradients and insured uniform suspension of bacteria for population density determinations. An agar salt bridge and calomel cell were mounted in one opening and a glass and a platinum electrode were placed in the third neck. The calomel and glass electrodes were

used for recording the pH values. This entire biological half cell was mounted in a constant temperature bath (29°). Prior to use of this bath the electrochemical voltages were observed to fluctuate in a cyclic manner with the room temperature whenever T. thiooxidans was present. This behavior was especially evident when platinum electrodes were used.

The agar salt bridge, mounted in one opening of the round-bottom flask, was led to a test tube containing saturated KCl maintained at the same temperature as the biological half cell. A standard calomel electrode was mounted in the test tube as a reference half cell. Leads from the complete cell were connected to a K-3 potentiometer and a pH meter.

All parts of the above cells which could withstand high temperatures were sterilized by autoclaving. The other parts were sterilized by rinsing in ethanol followed by three rinses with sterile distilled water. Before sterilization the electrodes were cleaned with concentrated sulfuric acid and then washed with distilled water.

All experimentation was oriented towards obtaining zero-current potentials of complete as well as half-cell reactions. The initial electrochemical measurements were made with a Model K-3 Leeds-Northrup potentiometer. This instrument gave accurate voltage determinations when zero current conditions were established. However, while balancing the galvanometer to obtain zero current conditions,

power was drawn initially from the cell causing, in some cases, a loss of voltage. Since it was desirable to eliminate this probable contribution to variable results, a specially designed vacuum tube voltmeter was obtained for use with a recorder. Open circuit conditions were maintained by use of this vacuum tube voltmeter and continuous emf measurements could be taken with the recorder.

III. MICROBIOLOGICAL TECHNIQUES AND OBSERVATIONS

The application of microbiological techniques to support the electrochemical investigations was focused on two areas of study. The first involved obtaining reproducible bacterial growth. After such growth was established, less complex media were sought by removing individual constituents from the more complex formulae. The second area of study involved developing techniques for determining bacterial counts in the uniform biological half cell suspensions.

A. Growth of *Thiobacillus thiooxidans*

Successful growth of *T. thiooxidans* was studied primarily in three media. At first, reproducible growth of *T. thiooxidans* was obtained with shaker cultures using Skerman's⁸ basic mineral salts (17 salts). One per cent of sterile powdered sulfur was suspended in this medium. High yields of 10^9 organisms/ml were obtained with mature cultures (maximum population density) after five days incubation.

Since the interpretation of the emf measurements in the Skerman's medium was exceedingly difficult, a simpler medium was sought. A.T.C.C. medium,^d containing five salts plus 1 per cent sulfur, was studied as a growth nutrient. This medium gave mature cultures with populations of 10^8 bacteria/ml. A further consideration of reducing the salts, comprising A.T.C.C., brought about the removal of ferrous sulfate. The concentration of T. thiooxidans in mature cultures in this medium, A.T.C.C.(-), was approximately 1×10^7 organisms/ml.

The bacteria to be used with the biocells usually were taken from five-day-old mature shaker cultures. These cultures were grown in 250 ml Erlenmeyer flasks with 30 ml of medium in each flask. Incubation was either at 29° or at room temperature.

The mature cultures were harvested from the shaker flasks by centrifuging the organisms at 9,000 rpm for 5 min in a Lourdes centrifuge. After decanting the supernatant, the cells were then washed twice with sterile medium and were suspended in the various volumes of the sterile medium for the particular bacterial concentration to be used for biological half-cell studies.

^d Milligrams/100 cc: $(\text{NH}_4)_2\text{SO}_4$, 20.0; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 50.0; CaCl_2 , 25.0;
 FeSO_4 , 5.0; KH_2PO_4 , 300.0.

B. Bacterial Counts

Two methods were applied for obtaining the bacterial populations of the cultures in the preparation and operation of the biocells. These methods were the Petroff-Hausser chamber count and the micro-Kjeldahl analysis⁹ for total nitrogen content of the bacteria.

T. Thiooxidans was removed from the medium by filtration before a modified micro-Kjeldahl analysis was applied. Turbidity determinations for bacterial counts were not practical because of the presence of powdered sulfur. Pour and spread plate counts were discarded after obtaining irregular and time consuming results. The Petroff-Hausser chamber counts were used to calibrate the nitrogen content from the bacterial with their concentration in the medium.

The micro-Kjeldahl technique was only used when appreciable volumes of samples were available and concentrations of bacteria were approximately 1×10^7 /ml or greater. Use of the Petroff-Hausser counting chamber technique was preferred for lower concentrations of bacteria and experiments where less than 1 ml of sample was available. The latter technique was adopted completely after the earlier phases of investigation in order to minimize disturbing the biological half-cell ecology. The total amount of liquid required for the samples by this technique was negligible compared to the large biocell volume.

C. General Microbiological Observations

Mature populations obtained in the large biological half cell (Table III) with Skerman's medium were consistently less than observed in the shaker cultures. Subsequent experimentation with growth of T. thiooxidans in less complex media showed that this difference in population density decreased with A.T.C.C. and disappeared when A.T.C.C.(-) was used.

TABLE III

TYPICAL MATURE POPULATION DENSITIES

<u>Medium</u>	<u>Shaker Culture</u> (organisms/ml)	<u>Large Biological</u> <u>Half Cell</u> (organisms/ml)	<u>Number of</u> <u>Salts</u>
Skerman's	1×10^9	1×10^8	17
A.T.C.C.	1×10^8	1 to 5×10^7	5
A.T.C.C.(-)	1 to 5×10^7	1 to 5×10^7	4

Experiments with growth of T. thiooxidans in both shaker and biocell cultures using Skerman's and A.T.C.C. as growth media showed that the reduction in the number of mineral salts caused a decrease in the mature population density. Two typical biocells (Fig. 3) were started with similar inoculi and similar volumes of medium. The temperature for both biocells was held at 29°. Similar lag periods were observed during the first day, followed by a rise in bacterial concentrations during the second day. During the third day of incubation,

the bacteria concentrations were observed to rise more rapidly in Skerman's than in A.T.C.C. After this period the population density appeared to stabilize at 1×10^8 for Skerman's and 4×10^7 bacteria/ml for the A.T.C.C. media.

IV. ELECTROCHEMICAL OBSERVATIONS WITH T. THIOOXIDANS

The initial investigations were concerned with establishing that an electrochemical potential, different from that of a control cell, exists when T. thiooxidans utilizes sulfur. The U-tube was chosen for these studies. Each side of the U-tube comprised a half cell, one biological and the other an oxygen-carbon reference electrode. Duplicate U-tubes were prepared with only one difference. T. thiooxidans was present in the arm of one of them. The other half cell had only sulfur suspended in Skerman's medium. Figure 4 shows that a significant difference exists between the complete cell emf's of the inoculated and control cell. Such results were typical both when carbon and when platinum electrodes were used in the cells. The increase in the difference with time suggests that after acclimation to the cell, T. thiooxidans became active and started to utilize the sulfur which in turn changed the electrochemical nature of the half cell.

Biological and control half cell emf's, using platinum electrodes, were measured as a function of time in the modified U-tube design (Fig. 1). The biological half cells consistently gave voltages

which were lower than their controls, however, leaks through the agar plug at various times after cell preparation encouraged pursuing experimentation with a better cell design.

The remaining experiments were conducted with large biological half cells using platinum in preference to the slower responding carbon electrodes. Since quantitative data were expected from the use of this new cell design, a check was made on how increases in hydrogen and sulfate ion would affect the half cell electrochemical emf as measured by this electrode. Sulfuric acid was added separately in approximately 20 increments to 1 per cent suspensions of powdered sulfur in each of the three media under consideration for studying growth of T. thiooxidans. The initial pH values of approximately 5.0 gave way upon additions of the acid to values of 1.0. ~~Each addition of acid simulated production of~~ sulfuric acid by T. thiooxidans. The maximum variation in half cell emf's with Skerman's medium experiencing these changes in pH was 0.012 V. Subsequent experimentation with A.T.C.C. and A.T.C.C.(-) media gave a smaller maximum variation for the same total change in pH. Thus, the electrochemical background fluctuations to be expected when sulfate ion is produced by T. thiooxidans were identified.

A. Medium Effect on Biocell Activity

Shaker cultures of T. thiooxidans were grown in Skerman's, A.T.C.C., and A.T.C.C.(-) media under similar conditions. The preparations for centrifuging, washing, and resuspension in fresh sterile

media, were planned to give populations having a concentration of 1×10^7 bacteria/ml. However, the indeterminate losses in the transfers gave rise to a slight variance in initial bacterial suspensions in the large biological half cells. The suspension in A.T.C.C.(-) was 1.2×10^7 . The value for Skerman's was 1×10^7 and for A.T.C.C., 2×10^6 bacteria/ml.

After inoculation, a lag phase was observed to take place during growth in each medium (Fig. 5). The values for bacterial population counts under the dotted line in Fig. 5 were below the micro-Kjeldahl analysis so they were estimated (Petroff-Hausser Count). After 70 hr, the population in the Skerman's salts, which was initially similar to the other two media, was now greater. Subsequent bacterial counts showed that the populations stabilized and after eight days the Skerman's medium had a population of 1×10^8 while both A.T.C.C. media had approximately 5×10^7 bacteria/ml. These saturation populations were typical of large biocell experiments with these three media (Table III).

If the variation in the initial concentration in bacteria can be ignored the amount of total acid produced by T. thiooxidans seemed to depend upon the medium in which it grew. Since it was not possible to wash residual amounts of acid from the centrifuged cells, the initial pH values differed. Subsequent accumulation of acid is shown in Fig. 6. Of particular interest was the fact that T. thiooxidans produced less

acid (0.73 mmole) in the Skerman's medium in attaining a greater cell population, 7×10^7 bacteria/ml than in the other two media (3.66 mmole for A.T.C.C.(-) and 1.89 mmole for A.T.C.C.) for the initial 70-hr growth interval.

The growth of T. thiooxidans was followed by electrochemical measurements. The fact that the control half cells (Fig. 7) started at exactly the same emf was coincidental. They usually differed by small amounts. T. thiooxidans was observed as a contaminant (Fig. 7) in the control half cell for the Skerman's medium after the 50-hr measurements. The visual presence of this microorganism was supported by a corresponding change in pH due to acid production. This microbiological activity caused a decrease in half-cell voltage as indicated by the values at the 70-hr interval.

The change from the initial emf's for the Skerman's and A.T.C.C. half cell controls were typical for these media. Since their half-cell voltages usually stabilized after 20 hr, subsequent experimentation involved preparation and operation of two control half cells until voltage stability was observed. Then, one of the half cells was inoculated for comparison of their behavior as a function of time. These complications were minimized when working with the A.T.C.C.(-) medium. Its half-cell emf was less erratic and stabilized quite readily.

The inoculated A.T.C.C.(-) (Fig. 8) half cell had the same initial voltage as its control half cell, whereas the other inoculated half cells were higher than their controls. After 33 hr each of the inoculated half cells had voltages more negative than their control cells. The behavior of the inoculated A.T.C.C. and A.T.C.C.(-) half cells was comparable after 29 hr of operation. These cells gave lower voltages than the inoculated Skerman's half cell. The relatively small difference between the inoculated and control half cells comprising Skerman's medium pointed out the need to have a less complex medium for intimately following growth of T. thiooxidans electrochemically. Thus, subsequent studies were conducted with A.T.C.C. and A.T.C.C.(-) media. The biological half cell emf's with these media were found to be more stable and further removed from their control half-cell values. The removal of the ferrous sulfate from the A.T.C.C. medium offered additional improvement in control and biological half-cell stability and reproducibility.

B. Long Term Biological Half-Cell Activity

Special precautions were taken with the preparation of one experiment which was allowed to run for an extended period of time. Emphasis was placed on minimizing external contact with the medium to eliminate the possibility of contamination and to increase the probability of long life. The supply of oxygen and carbon dioxide for this

biological half cell came from the atmosphere through sterile cotton plugs placed in the small air gaps around the wires leading into the cell through the rubber stoppers. The large biological half cell was used with its Teflon stirrer, pH meter, and the platinum electrode attachments. It was filled with sterile A.T.C.C. medium, characterized (pH, emf, sterility) and then inoculated with T. thiooxidans to give an initial concentration of approximately 7×10^7 bacteria/ml. Sterile sulfur was used as the energy source in this medium. The cell count, pH and biological half-cell behavior for a 16-day period are shown in Fig. 9. Table IV describes the behavior of this cell approaching the 111th day. Between these times the emf and the bacterial counts fluctuated slightly with a fairly uniform accumulation of acid. The general trend of the half cell potential was to become more negative. After the 111 days, the biological half cell was observed to be contaminated with bacteria other than T. thiooxidans. The half-cell emf was observed to become more positive after becoming contaminated. This observation was consistent with that obtained from other inoculated biological half cells which became contaminated with foreign bacteria.

TABLE IV

SUPPLEMENTARY DATA FOR LONG TERM BIOLOGICAL HALF CELL(T. thiooxidans Utilizing Sulfur)

<u>Time Period</u> (days)	<u>Half-Cell emf</u> (V)	<u>pH</u>	<u>Bacteria/ml</u>
72	-0.61	1.18	5×10^7
76	-0.62	1.09	5×10^7
79	-0.61	1.05	3×10^7
83	-0.62	0.97	4×10^7
90	-0.63	0.92	4×10^7
99	-0.63	0.91	6×10^7
106	-0.64	0.95	4×10^7
111	-0.64	0.92	3×10^7

Several interesting features were worth noting in this long term experiment. The initial inoculation gave a population of 7×10^7 T. thiooxidans/ml. Death occurred lowering the population below the level of detection by the micro-Kjeldahl analysis. At the end of the initial day of operation, the population was observed to increase to 1.5×10^7 . Fluctuations between this value and 3×10^7 were observed throughout the 17-day interval shown in Fig. 9. Between the end of this period and the 111th day, a maximum count of 5×10^7 was obtained. The population dropped to 3×10^7 bacteria/ml towards the end of the experiment. The ecological factors effecting bacterial growth kept the cell population in this range. The pH of the medium changed from an initial value of 4.85 to about 1.7 units after 15 days. Changes in its value after this time were small since appreciable amounts of acids had to be

produced relative to the total amount present in order to bring about a change in pH. The fact that T. thiooxidans was not increasing its population suggests inactivity but the increase in sulfuric acid concentration indicates that a constant cell division and death rate existed. The general trend in the half-cell emf throughout this time period was to become more negative.

The consistent trend in the curves obtained in this experiment indicated that many of the past variations in half cell potentials were no longer present and that reproducibility of subsequent biological half cells should be expected. One exception to this consistent trend toward a lower half cell potential may be significant. A noticeable decrease in cell population appeared to take place after the eighth day. This dip and later recovery seemed to cause a simultaneous change in the electrochemical potential.

C. Reproducibility of Biological Half Cell

Several biological half cells were prepared with T. thiooxidans utilizing sulfur in the A.T.C.C.(-) medium to determine reproducibility of results. Figure 10 records their biological half-cell emf for a five-day period. Initially, the cells started at approximately the same voltage followed by a slight rise and then a decrease. A slight divergence in values then takes place for one and one-half days. After this interval they gave equivalent results. The effect

of higher temperature is shown in Series XLVI when after three days the emf decreases markedly. At this time the thermostat for its bath malfunctioned and temperatures exceeding 60° were probably experienced during the evening period. This irreparable damage caused formation of a lower half-cell emf, measured upon return and subsequent control at 29°.

Figure 11 records the change in pH of each of these half cells. One can readily see that after the initial inoculation, a slight divergence takes place. This divergence disappears after about one day, giving rise to similar amounts of acid formed in each of the cells.

The divergence in the microorganisms' contribution to emf and acid formation between the one-fourth and one and one-half day period appears to be consistent with the microorganism experiencing a lag phase in each of the biological half cells throughout this time interval. Figure 12 shows the bacterial cell count per milliliter as a function of time. A lag phase existed in each cell with a slight inconsistency in the recovery time. In general, the cell populations duplicated themselves in each of the cells. The last two values for the cell count in Series XLVI reflect the damage caused by the temperature of the bath exceeding the control value.

V. DISCUSSION

The existence of an electrochemical contribution from T. thiooxidans was established during the initial investigations with combined half cells comprising biological and control half cells with either a calomel or oxygen-carbon electrode. The cell comprising T. thiooxidans utilizing sulfur made contributions to an electrochemical potential which reflected microbiological activity. Subsequent experiments with well defined biological half cells, comprising emf, pH, and bacterial count determinations, supported the initial observations. The interaction of T. thiooxidans with the various nutrients as well as sulfur gave half cell emf's which were distinctly different than control half cells operated for comparable periods of time under identical experimental conditions. The reduction in the number of nutrient ions for growth of T. thiooxidans improved one's ability to measure its more intimate contributions to an electrochemical emf. However, since the measured half cell emf is an algebraic resultant of each electrochemical contribution in the half cell, it is not possible at this time to say how T. thiooxidans is implicated. If sufficient activity coefficients at these ionic concentrations would be available, the theoretical contributions of the constituents of the media could be calculated so the remaining biological contribution could be identified. This calculation is much too complicated at present. However, as further

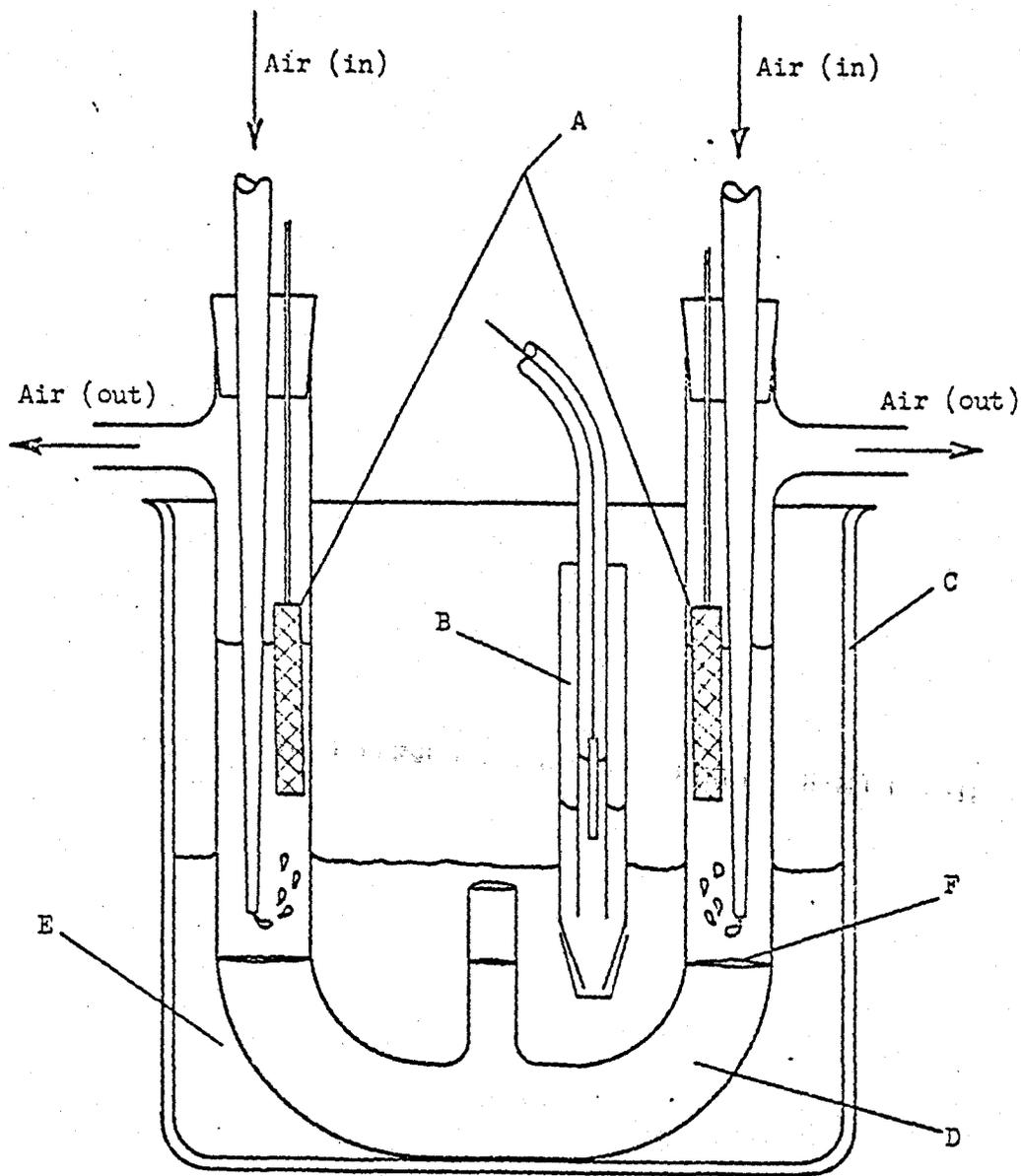
experimentation progresses towards finding the minimum number of minerals necessary for growth of T. thiooxidans utilizing sulfur, the possibility of calculating the actual contribution of each constituent and thereby the specific contribution of T. thiooxidans becomes much greater. The premise that the study of electrochemical behavior of autotrophs, especially Thiobacillus sp., may be simpler than heterotrophs has not been resolved in these investigations. Additional study with both types of species will be needed before a conclusion can be made that one or the other will offer the simpler approach to gaining a better understanding of electrochemical processes that take place during microbiological growth.

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microbiological growth.

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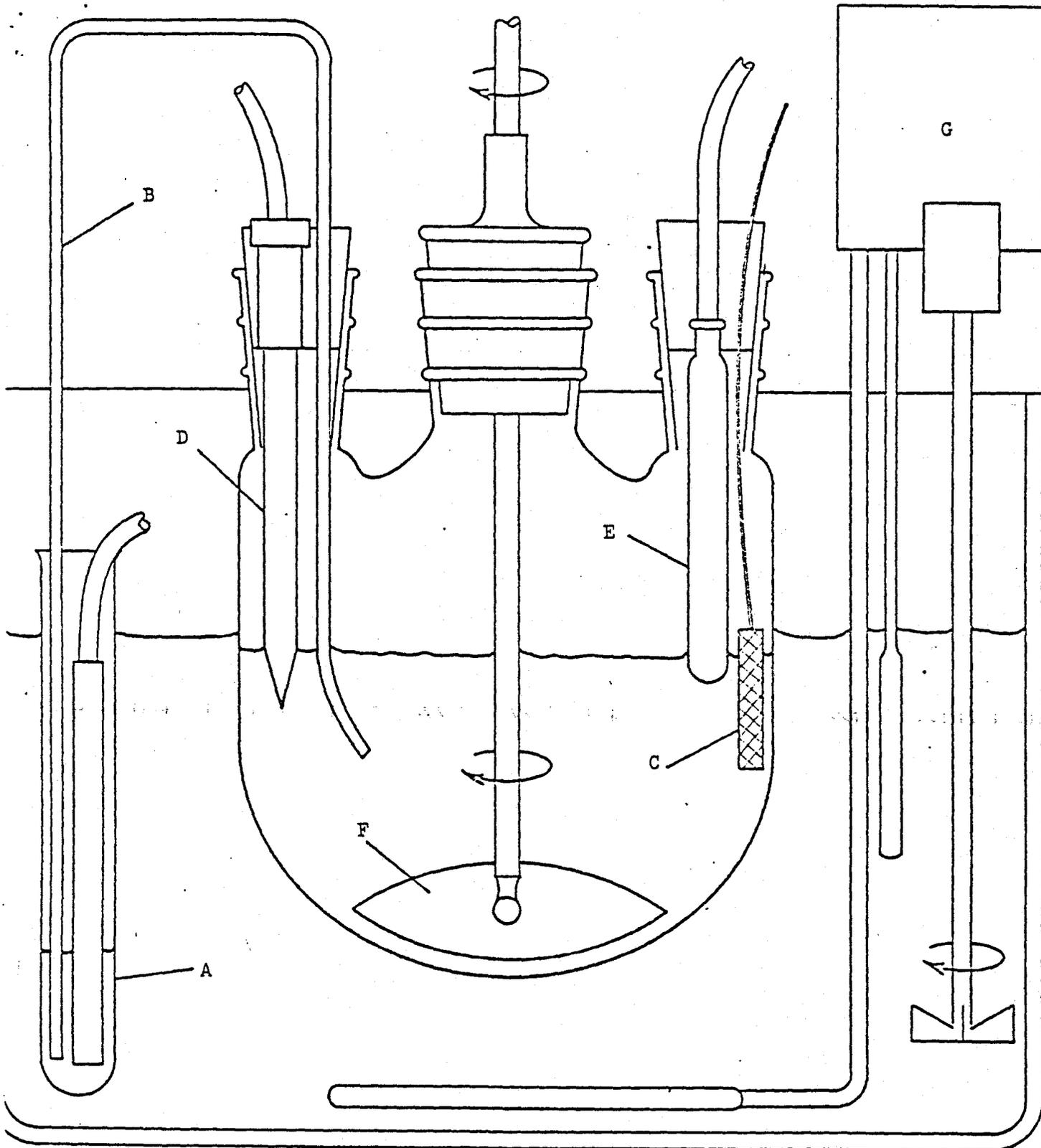
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U-Tube Biocell Key

- A - Platinum or carbon electrodes
- B - Calomel reference electrode
- C - 1,000 ml beaker
- D - Agar salt bridge
- E - Saturated KCl solution
- F - Bridge - biocell interface

Fig. 1 - Modified U-Tube for Use with Calomel Electrode



Key for Large Biological Half Cell

- A - Calomel reference electrode
- B - Agar salt bridge
- C - Platinum electrode
(biological half cell)

- D - Glass electrode (pH meter)
- E - Calomel electrode (pH meter)
- F - Teflon stirrer
- G - Constant temperature apparatus

Fig. 2 - Large Volume Biological Half Cell

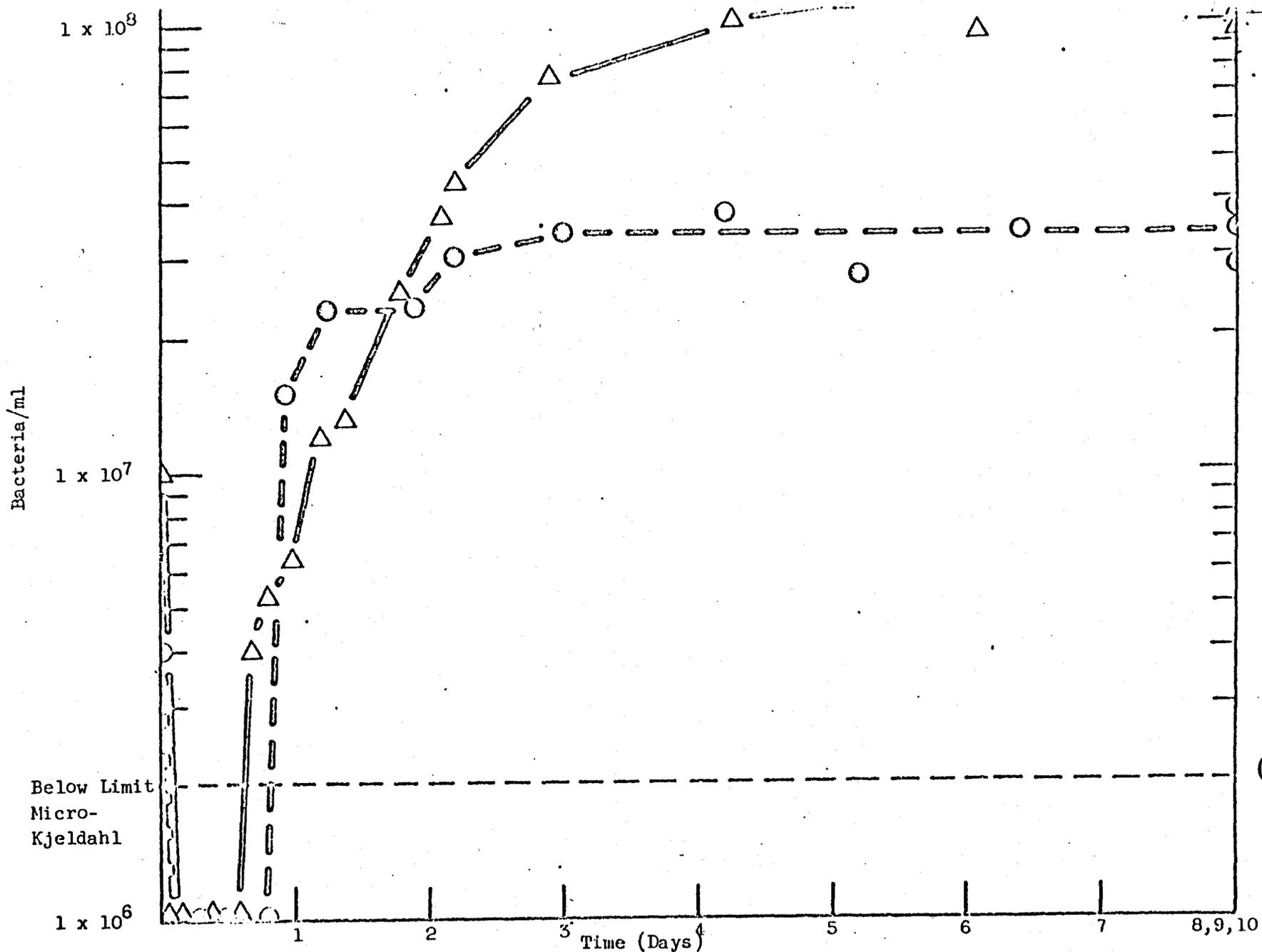


Fig. 3 - Bacterial Growth in A.T.C.C. and Skerman's Media

○-----○ A.T.C.C.
 △-----△ Skerman's

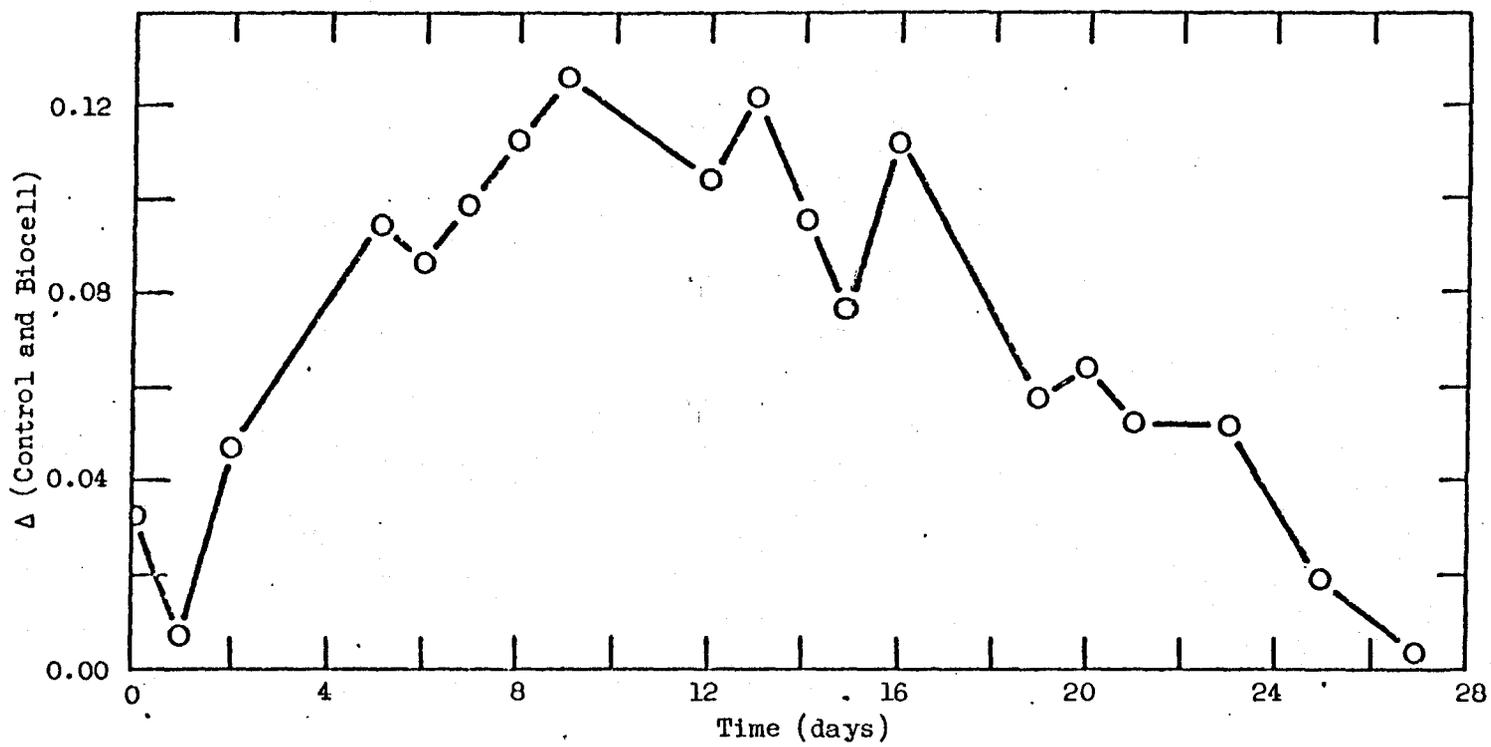


Fig. 4 - Voltage Difference Between Control and Biocell, U-Tubes

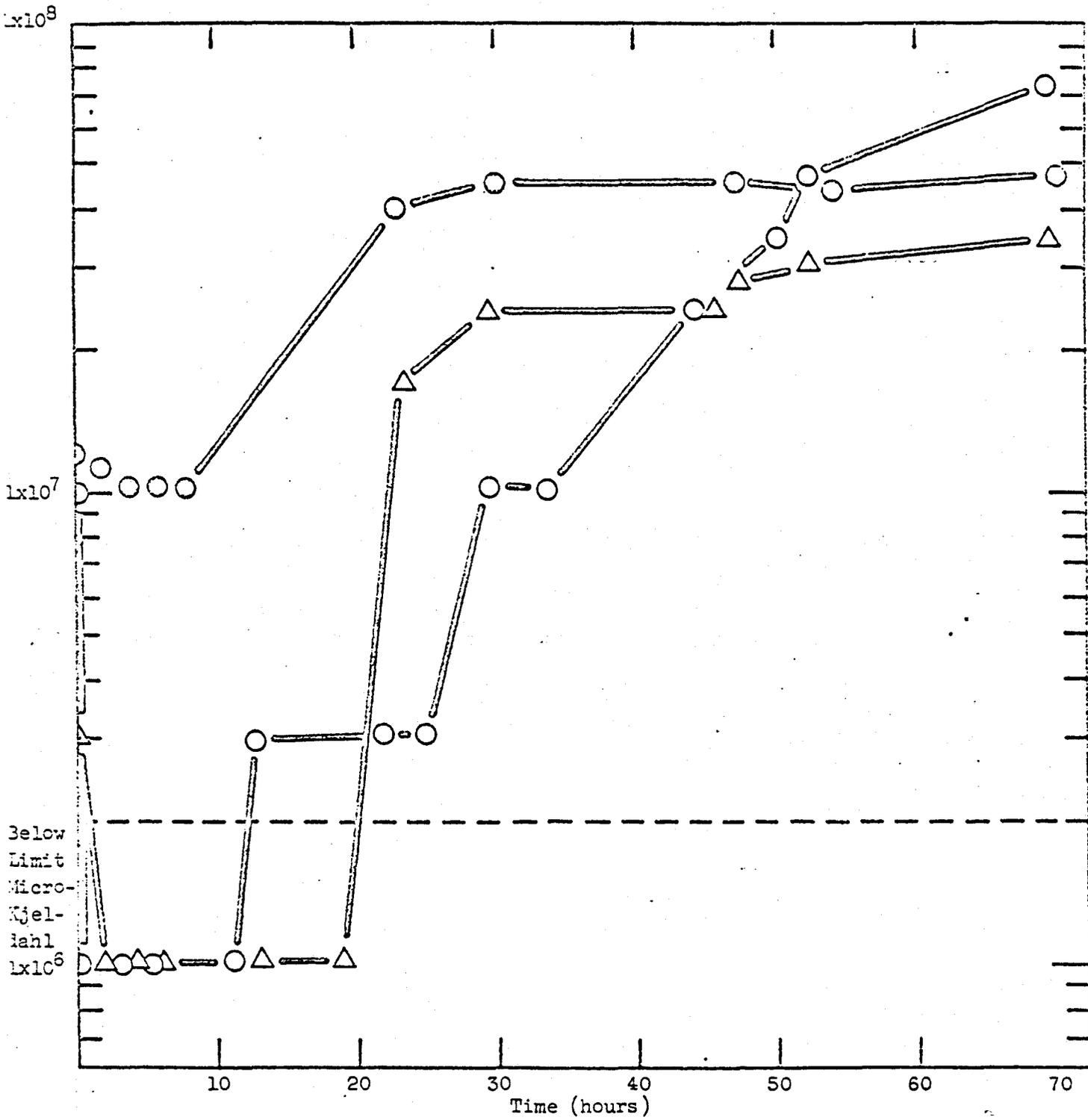


Fig. 5 - Bacterial Growth in Different Media
(*T. thiooxidans* utilizing sulfur)

○—○ A.T.C.C.(-)
 △—△ A.T.C.C.
 ○—○ Skerman's

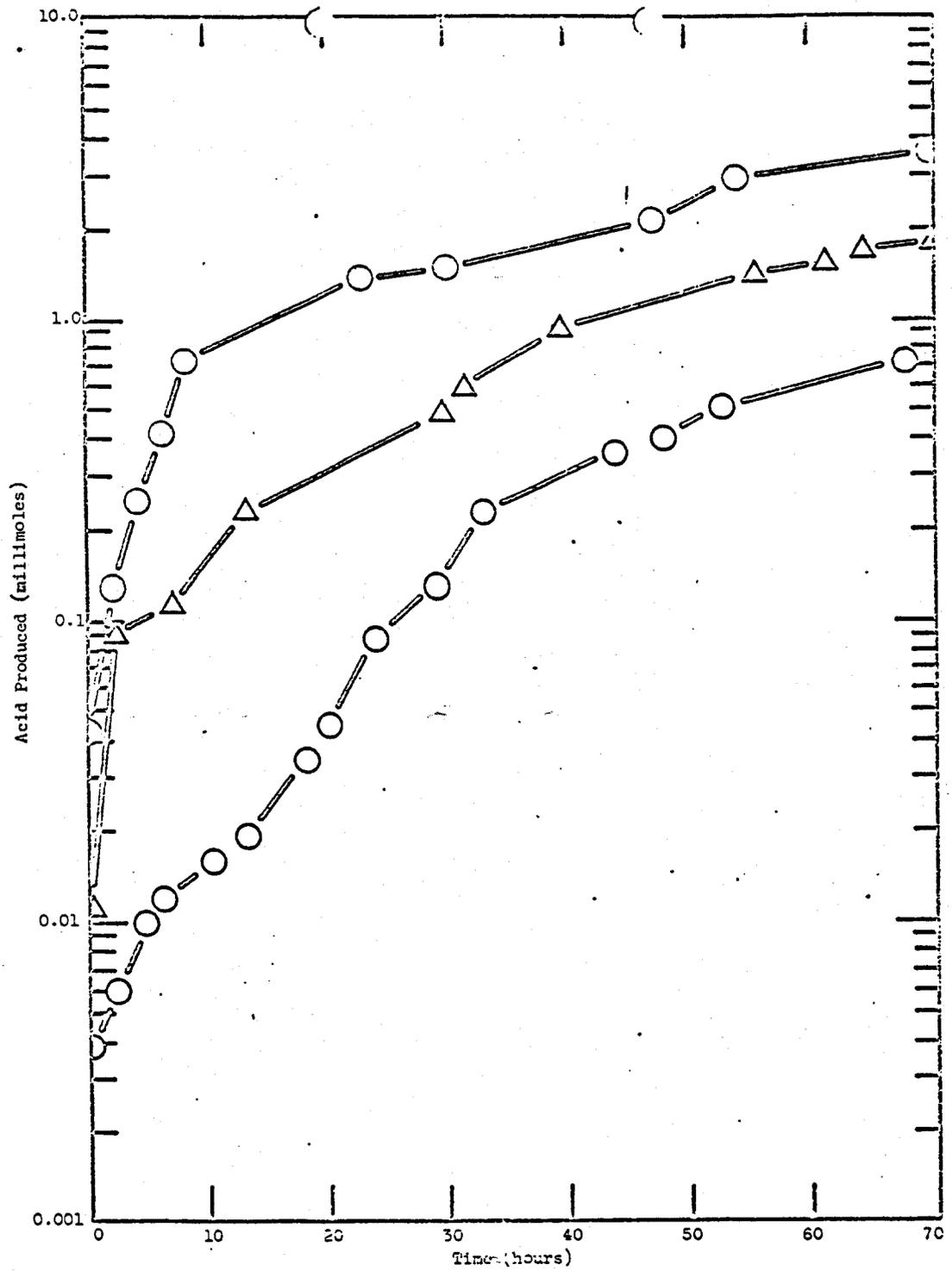


Fig. 6 - Acid Produced in Different Media
(*T. thiooxidans* utilizing sulfur)

- A.T.C.C. (-)
- △—△ A.T.C.C.
- Skerman's

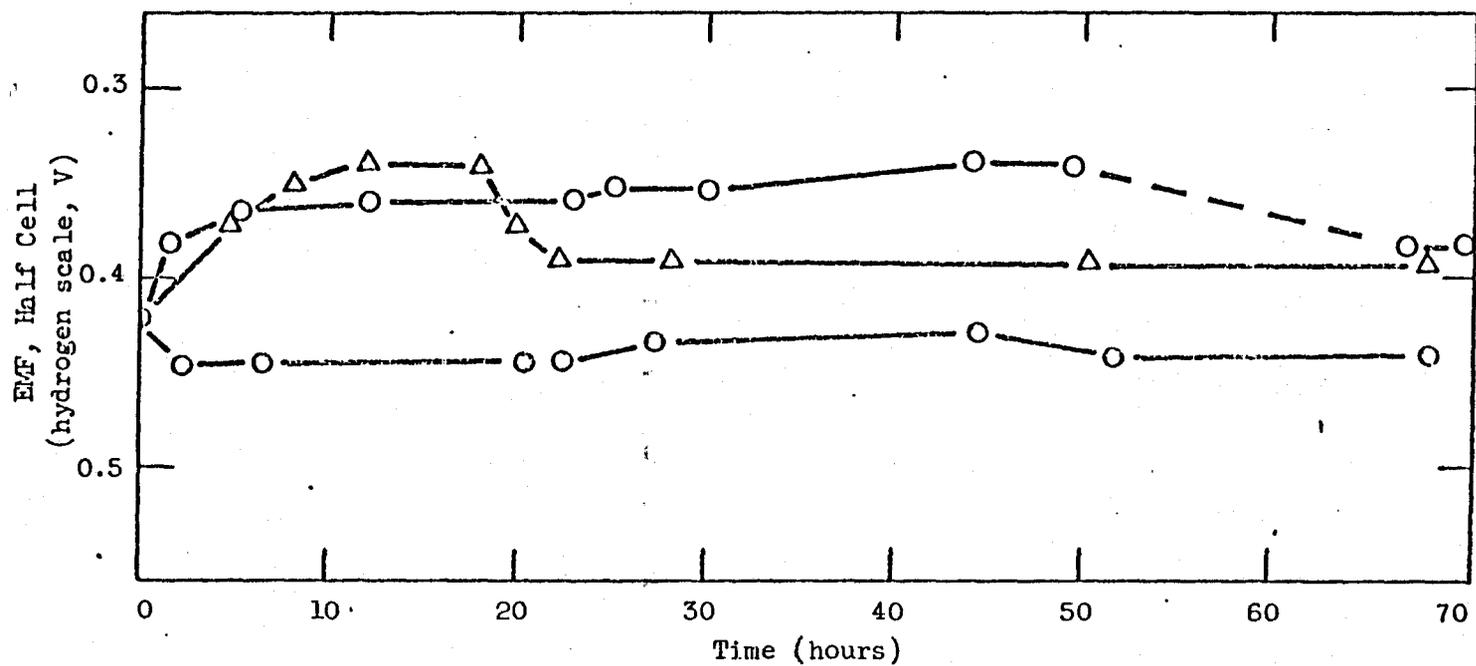


Fig. 7 - Control Half Cells with Different Media
(sulfur suspensions)

○—○ A.T.C.C.(-)
 △—△ A.T.C.C.
 ○—○ Skerman's

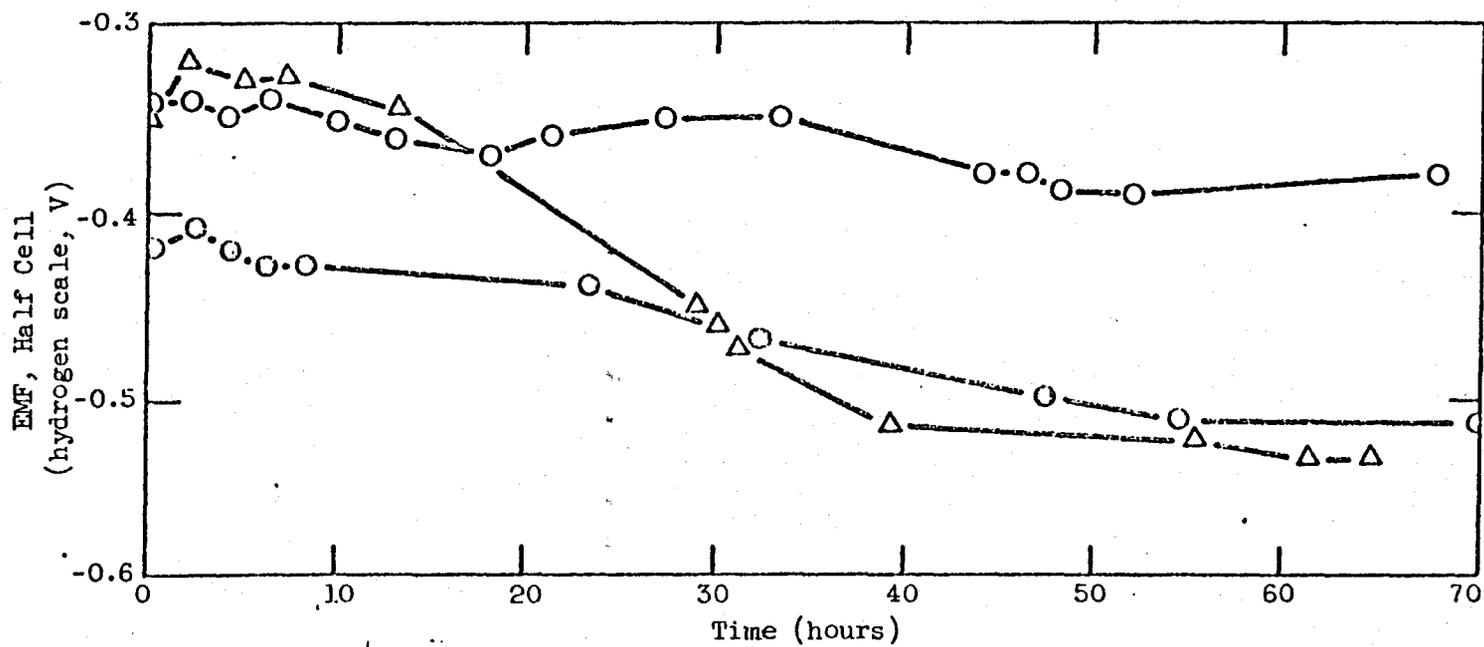


Fig. 8 - Inoculated Half Cells with Different Media
(*T. thiooxidans* utilizing sulfur)

○ — ○ A.T.C.C. (-)
 △ — △ A.T.C.C.
 ○ — ○ Skerman's

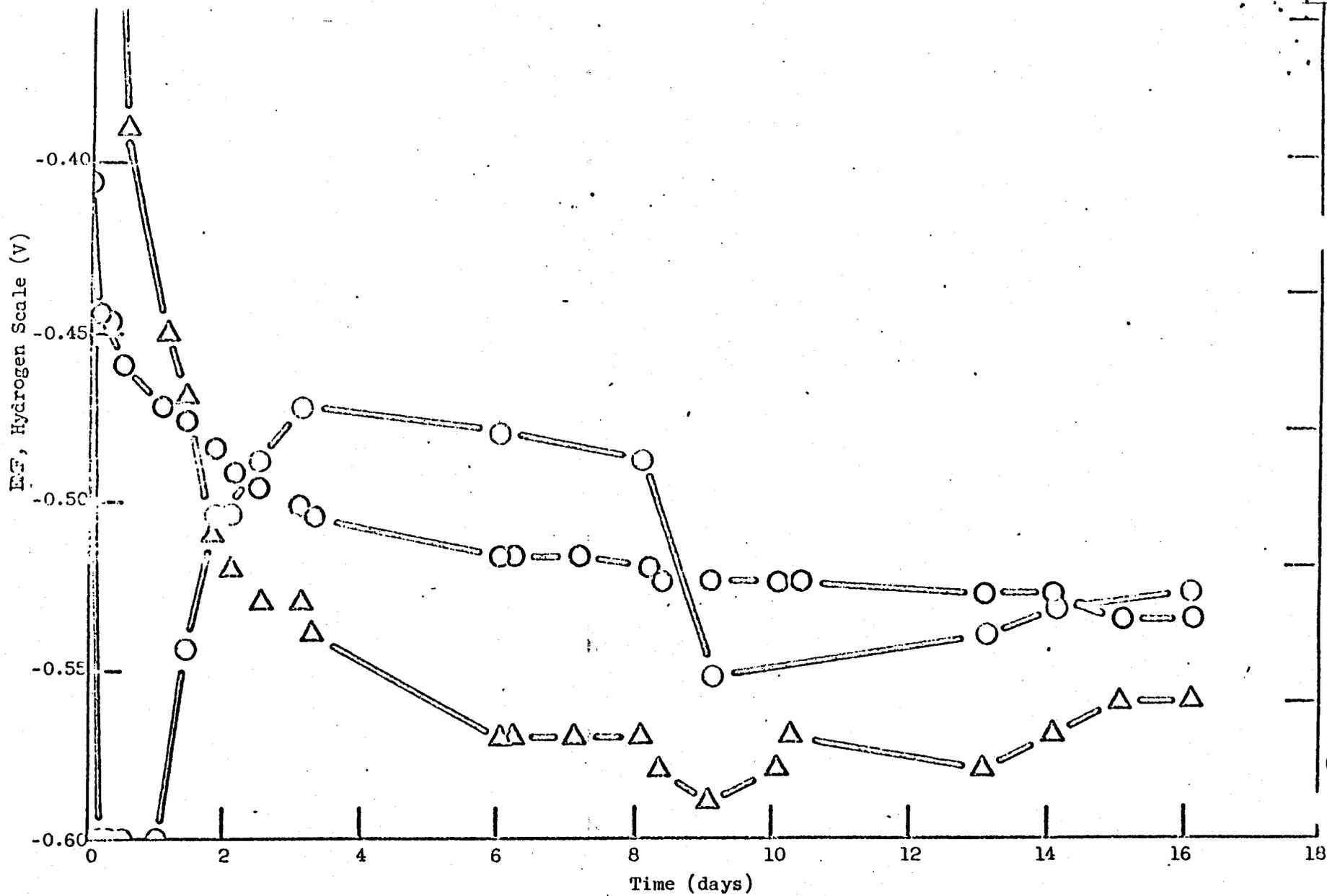


Fig. 9 - Long Term Biological Half Cell Behavior
(*T. thiooxidans*, Sulfur, A.T.C.C.)

○ — ○ pH
○ — ○ Bacteria/ml

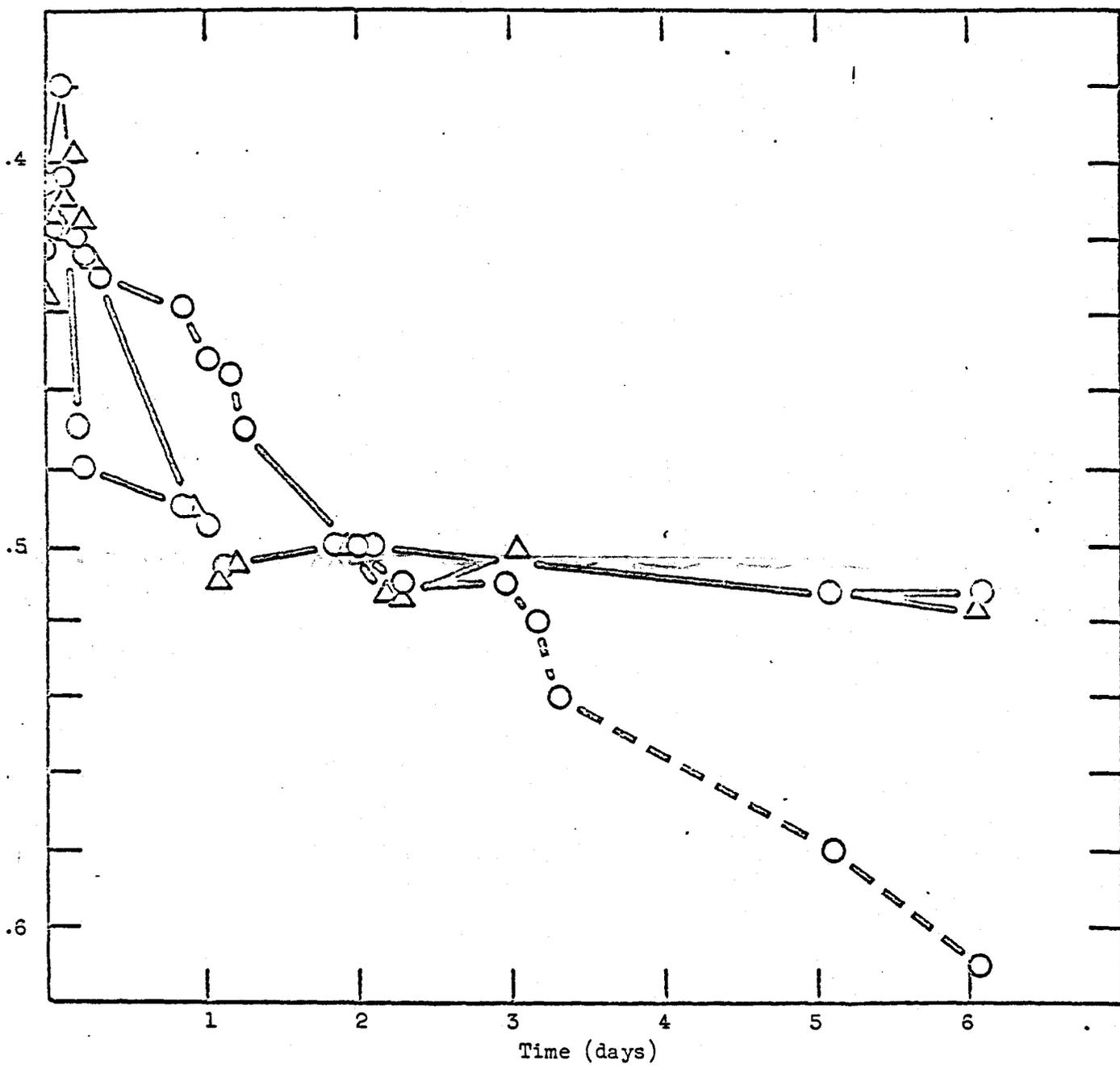


Fig. 10 - Reproducibility of Biological Half Cell, emf
 (T. thiooxidans, A.T.C.C.(-), sulfur)

- Series XLVI
- △—△ Series XLVII
- Series LIII

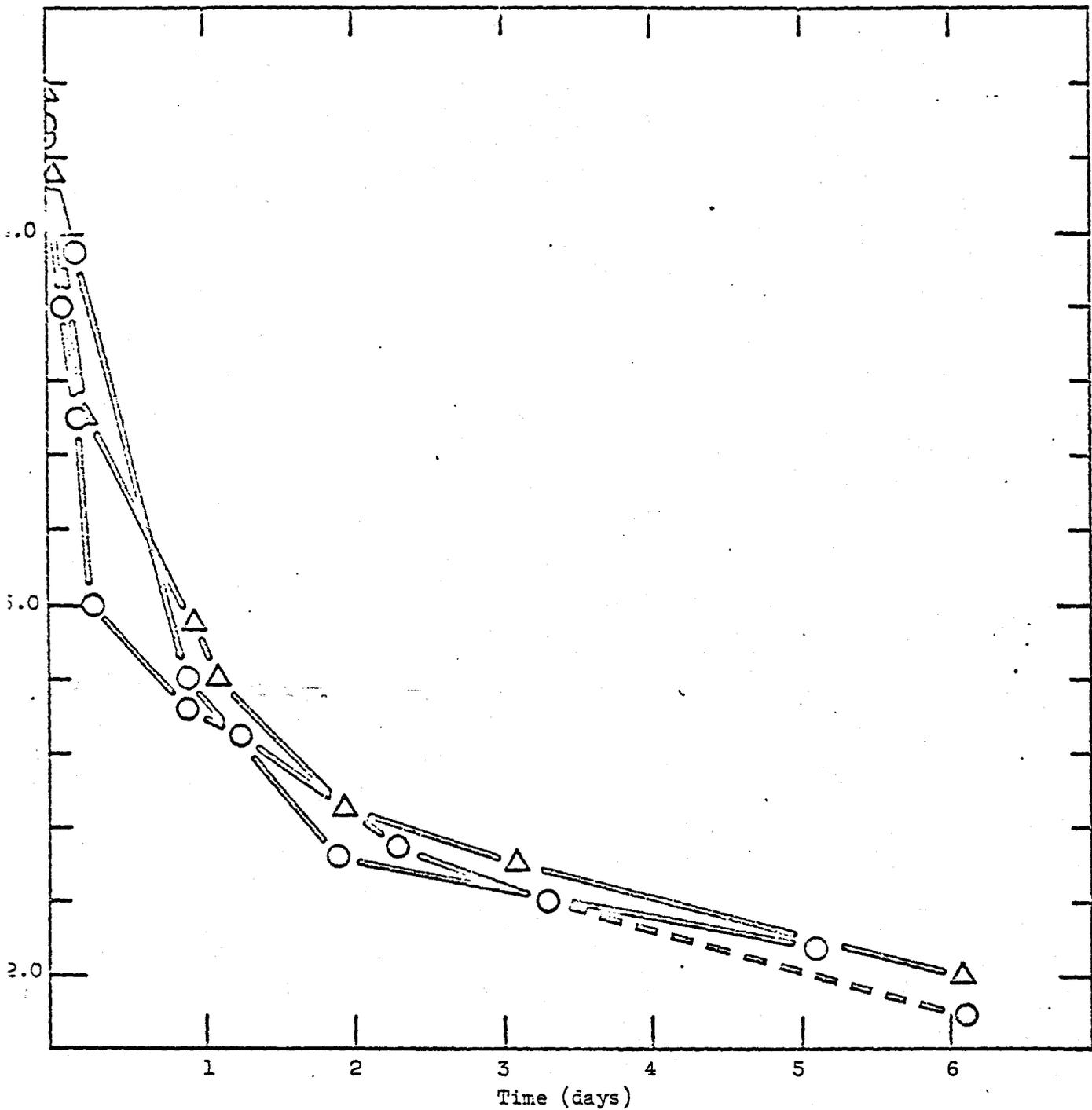


Fig. 11 - Reproducibility of Biological Half Cell, pH
 (T. thiooxidans, A.T.C.C.(-), sulfur)

- Series XLVI
- △—△ Series XLVII
- Series LII

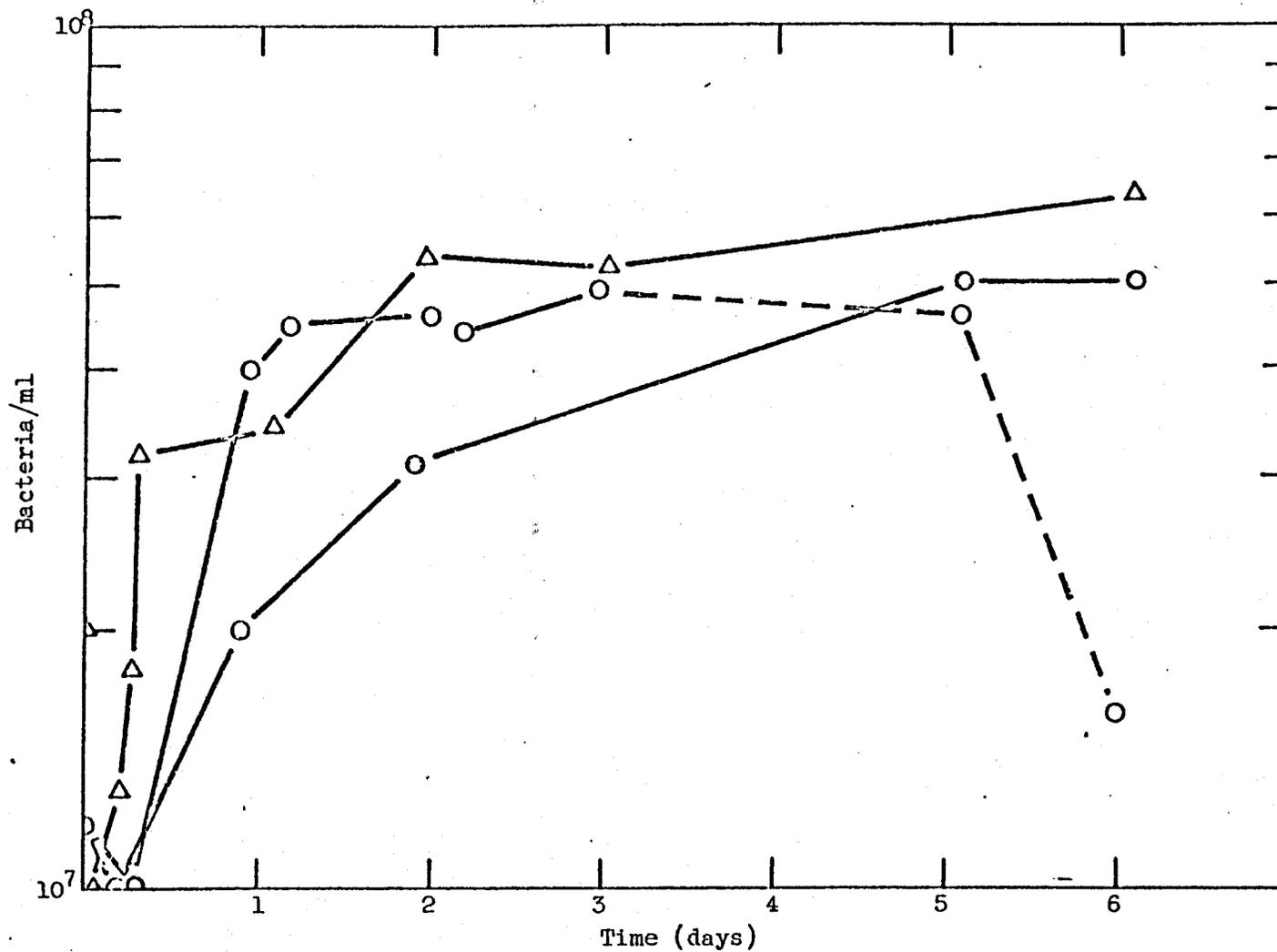


Fig. 12 - Reproducibility of Bacterial Growth in Biological Half Cell
 (*T. thiooxidans*, A.T.C.C.(-), sulfur)

○—○ Series XLVI
 △—△ Series XLVII
 ○—○ Series LII

July 1, 1965

Dear _____

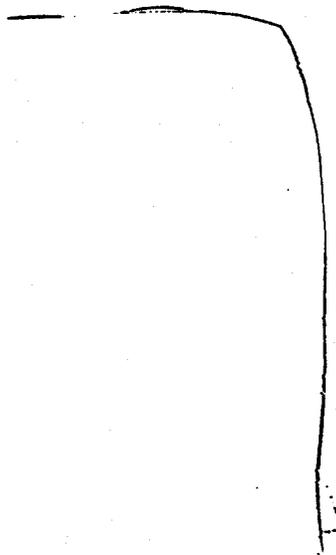
Enclosed are two copies of our paper "Electrochemical Observations in Microbiological Processes. II. Growth of Thiobacillus Thiooxidans." I would appreciate your making arrangements for release of this paper for publication. Any comments that you or your associates may have for improving the paper would be well received.

Thank you for your kind assistance in getting this paper released.

Very truly yours,

ELECTROCHEMICAL OBSERVATIONS IN MICROBIOLOGICAL PROCESSES. II.

GROWTH OF THIORACILLUS THICOXIDANS



SUMMARY

The electrochemical activity of the individual chemicals in the nutrient medium for growth of Thiobacillus thiooxidans was studied along with the effect of the gases in equilibrium with their solutions. Several chemicals were active individually, however the magnitudes, as measured by changes in half-cell potential, were less than that observed when T. thiooxidans was present in their composite mixture. Sterilized Skerman's mineral salts and the American Type Culture Collection (without ferrous sulfate) media were not sensitive electrochemically to changes from pure oxygen to nitrogen atmospheres. When T. thiooxidans was present in these media, the biological half-cell emf became sensitive to changes in the oxygen content of the atmosphere in equilibrium with the organism and nutrient. The ability of T. thiooxidans to make an electrochemical contribution, as registered by a platinum electrode, is substantiated further by those investigations.

I. Introduction

The existence of an electrochemical contribution by Thiobacillus thiooxidans utilizing sulfur during growth was previously established.¹ The fact, that this electrochemical behavior existed, raised several questions about its origin. A review of the possible half-cell emf's² which may exist when sulfur is oxidized in this system to sulfite, thiosulfate, polythionate, or sulfate, suggests that the previously measured emf may be the resultant of several potentials due to the steady state concentrations of such intermediates. Even though Starkey³ and subsequent workers found that only sulfate was formed by T. thiooxidans utilizing sulfur, the magnitude of the observed biological half-cell emf was such that it could rise from the presence of small amounts of intermediate products in steady state ratios. Therefore, microtechniques of analysis were employed to check whether or not any heretofore undetected oxidation-reduction reactions contributed to the observed biological half-cell emf's.

Since T. thiooxidans is an aerobe, its growth depends upon the availability of oxygen. The effect of oxygen or the lack of it on the electrochemical character of control and inoculated half cells, was investigated also to isolate T. thiooxidans' contribution to a half cell potential.

II. Electrochemical Accessories

The electrochemical apparatus used in these studies was equivalent to that described in the previous paper¹ except for some minor additions. Of necessity, the vacuum tube voltmeter and recorder combination were used to measure the immediate electrochemical response to changes from aerobic to anaerobic atmospheres above the half cells. Figure 1 shows the essential elements of the circuitry comprising the electrochemical measurements. The voltage output on the output resistor was fed as an input signal to a Daystrom-Weston Model 6701, Type 1 continuous chart recorder. This recorder (an automatic recording potentiometer) remained continually balanced with the potential input from the voltmeter. The voltmeter output current was the direct result of the difference between the biological half-cell and the calomel reference half-cell potentials. Open circuit conditions were maintained by use of this vacuum tube voltmeter. Calibrations with a standard cell via a Leeds-Northrup K-3 potentiometer before and after experimentation insured correct recorder measurements of half-cell potentials. All half-cell emf's quoted in this paper are based on the standard hydrogen electrode.

The large volume biological half cell, utilizing the platinum, glass, and calomel electrodes, agar-KCl salt bridge, and Teflon stirrer, was used for these investigations. The preparation,

inoculation, and operation of this cell duplicated our earlier investigations¹ except for one addition to the cell design. A gas sparger was inserted into one of the five necks of the one liter flask. One of the rubber stoppers in an adjacent neck was perforated to provide an exhaust vent for the gases bubbled into the half cell. This vent insured that the total pressure of the gases above the nutrient would not exceed that of the outside atmosphere.

A Precision Scientific "Wet Test Meter" was used to measure the amount and flow of various gases into the half cells. This meter also saturated each gas with water vapor, thus reducing losses by evaporation from the biocell.

The effects of stirring the biocell and bubbling gases into the media were determined for air, nitrogen, and oxygen. The changes in magnitude of the half-cell voltage for pure nitrogen and oxygen for each of the media were equivalent to those reported for air in the earlier paper, i.e., static atmosphere versus sparger bubbling, less than one millivolt; on-off stirring action, less than two millivolts for each of the media considered in these studies.

III. Microbiological Preparations and Observations

T. thiooxidans for these biocell investigations were taken from five-day-old mature shaker cultures grown at room temperatures.

These cultures, comprising separately Skerman's⁴ ATCC^a and ATCC(-)^b mineral salts, were grown in 250 ml Erlenmeyer flasks with 1% sulfur for an energy source. The mature cultures were harvested by centrifuging the organisms at 9 000 rpm for 5 min. The supernatant was then decanted, the cells washed with fresh sterile medium, resuspended and recentrifuged. After another washing, they were resuspended in the chosen sterile medium controlled at 29°C for the biological half-cell investigations:

IV. Analysis for Sulfur Intermediates

The desire to relate emf changes to specific oxidation-reduction states within the sulfur system, made it necessary to investigate the possible existence of sulfur intermediates and their changes with time. The approach to the analysis of the biocells was initially divided into two phases.

Phase one was concerned with the gross description of the cell medium at increasing time intervals during the experiments. Phase two concerned itself with the measurement of the amount of intermediate products formed during biocell operation.

-
- a. American Type Culture Medium, mg/100 cc: $(\text{NH}_4)_2\text{SO}_4$, 20.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50.0; CaCl_2 , 25.0; FeSO_4 , 5.0; KH_2PO_4 , 300.0.
b. Same as (a) without FeSO_4 .

During the investigations, the ~~description of the biocell~~
was ^{characterized} ~~made~~ by titrating with standard bases and oxidizing agents.

Samples of the medium after different periods of growth showed that the presence of strong acid (sulfuric) could be assayed by titration with alkali to a pH of 5.0. An extension of the titration to a pH of 8.5 was employed to detect the presence of weak acids. These pH values were chosen from inflection points observed during titrating samples from the biocell. Since several of the possible sulfur intermediates that could be formed react with iodine, independent and simultaneous iodine titrations were also conducted.

Phase two concerned the development and application of a column chromatographic separation for the potential metabolic intermediates. This method was derived from an adaption of Trudinger's⁵ work on the medium of Thiobacillus X, and Iguchi's⁶ methods of anion separation with anion-exchange resins.

Approximately 10 ml samples of cultures were removed from the biological half cell at prescribed intervals, and after filtration to remove all sulfur and bacteria, an exact volume of 10 ml was eluted through a column of 3 g washed Dowex 1 x 4 anion exchange resin contained in an 8 mm O.D. glass tube fitted with a glass stopcock for flow control. The various fractions were eluted as follows:

1. The sulfite and sulfide fractions were eluted with 20 ml of 0.5 M ammonium acetate, pH 5.

2. The thiosulfate was eluted with 10 ml of 2 M ammonium acetate, pH 5.

3. The polythionate fraction was removed in the following manner. A mixture of 1 ml of 1 per cent sodium cyanide and 2 ml of 1:10 ammonium hydroxide was added to the column and allowed to react with the remaining constituents for 5 min. The solution was eluted and the resin was washed thoroughly with 20 ml distilled water. Two milliliters of 1:10 nitric acid was added to the column and allowed to react for 5 min. The eluate was then washed through with 1 ml of distilled water and collected in a graduated tube. The eluate and washings were then brought up to a total volume of 5 ml with distilled water.

The amounts of sulfite and thiosulfate eluted were determined by iodine titration. The polythionates were assayed after the addition of two drops of 10 per cent ferric ammonium sulfate to the prepared nitric acid eluates. The amount of color developed was determined by absorbance measurements of 460 m μ with a Beckman DU spectrophotometer. These procedures were checked with known mixtures of the sulfur intermediates in cell media with and without T. thiooxidans before being applied to the analysis of biological half cells.

The results from the analysis for the gross description of the shaker and biocell cultures are summarized in Tables I and II for typical changes in compositions as a function of time. Table III represents typical analyses for the specific sulfur intermediates. These results are consistent with earlier observations with more rapid accumulation of acid in the shaker culture as compared to the large biocells. Specific observations governing the data in these tables are as follows:

1. The accumulation of strong acid, indicated by alkaline titration to pH 5, was shown by subsequent precipitation with barium chloride to be a direct result of the formation of sulfate ions. It was not unusual to obtain a pH of 1.0 within several weeks of operation. For a 600 ml biocell, this would give a total of 60 mM of hydrogen ion or 30 mM of sulfate ion.

2. The concentration of the weak acids, indicated by the second titration to pH 8.5, was very small, below 0.04 meq/ml. A significant increase during growth of T. thiooxidans was not observed.

3. The total oxidizable intermediates of the inoculated cells (Tables I and II) was initially very small, below 0.0002 meq/ml. This concentration did not increase as a function of the time. The uninoculated controls showed essentially the same concentrations of these products as did the biocells.

TABLE I

GROSS DESCRIPTION OF ACIDS AND OXIDIZABLE INTERMEDIATES
FORMED DURING LARGE SHAKER CULTURE GROWTH

(Medium, ATCC)

<u>Time</u> <u>(days)</u>	<u>pH</u>	<u>Titration (milliequivalents) For 10 ml Samples</u>		
		<u>NaOH to pH = 5</u>	<u>NaOH to pH = 8.5</u>	<u>Iodine</u>
0	2.20	0.27	0.25	0.002
1	2.16	0.30	0.26	0.003
4	1.52	1.41	0.27	0.003
5	1.40	1.71	0.27	0.004
6	1.38	2.19	0.28	0.004
8	1.30	2.97	0.31	0.002
10	1.07	3.85	0.29	0.004
12	1.04	4.40	0.25	0.003

TABLE II

GROSS DESCRIPTION OF ACIDS AND OXIDIZABLE INTERMEDIATES
FORMED DURING BIOLOGICAL HALF-CELL GROWTH

(Medium, ATCC)

<u>Time</u>	<u>pH</u>	<u>Titration (Milliequivalents) For 10 ml Samples</u>		
		<u>NaOH to pH = 5</u>	<u>NaOH to pH = 8.5</u>	<u>Iodine</u>
0	4.45	0.01	0.24	0.002
4 hr	4.00	0.01	0.30	0.002
7 hr	3.68	0.01	0.36	0.002
1 day	3.00	0.05	0.30	0.002
2 days	2.42	0.21	0.26	0.002
3 days	2.13	0.37	0.26	0.0015
5 days	1.95	0.49	0.26	0.002
7 days	1.94	0.62	0.24	0.002
9 days	1.94	0.68	0.23	0.002
13 days	1.84	0.65	0.26	0.001

TABLE III

ANALYSIS FOR OXIDIZABLE INTERMEDIATES
FORMED DURING BIOLOGICAL HALF-CELL GROWTH

Time (days)	pH		Titrations (Milliequivalents) for 10 ml Samples					
	C*1	B*2	Sulfite		Thiosulfate		Polythionates	
			<u>C</u>	<u>B</u>	<u>C</u>	<u>B</u>	<u>C</u>	<u>B</u>
0	4.90	4.80	0.0012	0.0012	0.0022	0.0022	0.022	0.022
1	4.90	2.72	0.0012	0.0017	0.0005	0.0007	0.0190	0.026
2	4.90	2.35	0.0010	0.0010	0.0007	0.0007	0.0190	0.026
3	4.90	2.20	0.0010	0.0010	0.0007	0.0007	0.0190	0.029
6	5.00	1.90	0.0012	0.0015	0.0007	0.0007	0.0190	0.019
8	5.00	1.87	0.0012	0.0015	0.0007	0.0007	0.0190	0.019
9	5.00	1.50	0.0012	0.0015	0.0007	0.0007	0.0190	0.019

*1 Control half cell

*2 Biological half cell

4. Evidence for the accumulation of either sulfite or thiosulfate ions over and above the control concentration which was below 0.00015 meq/ml (Table III) was not obtained.

5. There may have been a slight increase in the polythionate concentration in one biocell (Table III) during the second day of incubation. The concentration went from 0.0022 meq/ml to 0.0026 meq/ml on this day. This change may be a significant increase. The control did not show a similar gain. This polythionate concentration in the biocell subsequently rose to a high of 0.0029 meq/ml by the fourth day and then returned to control levels of 0.0019 meq/ml.

These analytical results show that T. thiooxidans utilization of sulfur gave rise primarily to accumulation of sulfate ion. If other intermediates are formed, their concentration levels are essentially below the limit of these methods of inorganic analyses. Future analyses should be made for these intermediates on the surface or inside of the cell wall of these bacteria since they do not appear to accumulate as metabolic products in the nutrient medium.

V. Electrochemical Observations

Skerman's medium with 1 per cent sulfur was placed in the biological half cell which in turn was set in a constant temperature

bath. The Daystrom-Weston recorder was connected to determine instantaneously changes in the half-cell emf. After an initial observation period which showed the half-cell emf to be constant (the air bubbling into the cell), the air was replaced with pure nitrogen. The top curve in Fig. 2 shows that the presence of pure nitrogen bubbling into the cell had little effect on the half-cell potential during this time period. This curve as well as those that follow are direct tracings from the recorder paper. A similar experiment in which the air in the bubbler was switched to pure oxygen indicated a slight, negligible reduction in the half-cell voltage.

After the Skerman's medium had been inoculated with T. thiooxidans and equilibrated with the air bubbling into the cell, a change from this air to pure oxygen gave an immediate change in the half-cell emf (third graph, Fig. 2). The remaining curves on this page show that a change to air and nitrogen after the pure oxygen has been bubbled into inoculated medium for a time, also gave changes in the biological half-cell emf. Figure 3 shows the reproducibility of the oxygen effect in the inoculated Skerman's medium. These two curves were taken after the biocell's voltage had been stabilized under pure nitrogen approximately 3 hr. Pure nitrogen raised and oxygen lowered the inoculated half-cell emf. The extent of the gas effect under prolonged experimentation was observed

to depend upon the concentration of the inoculum as well as the time period after inoculation. Figure 4 gives an example of initial and subsequent oxygen effects observed in an inoculated Skerman's medium.

The majority of the effects, obtained from using the pure gases, took place over a period of several minutes in the inoculated Skerman's medium. Appreciable oxygen, nitrogen, and carbon dioxide effects on the biological half-cell potential were observed only after the centrifuged, washed T. thiooxidans were placed in the medium. The latter effect, carbon dioxide, was not shown in the graphs; however, it essentially duplicates the effect observed with nitrogen.

~~The effect of changing the composition of the gaseous~~
atmosphere in equilibrium with the biological half cell was found to be somewhat more complex when Skerman's was replaced with ATCC medium. Switching from pure oxygen to nitrogen caused changes in the emf of both control and biological half cells. The changes in emf of the biological half cell were much greater than that observed with the control half cell. The following series of experiments were initiated to individually, and then collectively, consider the degree of the gas effect for each constituent in the ATCC medium.

The large biological half cell with the platinum electrode was filled with approximately 600 ml of sterile distilled water. High voltages of approximately -0.24 v were obtained for a pure nitrogen atmosphere and a low of -0.30 v for pure oxygen atmosphere were obtained with this electrode. The half-cell emf was allowed to stabilize with pure oxygen bubbling into it before the individual constituents of ATCC were added. After the addition of an individual compound, the effects of the pure gases were determined by allowing the voltage to stabilize. This stability was identified by recording voltages until no change in half-cell emf was observed over a 10 min period. Then, the biocell was emptied and filled with fresh sterile distilled water for the next constituent. Table IV summarizes in part the results obtained from these investigations.

These individual constituents make electrochemical contributions which in an oxygen atmosphere may be as high as -0.13 and as low as -0.46 v depending upon which one is selected. Except for calcium chloride and ferrous sulfate, the nitrogen and carbon dioxide effects almost duplicate themselves. Furthermore, it is of interest to note that there was relatively little difference in the nitrogen and oxygen effects except for KH_2PO_4 . To summarize, variations in partial pressures of carbon dioxide may affect the CaCl_2 and FeSO_4 contributions to emf, and variations in the nitrogen-oxygen

TABLE IV

CASEOUS EFFECTS ON ATCC CONSTITUENTS AS MEASURED
BY HALF-CELL EMF

<u>Constituent</u>	<u>Concentration</u> <u>(gm/l)</u>	<u>Stabilized Half Cell EMF</u> <u>Pure Gases, (v)</u>		
		<u>Carbon Dioxide</u>	<u>Nitrogen</u>	<u>Oxygen</u>
MgSO ₄ ·7H ₂ O	0.5	-0.25	-0.20	-0.23
CaCl ₂	0.25	-0.19	-0.10	-0.13
FeSO ₄	0.03	-0.72	-0.41	-0.42
KH ₂ PO ₄	3.0	-0.32	-0.38	-0.46
(NH ₄) ₂ SO ₄	0.2	-0.23	-0.22	-0.24

pressures may affect the KH_2PO_4 contribution to an emf. Each of these compounds and pure water, except for FeSO_4 , contribute electrochemical voltages which were found to be significantly higher in the hydrogen scale series for half-cell potentials than have been observed with T. thiooxidans oxidizing sulfur in the presence of air, i.e., -0.6 to -0.64 v.¹ The half-cell sensitivity to the FeSO_4 and carbon dioxide interaction were part of the reason for eliminating FeSO_4 from the ATCC medium in subsequent studies.

The individual behavior of the constituents in ATCC toward the various gases does not mean that such behavior is carried over in the complex medium. In order to further obtain data on how the half-cell emf may be affected by these constituents, an experiment was conducted in which the half-cell emf was recorded during an arbitrary sequence of adding each constituent to sterile distilled water while bubbling air into the mixture. Table V shows the stabilized voltages that were obtained after the sequential additions, starting with FeSO_4 . During the experiment, the time for the half-cell voltage to stabilize was observed to vary with the individual components. Approximately 47 min was necessary for the FeSO_4 and approximately 3-1/2 hr for the KH_2PO_4 addition to stabilize. These variations in voltage suggest that the initial electrochemical behavior of an ATCC medium may change if the time factor is not

TABLE V

HALF-CELL EMF'S DURING SEQUENTIAL ADDITIONS
OF ATCC CONSTITUENTS (AIR ATMOSPHERE)

<u>Sequential Additions to</u> <u>Distilled Water</u>	<u>Sequential Stabilized Half-</u> <u>Cell EMF's (</u>
FeSO ₄	-0.36
MgSO ₄	-0.33
(NH ₄) ₂ SO ₄	-0.34
CaCl ₂	-0.29
KH ₂ PO ₄	-0.42

sufficient for obtaining stability. This fact may explain some of the variations that have been experienced in the past except for one additional step in the procedure which should contribute towards stability. After the media were prepared and thoroughly mixed, they were autoclaved for sterilization. This step should act as a stabilizing or uniform aging factor.

In general, a review of the stabilized voltages obtained in this sequence shows that the resulting voltages are close together having a low of -0.42 and a high of -0.29 v. The addition of sterile powdered sulfur to the final mixture and subsequent sterilized media, did not change the electrochemical potential of the media in the half cells.

An assessment of the previous experimental results directed a study of changes in atmospheric composition in equilibrium with the ATCC medium without its ferrous sulfate constituent, ATCC(-). Figure 5 (top) shows that the short and long term operation of the sterile ATCC(-) medium under an atmosphere of pure nitrogen resulted in a relatively small and negligible change in half-cell emf as compared to air. After this determination, the nitrogen in the sparger was switched to oxygen (Fig. 5, bottom) and again a negligible change in half-cell emf was observed for the short and long term treatment. Thus, this medium responded to these two pure gases like the Skerman's and not like the original ATCC medium.

The next experimental sequence involved studying the specific gas effects on the inoculated ATCC(-) medium. Figure 6 shows that the presence of T. thiooxidans utilizing sulfur causes an instantaneous decrease in the half-cell emf when the air in equilibrium with the cell was changed to pure oxygen. This effect was similar to those observed in both the Skerman's and ATCC media. The half-cell voltage, after 1, 2, and 4 hr, was higher than its original value and appeared to stabilize at -0.30 v. Similar treatment with the inoculated Skerman's medium reduced the half-cell voltage and caused it to remain below its original value.

In another inoculated biological half cell (Series LII) that had been operating under a normal air atmosphere, the air was changed to pure nitrogen (Fig. 7). The short term effect of this change was not noticeable on the half-cell emf; however, the longer term effect, 1 and 5 hr, was appreciable. After this half-cell emf had stabilized in the presence of pure nitrogen, a change to pure oxygen was made (Fig. 8). In a relatively short period of time, the half-cell voltage dropped to a value slightly below that experienced in Series XLVII (Fig. 6). After 2 hr this stabilized half-cell emf obtained from this switch to oxygen (i.e., after a long term treatment with pure nitrogen) appeared to be different than

the oxygen treatment of the air stabilized biological half cell (Series LXVII). The latter gave a value of -0.35 v which was above its initial cell emf while the former (Series LII) gave a value which was lower, -0.55 v. Thus, the prolonged treatment with nitrogen on this biological half cell (Series LII) suggests that a significant change from its original character took place.

VI. Discussion

The analysis for the existence and accumulation of partially oxidized sulfur intermediates did not reveal any concrete evidence for explaining the electrochemical contribution that T. thiooxidans appears to make when added to sterile media. If such intermediates exist, their concentrations are at or below the limit of analyses developed in this study. Other sources, such as the surface or inside of the T. thiooxidans' cell wall may make electrochemical contributions. Justification or encouragement to explore such possibilities exist from this study. The investigations on individual and combined electrochemical investigations on the compounds comprising the ATCC and ATCC(-) identify their electrochemical activity. The activity of these ions reflected by their half-cell emf's especially with the ferrous sulfate eliminated from the nutrient, is higher in the hydrogen electromotive series scale than experimentally observed during

active biological half-cell operation with T. thiooxidans. Thus the fact that this microorganism by its presence makes an electrochemical contribution to the half cell is further substantiated through these investigations.

The greater sensitivity of the biological than the control half-cell potential to changes in the oxygen content of the atmosphere and nutrient further suggests that viable T. thiooxidans is electrochemically active. The actual individual or group of oxidized and reduced compounds contributing to this electrochemical activity must still be isolated.

Acknowledgment

The authors are indebted to Dr. _____
_____ } who acted as consultants
for identifying the electrochemical characteristics associated with
T. thiooxidans utilizing sulfur.

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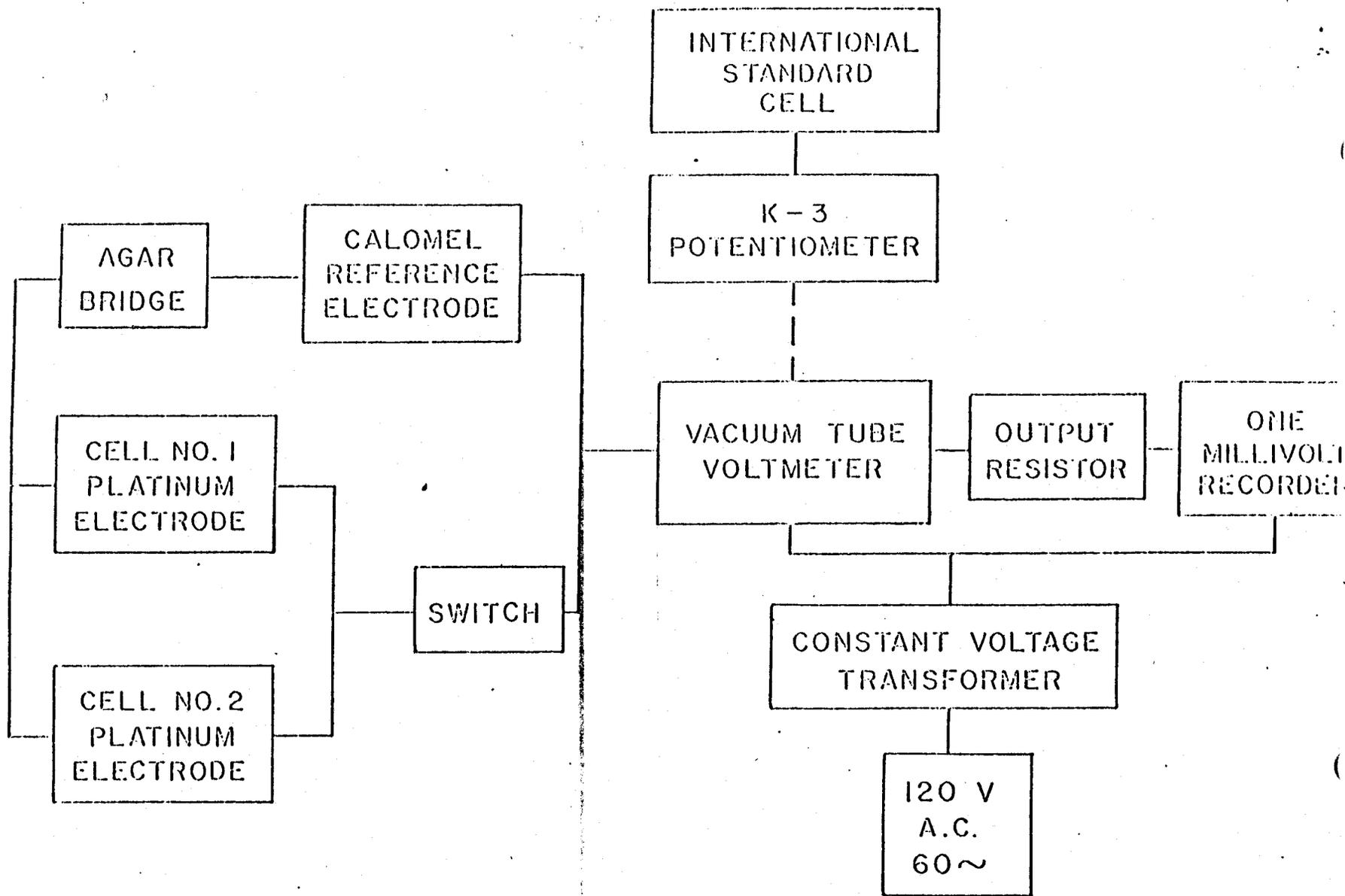
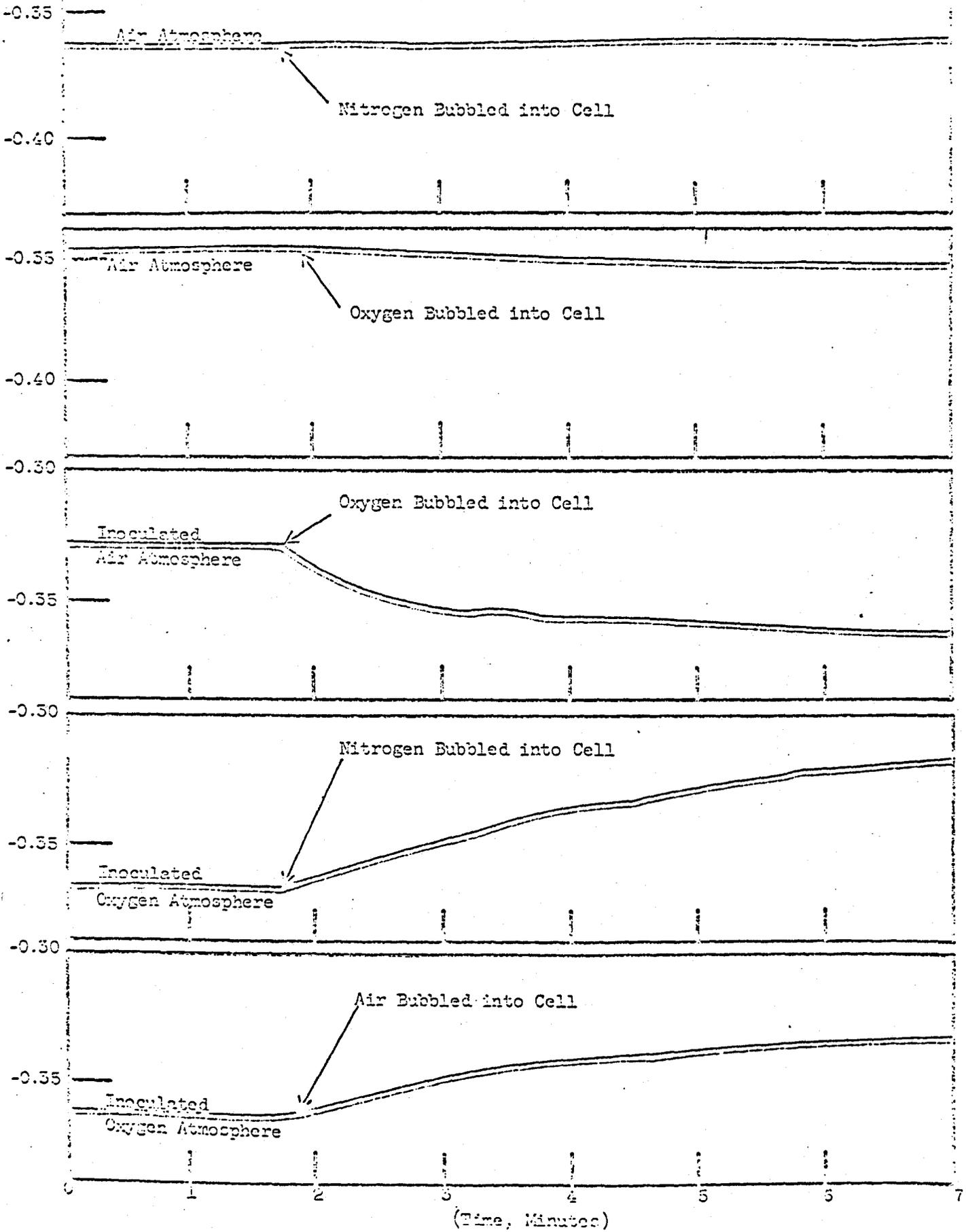


Fig. 1 - Block Diagram for EME Circuitry

and Inoculated Sherman's Medium



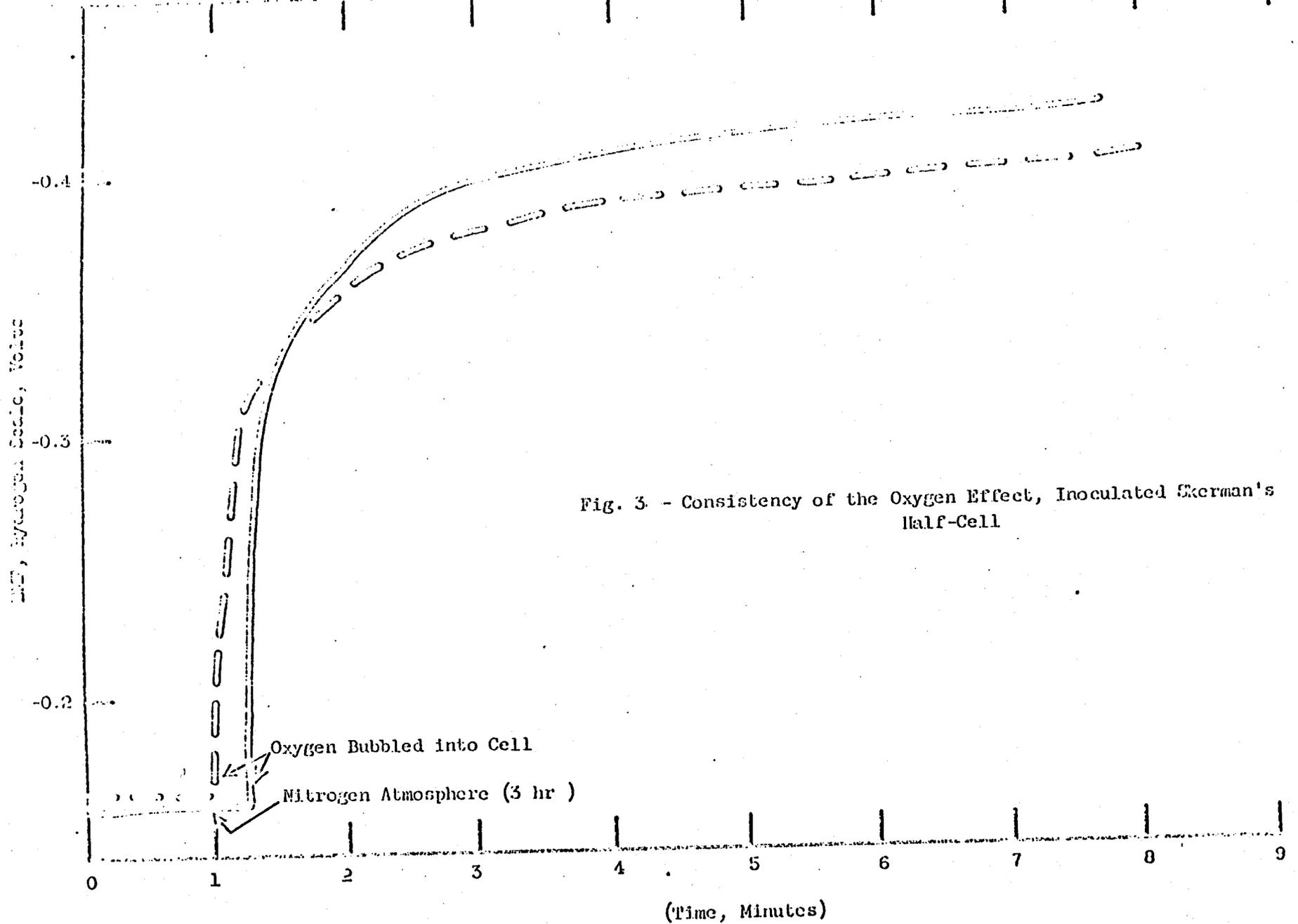
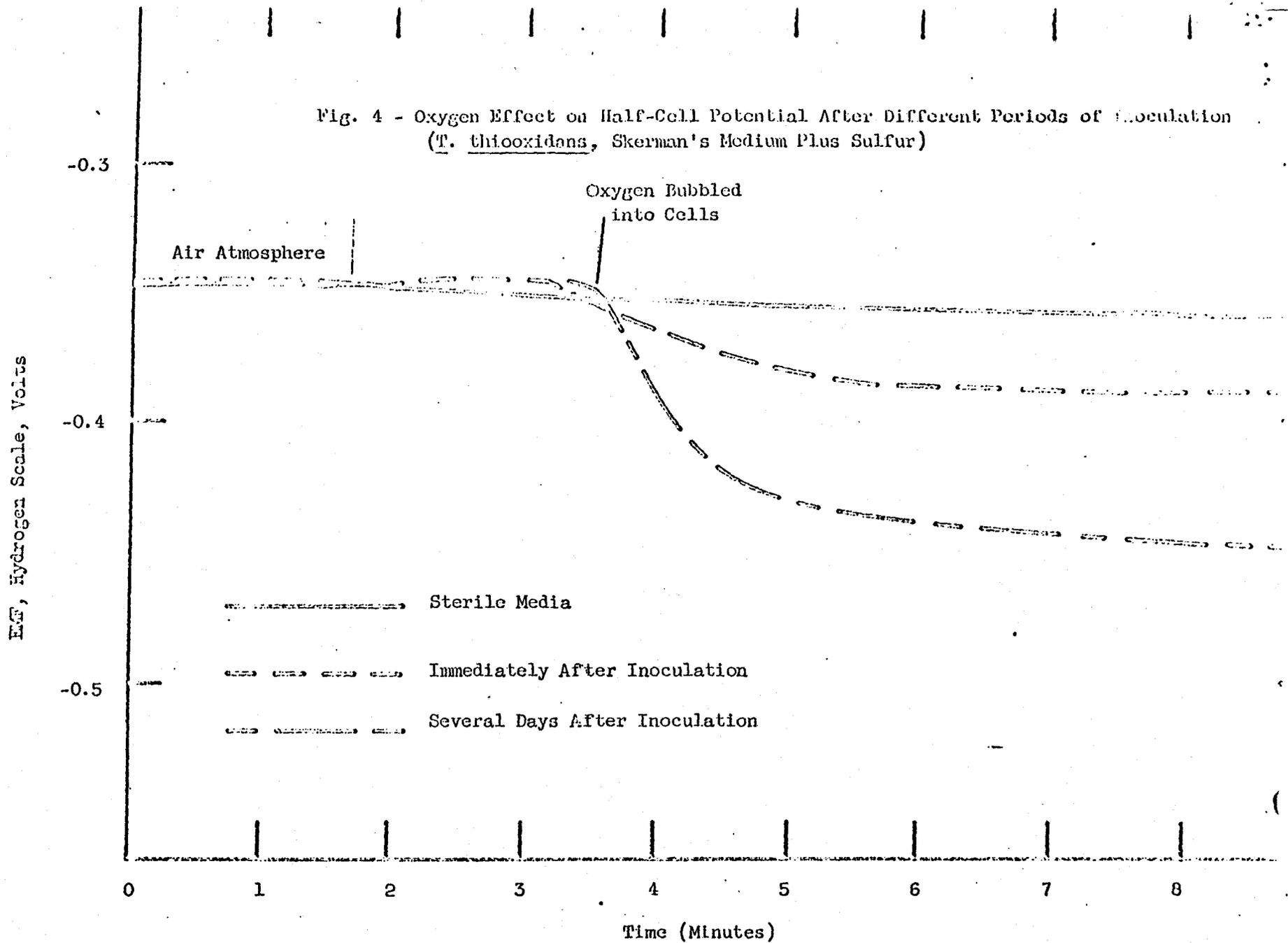


Fig. 3 - Consistency of the Oxygen Effect, Inoculated Skerman's Half-Cell

Fig. 4 - Oxygen Effect on Half-Cell Potential After Different Periods of Inoculation
(*T. thiooxidans*, Skerman's Medium Plus Sulfur)



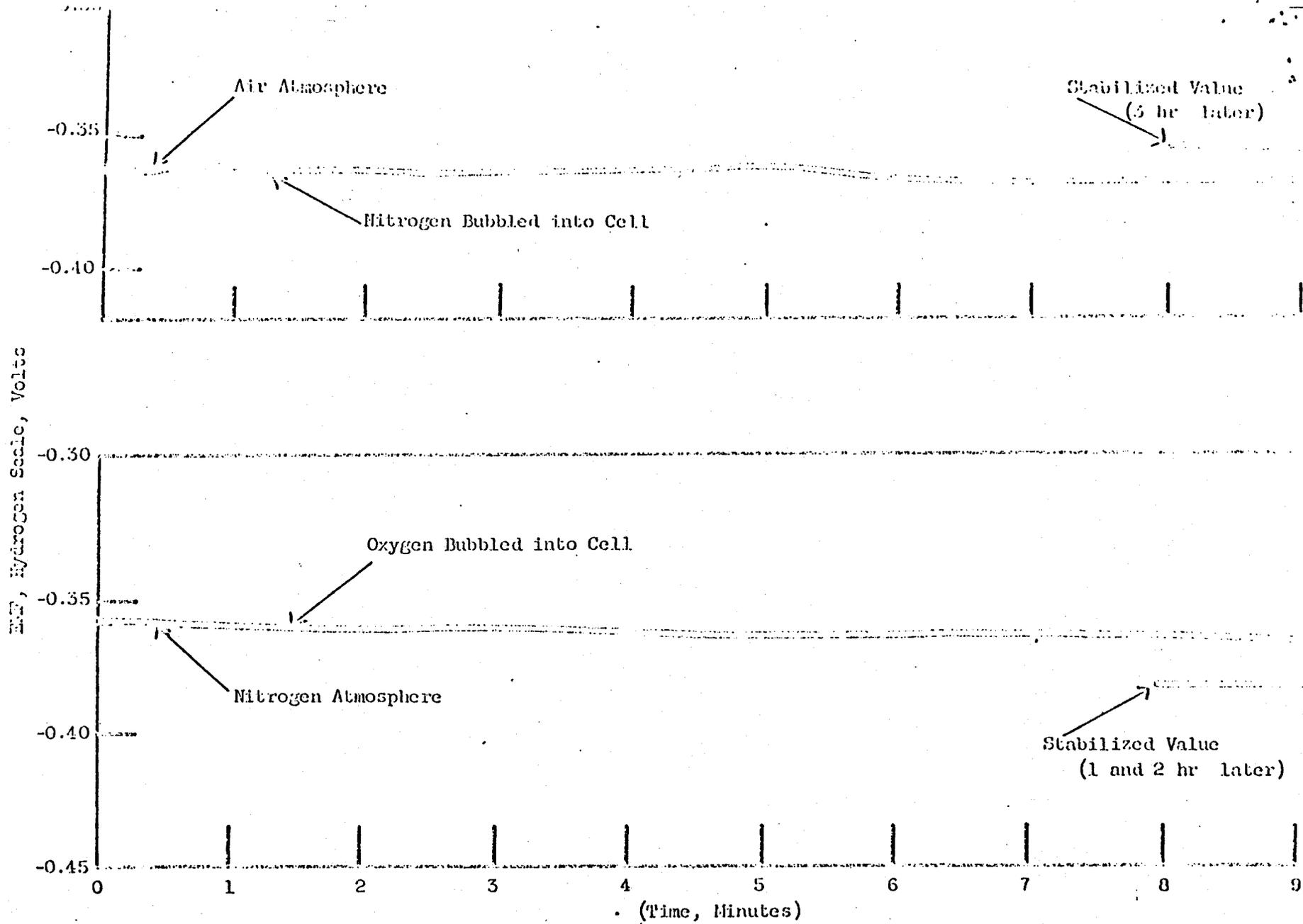


Fig. 5 - Gas Effects in Sterile A.T.C.C.(-) Medium

Fig. 6 - Oxygen Effect in Inoculated A.P.C.C. (-) Medium
(Series XLVII, Age - One Month)

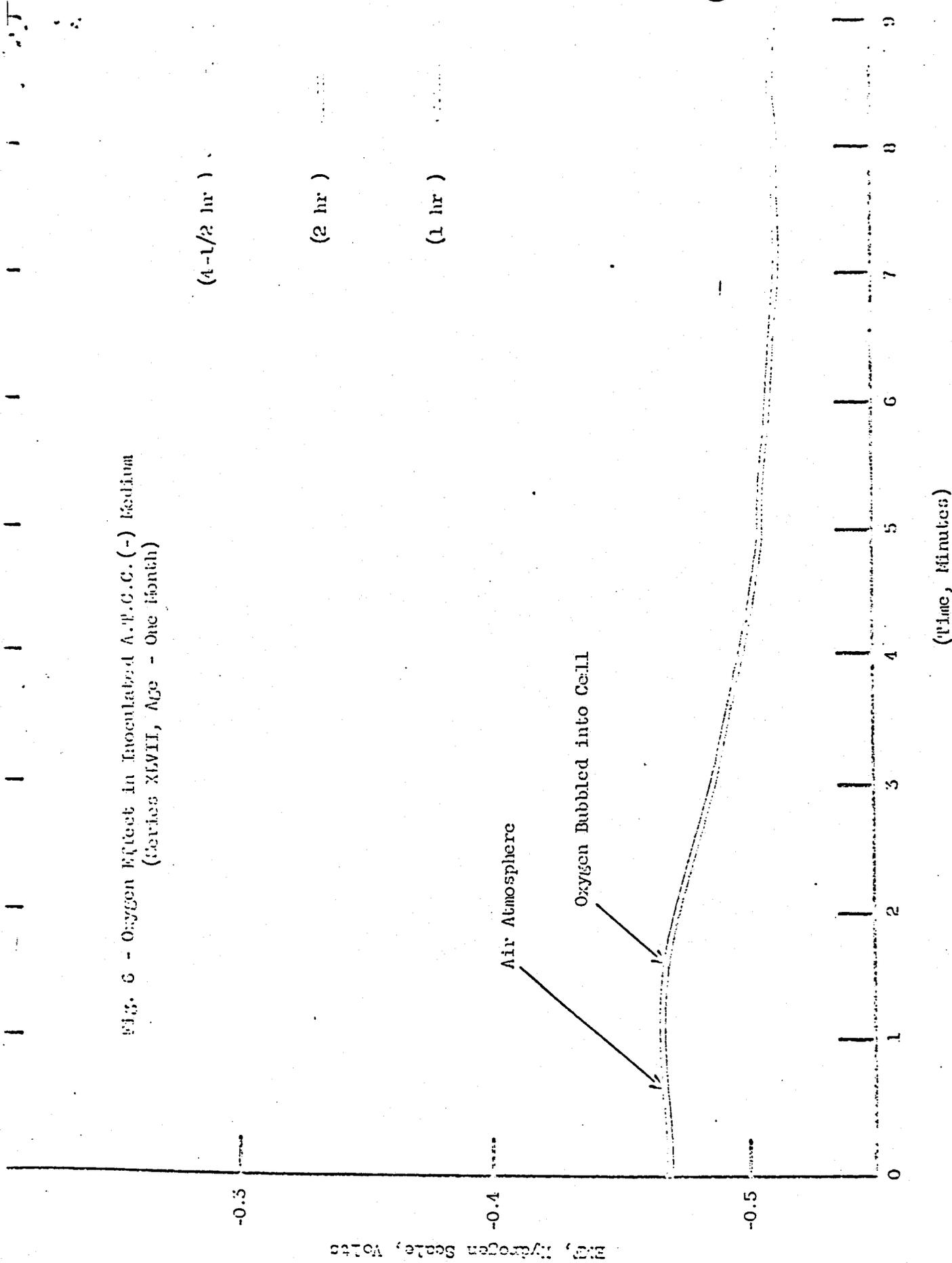
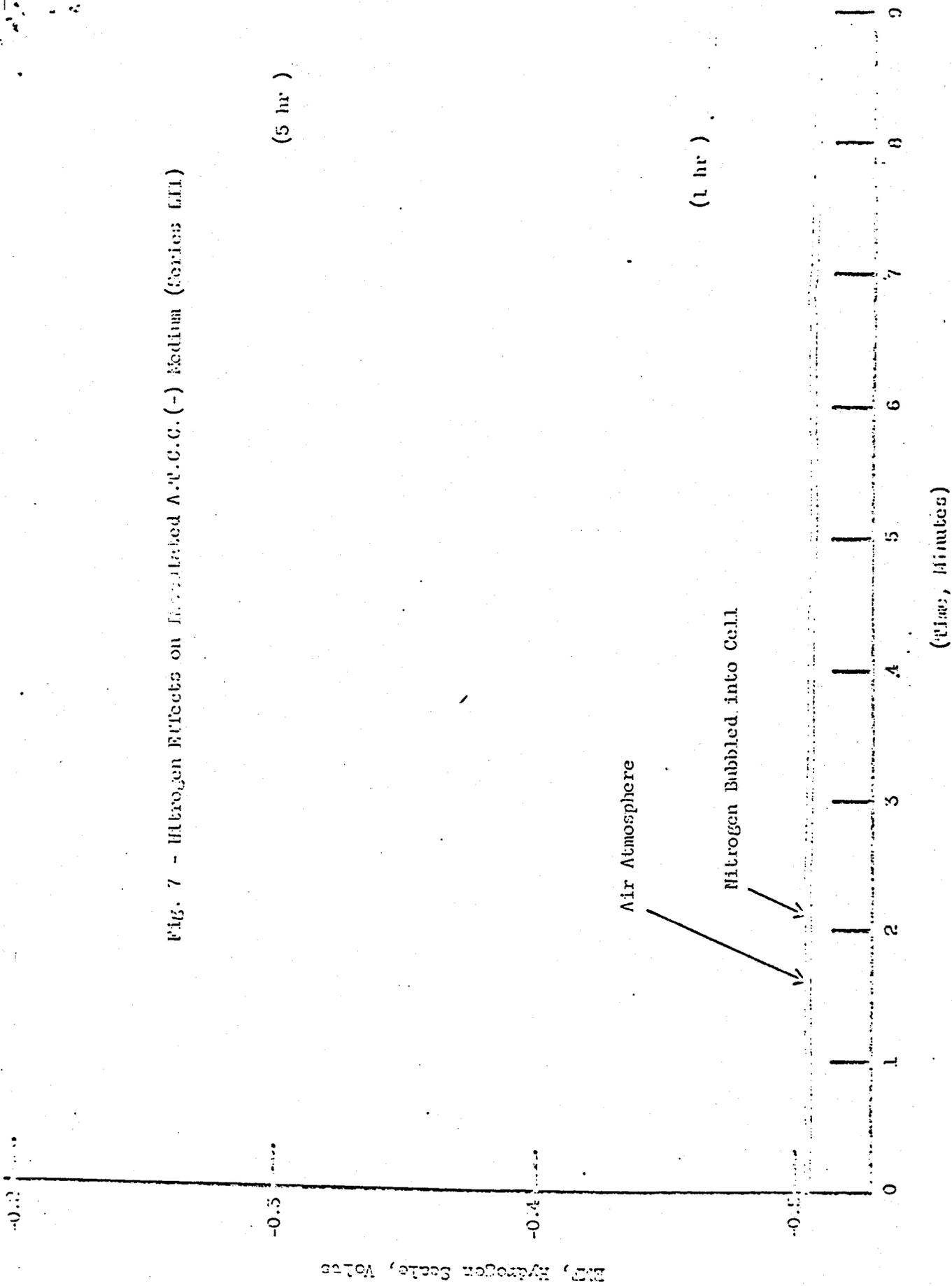


Fig. 7 - Hydrogen Effects on Irradiated A.T.C.C. (-) Medium (Series LII)



(5 hr)

(1 hr)

Air Atmosphere

Nitrogen Bubbled into Cell

(Time, Minutes)

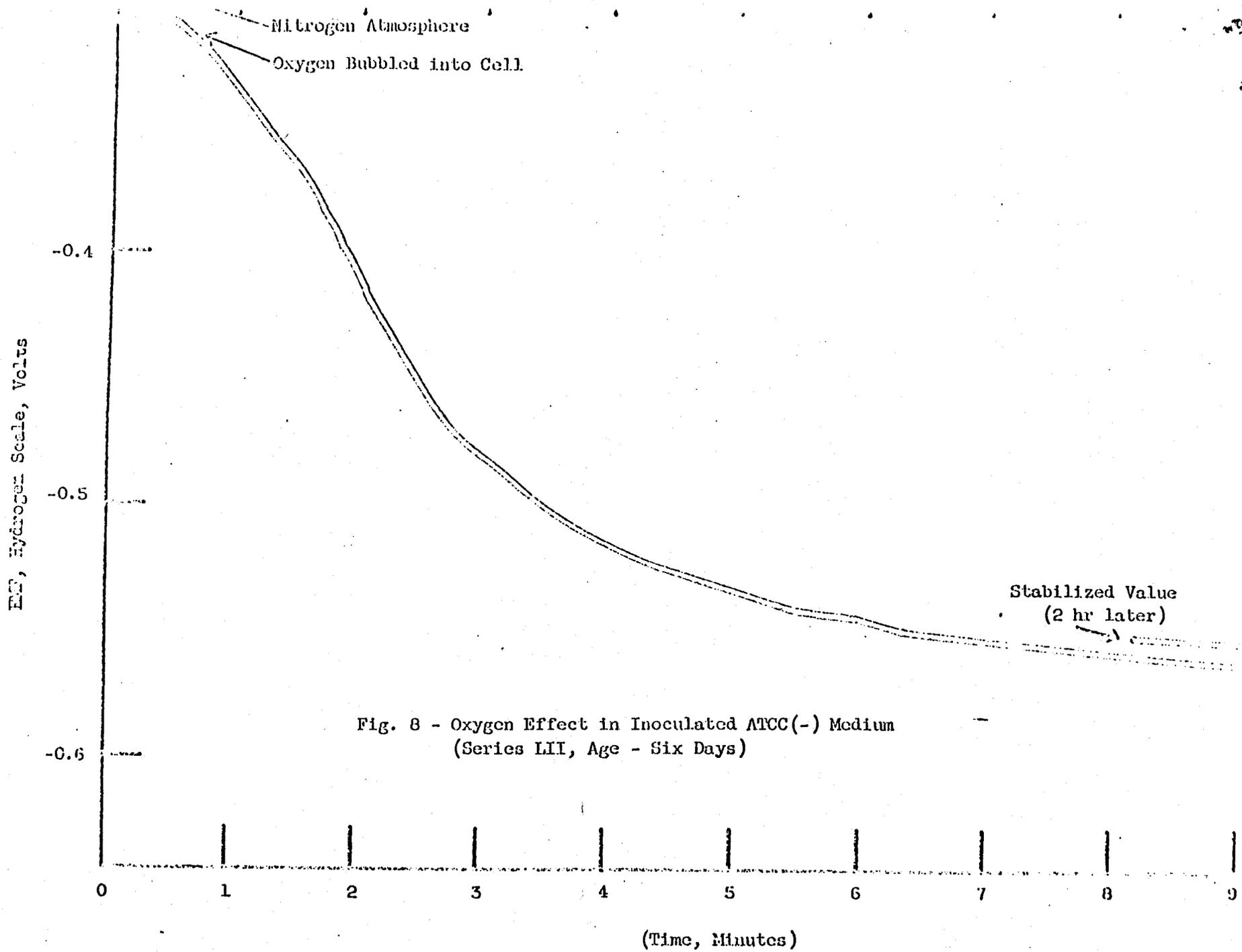


Fig. 8 - Oxygen Effect in Inoculated ATCC(-) Medium
(Series LII, Age - Six Days)