FINAL REPORT

Nonr-G-0006-60

THE BIOCHEMISTRY AND PHYSIOLOGY OF NITRIFYING BACTERIA

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by

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Papers describing research

Silver, W. S. and D. J. D. Nicholas
"Nitrification"
Bacteriological reviews. In preparation.

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"Kinetic studies of a chemical reaction between ferrocytochrome c and nitrite"
In preparation.

Introduction

Previous work in this laboratory has established that washed cell suspensions of Nitrobacter can oxidize formate since the addition of this substrate caused the appearance of reduced cytochrome bands in the reversion spectroscope and oxygen uptake in the Warburg respirometer (see Progress Reports Nonr-2561(00)). One of the main goals of the grant with which this report is concerned was to compare the labling patterns from $C^{14}O_2$ and $HC^{14}OOH$. This study was not successfully completed due to cultural difficulties (see I below).

A rapid recording platinum microelectrode was devised with the ultimate aim of using this device for studies of oxidative phosphorylation. The device was successfully used for observing nitrite oxidation by washed cells, but again cultural problems prevented full exploitation of the apparatus.

Further studies have been made and are continuing on the model reaction between ferrocytochrome c and nitrite. Preliminary observations of kinetics have been conducted and it appears to be feasable to detect nitric oxide directly by electron spin resonance technic (ESR).

I. Cultural Problems

A critical factor that has consistently plagued those of us who work with nitrifying bacteria has been to obtain and maintain pure cultures. Although certain biochemical experiments can be properly conducted with mixed cultures when the contamination level is quantitatively known (for example see Lees and Simpson, 1957), this is certainly not the case when the metabolic pathway of interest is not unique to the organism in question. In this laboratory it has been observed that washed cells of Nitrobacter, whether in the pure state or containing approximately 10% contamination (on a count basis), oxidize nitrite stoichimetrically to nitrate according to the equation $NO_2^- + 1/2\ O_2 \rightarrow NO_3^-$. It would seem then that investigations of this oxidation with whole cells could be conducted with mixed cultures without a great likelihood of being led astray. This would not necessarily be the case if enzyme preparations were employed in an attempt to elucidate the enzymatic mechanism of the oxidation for the contribution of the contaminant as a source of unrelated cofactors or enzymes could readily lead to misleading artifacts. Whether this has been considered in certain data in the literature on oxidative phosphorylation in Nitrobacter is equivocal (Aleem and Nason, 1960).

Initial studies in this laboratory were conducted with an apparently pure culture of Nitrobacter which was isolated from soil by a combination of antibiotic treatment and dilution to extinction. Purity was established by testing the isolatant on a wide variety of media of appropriate types in a manner that would have detected 10 contaminants per ml. in a population of

approximately 10^8 autotrophs. For a period of some six months there was no evidence for contaminants in the stock cultures although some large batch preparations (28 l.) did become contaminated. Subsequently, contamination of a type not previously seen in early enrichment cultures appeared at varying concentrations, and this contamination has to date not proven amenable to elimination by methods previously used for the initial successful isolation as well as by plating on washed agar mineral media.

When it became apparent that this culture could not be used for planned studies of $C^{14}O_2$ fixation, attempts were made to obtain better culture from other investigators. Two subcultures of the Lees strain of Nitrobacter were kindly supplied by Dr. Lees, University of Manitoba. One of the tube cultures was cautiously subcultured in a transfer hood under stringent aseptic conditions and initial testing of the contents of the original tube failed to reveal contamination. However, upon scaling up the culture to 250 ml. and 1000 ml. shake flasks, all cultures, as well as tube subcultures from the original culture were contaminated. Similar results were obtained with cultures established from the duplicate tube received from Dr. Lees. Thus, in our hands, the Lees strain has proven impure and the purity of the strain must be deemed equivocal.

Subcultures were also requested from Dr. M. Alexander, Cornell University and Dr. G. A. Zavarzin, Institute of Microbiology, Academy of Sciences, Moscow, U. S. S. R., since both of these investigators claim to have done their experiments with pure cultures. The Cornell strain was never received and

Dr. Zavarzin has informed me that it is the practice of his institution not to provide cultures for distribution. Thus, I have been unsuccessful in obtaining a pure culture of Nitrobacter and have had to abandon the planned comparative study of labeling patterns from $C^{14}O_2$ and $HC^{14}OOH$.

It is worthy of mention that when washed cell masses of the Lees strain were prepared (2% contamination in this batch) and the cells observed in the Hartridge reversion spectroscope, formate, as well as nitrite, caused the immediate appearances of reduced cytochrome bonds at 551 mμ. Thus, the previous report from this laboratory that formate is an oxidizable substrate for Nitrobacter has been confirmed, (Silver, 1960). It is unfortunate that a pure culture is not available for it is logical to expect that very large crops of Nitrobacter should be obtainable from culture media containing HCOOH, NO_2^-, and perhaps small quantities of NO_3^-. This very important point should be tested when such a culture is available.

It may be stated then that it is pointless to proceed with studies of C^{14} and N^{15} labeling patterns to determine the metabolic paths involved in carbon and nitrogen metabolism until a more suitable culture is available.

II. Determination of nitrite oxidation with a rapid recording platinum microelectrode

Since manometric assays of nitrite oxidation by whole cells and extracts of Nitrobacter are too insensitive to reveal any details of the early kinetics of the reaction (see Progress Reports of Nonr 2561(00) June 30, 1959 and December 15, 1959) it was deemed desirable to devise other methods for kinetic studies. Such instrumentation would be especially useful since a much smaller cell mass would be required for analyses than for conventional manometric technics.

A survey of the literature on such instrumentation indicated that the Davies and Brink stationary platinum microelectrode (Davies and Brink, 1942) should be readily adaptable to use with bacterial cells, and that Packers adaptation of this instrument would be useful for studies not only of nitrite oxidation but also of concomitant oxidative phosphorylation (Packer, 1958). A circuit for the polarizing voltage and sensitivity control was kindly provided by Dr. Packer and the device was constructed in the standards shop of the Department of Electrical Engineering. It was found, however, that Dr. Packer's circuit could not be used without modification under our instrumental conditions. The final circuit devised is shown in Figure 1;[1] and the complete apparatus in Figure 2. It should be noted in the latter figure that it was necessary to install a voltage divider between the D.C microvoltmeter and the chart recorder for proper pen response.

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[1]The invaluable assistance of Cmdr. A. R. McMullen, USN Ret. in working out this circuit is gratefully acknowledged.

The use of the instument for measuring oxygen uptake by cells suspensions was first tested with Pseudomonas aeruginosa in order to conserve the less readily obtainable Nitrobacter cell suspensions. A typical determination is shown in Figure 3. It should be noted that for a given cell suspension there is good reproduceability for successive runs. This is not true for determinations from day to day; thus, the system must be calibrated for each experiment.

A similar experiment conducted with washed Nitrobacter cells is shown in Figure 4. The endogenous respiration was extremely low and the addition of 5μm of $NaNO_2$ caused an immediate, marked, increase in oxygen uptake. The relative rates of respiration may be estimated from the slopes of the curves: endogenous, 0.1; + nitrite, 1.07. The endogenous respiration is probably due to contaminants known to be present in the culture. A grossly contaminated culture (see Section I) prevented further experimentation intended to measure oxidative phosphorylation concomitant with nitrite oxidation. The apparatus is of considerable general value and is currently being used in other work in this laboratory (NIH-RG-7991 and NIH-RG-8577)

III. Cytochrome c - nitrite reaction as a model system of nitrite metabolism

This laboratory has already reported some preliminary data on a very interesting *in vitro*, pH dependent, reaction between ferrocytochrome c and nitrite (see Previous Progress Reports of Nonr-2561(00) and Silver, 1961). This chemical reaction is of considerable interest for several reasons: (1) Small amounts of nitrite inhibit the respiration of heterotrophic microorganisms at an acid pH within seconds. (2) The rapid onset of inhibition of respiration suggests that a respiratory enzyme is involved. (3) The nitrite oxidase system of *Nitrobacter* is intimately linked with cellular cytochromes. (4) Nitric oxide (NO), an apparent intermediate of the reaction, has been recently implicated as a key intermediate in a variety of metabolic systems involving inorganic nitrogen (Fewson and Nicholas, 1960). (5) *Nitrobacter* oxidizes nitrite slowly at hydrogen ions concentrations which were markedly inhibitory for all other organisms tested. Thus, it was reasoned that further investigation of the model system might lead to a better understanding of the interaction of nitrite with cellular enzymes of the nitrifier as well as of heterotrophs.

The reaction sequence postulated for the ferrocytochrome c - nitrite system was as follows:

(1) Fe^{++} cyto c + NO_2^- + $2H^+$ -- Fe^{+++} cyto c + NO + H_2O

(2) NO + 1/2 O_2 -- NO_2

(3) Fe^{++} cyto c + NO_2 -- Fe^{+++} cyto c + ?

(4) Fe^{+++} cyto c + NO -- Fe^{+++} cyto c - NO complex

Thus far, there has been no direct evidence that free NO is an intermediate; however, NO in the form of ferrocytochrome c complex has been detected spectrophotometrically.

One method that offers promise for the detection of free NO is the Electron Spin Resonance technic (Ingram, 1958). In a system containing Fe^{++} cytochrome c, NO_2^-, Fe^{+++} cytochrome c, Fe^{+++} cytochrome c - NO complex, NO_2 and NO, the paramagnetic components would be Fe^{+++} cytochrome c, NO and NO_2. The latter substance would not be formed in an anaerobic system and NO should accumulate. It is anticipated that the g factors of NO and Fe^{+++} cytochrome c would be quite different, and that therefore the resonant field strengths sufficiently different to yield a good separation of the two spectra. The two signals should be readily identifiable for NO would give characteristic 1, 1, 1 triplet. These experiments which are to be carried out in collaboration with Dr. M. T. Emerson, Institute of Molecular Biophysics, the Florida State University, Tallahassee, have been temporarily delayed until he receives a special sample tube which is required for acqueous systems. There is every reason to believe that the experiment can be successfully carried out.

An experiment was done to assay the effect of nitrite concentration on the rate of fall of the 500 mμ maximum of ferrocytochrome c. The experimental protocol and results of an experiment conducted at pH 5.6 and 6.0 are shown in Figure 5. It may be noted that at the higher pH there was doubling of the decrease in absorption at 550 mμ for every doubling of (NO_2^-), but that this was not so at pH 5.6 over the wider range of nitrite concentrations tested. Inspection of the original traces revealed that at $(NO_2^-) \leq 30$ μm the reaction

was first order with respect to nitrite concentration. The significance of this is not clear.

It should prove interesting to compare the rate of appearance of NO, a postulated immediate product of the reaction, with the disappearance of ferrocytochrome c. Detailed kinetics of the reaction will probably only be meaningful when reaction (1) above is studied in a system divorced from subsequent reactions. This should be the case in an anaerobic system studied by the ESR method. Further experimentation along these lines is now underway.

IV. Conclusions and Summary

It was not possible to complete the original primary task of this project--namely to study the path of $C^{14}O_2$ and $HC^{14}OOH$ metabolism by Nitrobacter, because the culture isolated in this laboratory became contaminated and all efforts to effect a purification failed. Not only was it impossible to obtain a pure culture from other laboratories but the question as to whether there are any pure cultures of this organism, despite claims to the contrary, remains open.

It was possible to assay nitrite oxidation by a rapid recording platinum microelectrode device assembled in this laboratory. In impure culture it was found that the addition of nitrite to washed cell suspensions caused a rapid increase in oxygen uptake within the resolution time of the instrument (1 sec.). Thus the method, coupled with P^{32} incorporation, would be invaluable for studies of oxidative phosphorylation if cultures of high purity were available.

Further study of a chemical reaction between ferrocytochrome c and nitrite has been made. There is considerable indirect evidence that this reaction may be a model system of the reaction of nitrite with cellular constituents in heterotrophic microorganisms. The reaction was apparent first order at low nitrite concentrations, but seemed to be second order at 50 μm NO_2^-. There is good reason to believe that experiments planned to detect NO by ESR methods should prove invaluable in clarifying the nature of the intermediate products of this model system.

Addendum

Thesis work of Eugene C. Sommer

When difficulty in maintaining pure cultures of *Nitrobacter* was encountered it was apparent that a research problem with this organism would not be appropriate for the graduate student assigned to the grantee. Accordingly, this Master of Science candidate, Mr. Eugene C. Sommer, was assigned a problem relating to an interesting, blue pigmented *Pseudomonas* isolated in this laboratory. This research problem proved to be better suited to both the academic background of the student as well as the time available for the thesis problem. Since Mr. Sommer's summer salary was supported with funds from Nonr-G-0006-60, a brief summary of his research, exerpted from his thesis, is appended below. This research is described in more detail in the following:

Sommer, E. C. 1961
"Studies of a blue pigmented *Pseudomonas*," M. S. Thesis, University of Florida.

Sommer, E. C., W. S. Silver, and L. C. Vining
"Studies of pigmentation by *Pseudomonas indigofera*."
Can. J. Microbiol., 7, In press.

SUMMARY

The characteristics of a blue Pseudomonas were studied with the purpose of identifying both the pigment and the organism. The organism was found to be identical to Pseudomonas indigofera on the basis of a comparison of the physiological and morphological characteristics of the isolate to three cultures of Pseudomonas indigofera. The pigment of the isolate appeared to be similar to indigoidine on the basis of spectral data; however, no conclusive evidence was obtained and the possibility still remains that the pigment of the isolate could be a closely related compound.

Physiological studies included the effect of temperature, pH, versene, substrate, and gaseous atmosphere on growth and pigmentation; the effect of carry-over as an initiation for pigmentation was also studied. An inverse relation between the amount of yellow and blue pigments present was noted suggesting that the yellow substance might be a precursor of the blue pigment. A synthetic medium for the production of uniformly blue pigmented colonies was defined as a result of nutritional studies.

A study of the chemical nature of the pigment included a study of solubility, spectral, oxidation-reduction behavior, and other miscellaneous properties. All of the studies favored the identity of the pigment with indigoidine. A culture of the isolate was deposited with the American Type Culture Collection

Figure 1

POLARIZATION AND GAIN CONTROL

CIRCUIT FOR PLATINUM MICROELECTRODE

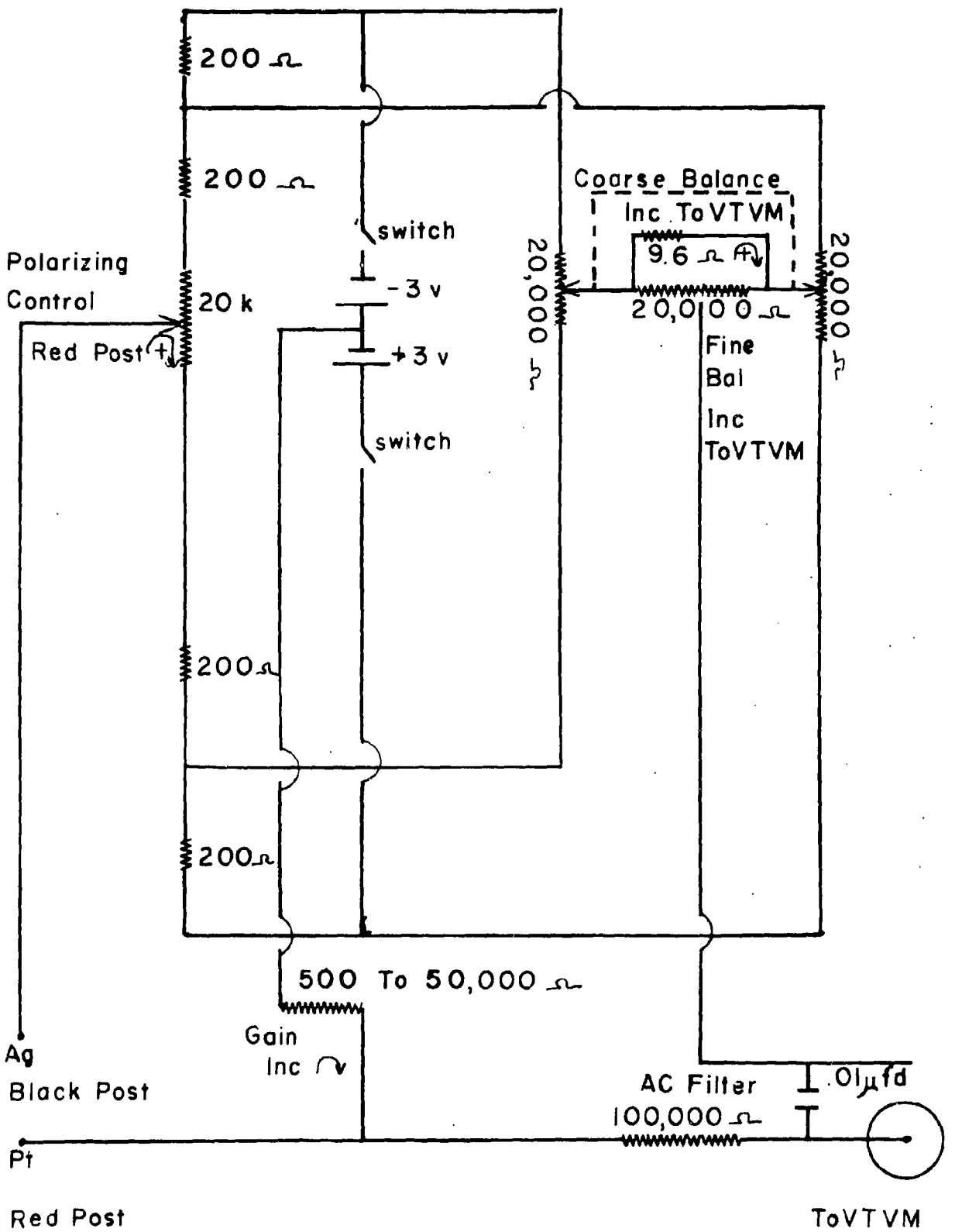

200 Ω
200 Ω
switch
Coarse Balance
Inc ToVTVM
9.6 Ω
20,000 Ω
Polarizing
Control
20 k
Red Post
-3 v
+3 v
switch
20,000 Ω
20,000 Ω
Fine
Bal
Inc
ToVTVM
200 Ω
200 Ω
500 To 50,000 Ω
Gain
Inc
Ag
Black Post
AC Filter
.01 μfd
100,000 Ω
Pt
Red Post
ToVTVM

Figure 2

SCHEMATIC DIAGRAM OF COMPLETE

APPARATUS FOR RAPID DETERMINATION OF RESPIRATION

A. Constant speed motor, 60 rpm

B. Nylon cuvette holder for 3 ml beaker

C. Ag, AgCl anode

D. Pt cathode: 25 μ Pt wite sealed flush in soft glass. Mercury contact to shielded cable.

E. Polarization and sensitivity control

F. Millivac D.C. microvoltmeter, model MV-27D

G. Voltage divider

H. Texas Instruments" "Serviriter" model PSR 1mv. (1 mv. full scale) with foot switch controlled marker pen.

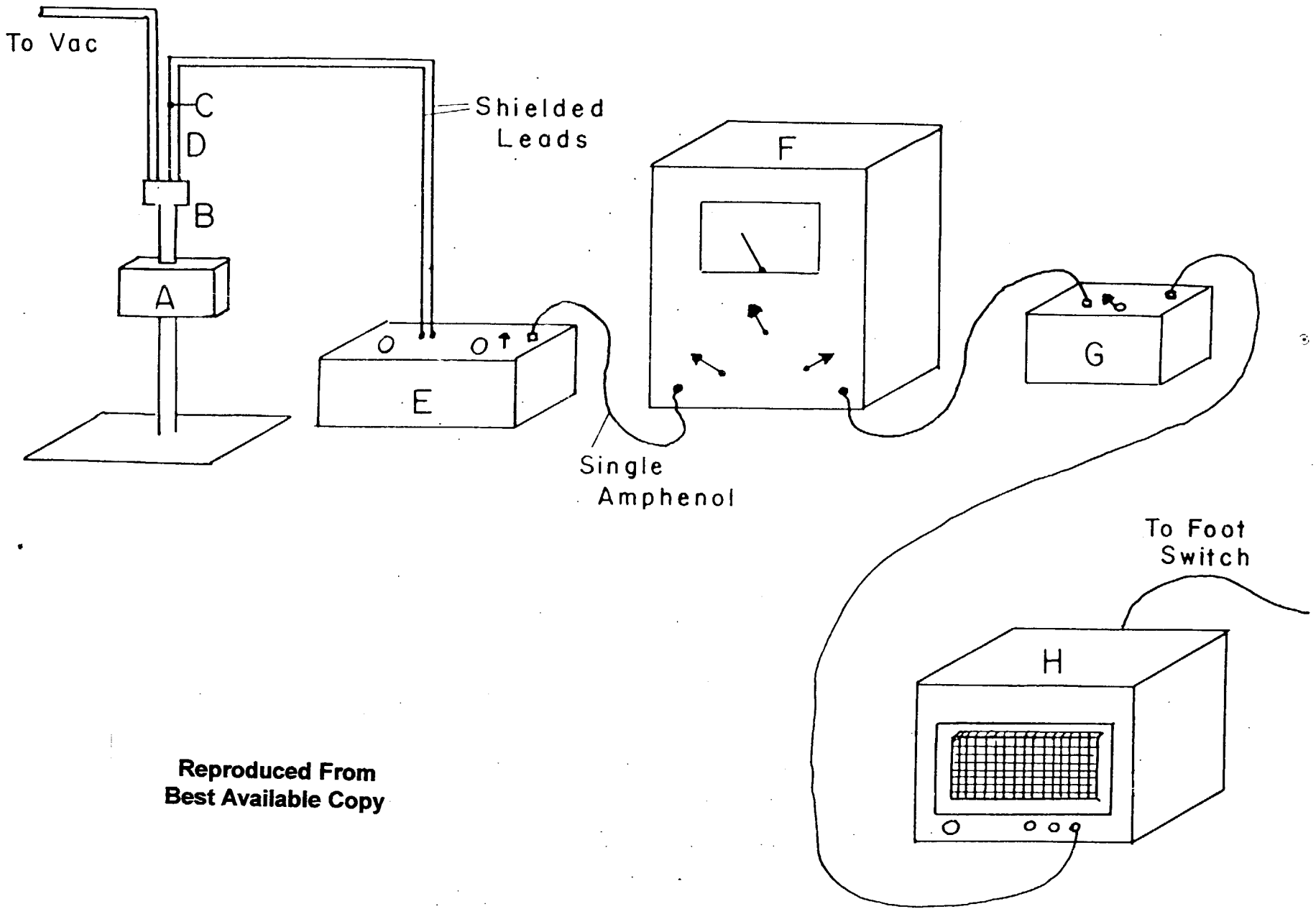


Figure 3

RAPID, MICROELECTRODE MEASUREMENT OF
OXYGEN UPTAKE BY CELLS SUSPENSIONS
OF PSEUDOMONAS AERUGINOSA

Protocol

Washed cells in phosphate buffer pH 7.0; 2ml

Chart speed 6 inches/min.

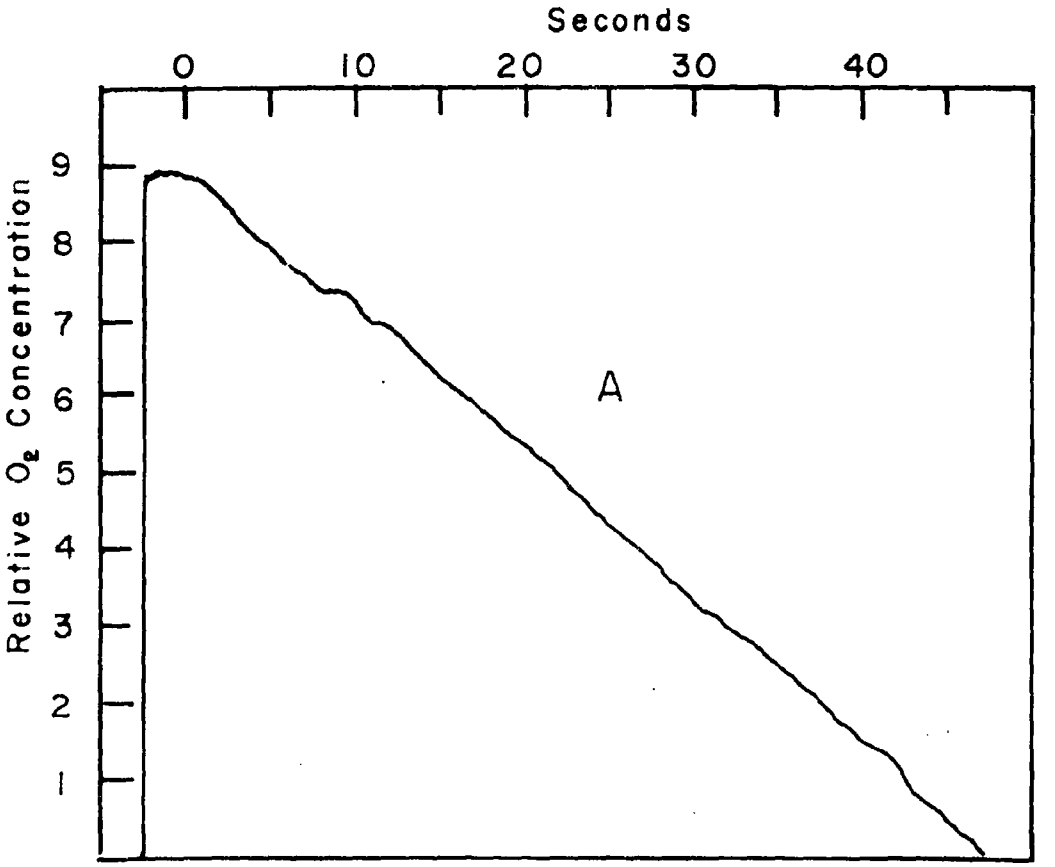
Fine balance 2.73

Polarization voltage 0.6 v.

Microvoltmeter 0.25 mv.

Attenuator 45Ω (out/in=1/10)

Since the endogenous respiration was very high, no substrate was added. Note that the slopes of the two determinations with the same cell suspensions are essentially identical (A: 1.06, B: 1.09). This is a copy of the original chart trace.


Seconds
0
10
20
30
40
Relative O_2 Concentration
9
8
7
6
5
4
3
2
1
A

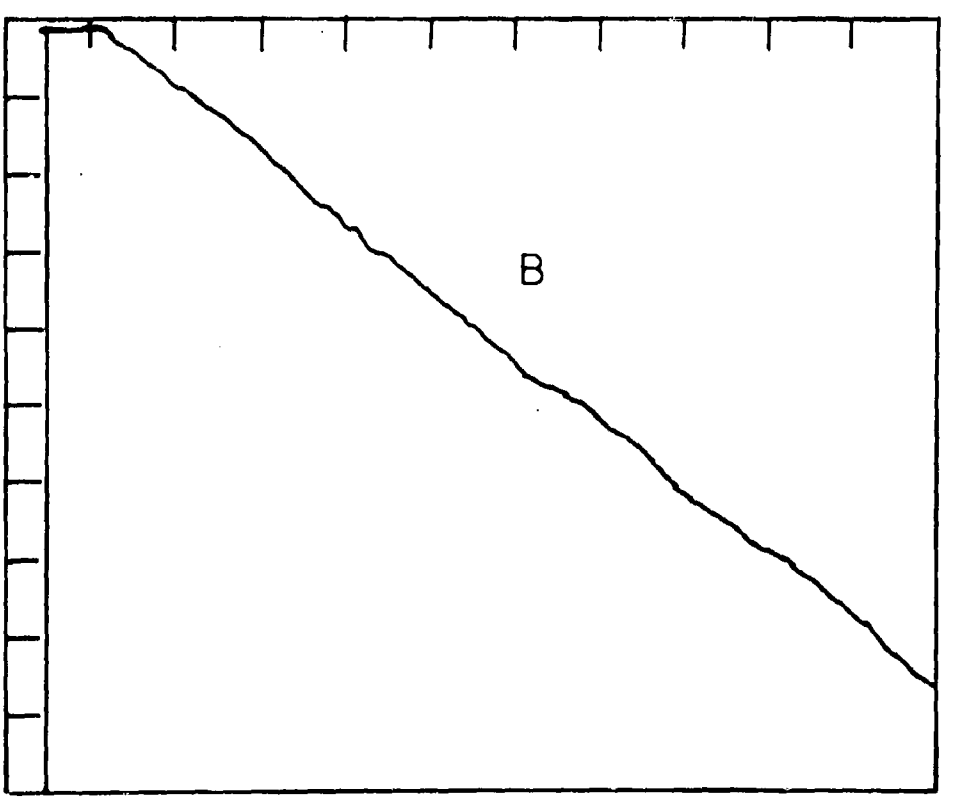

B

Figure 4

RAPID MICROELECTRODE MEASUREMENT OF
OXYGEN UPTAKE BY CELL
SUSPENSIONS OF NITROBACTER WINOGRADSKYI

Protocol

Conditions same as those of Figure 3 except chart speed was 3 inches/min.

Note

The cell suspension contained approximately 20% (by dilution count) contamination. The endogenous respiration is probably due to this contaminant rather than Nitrobacter.

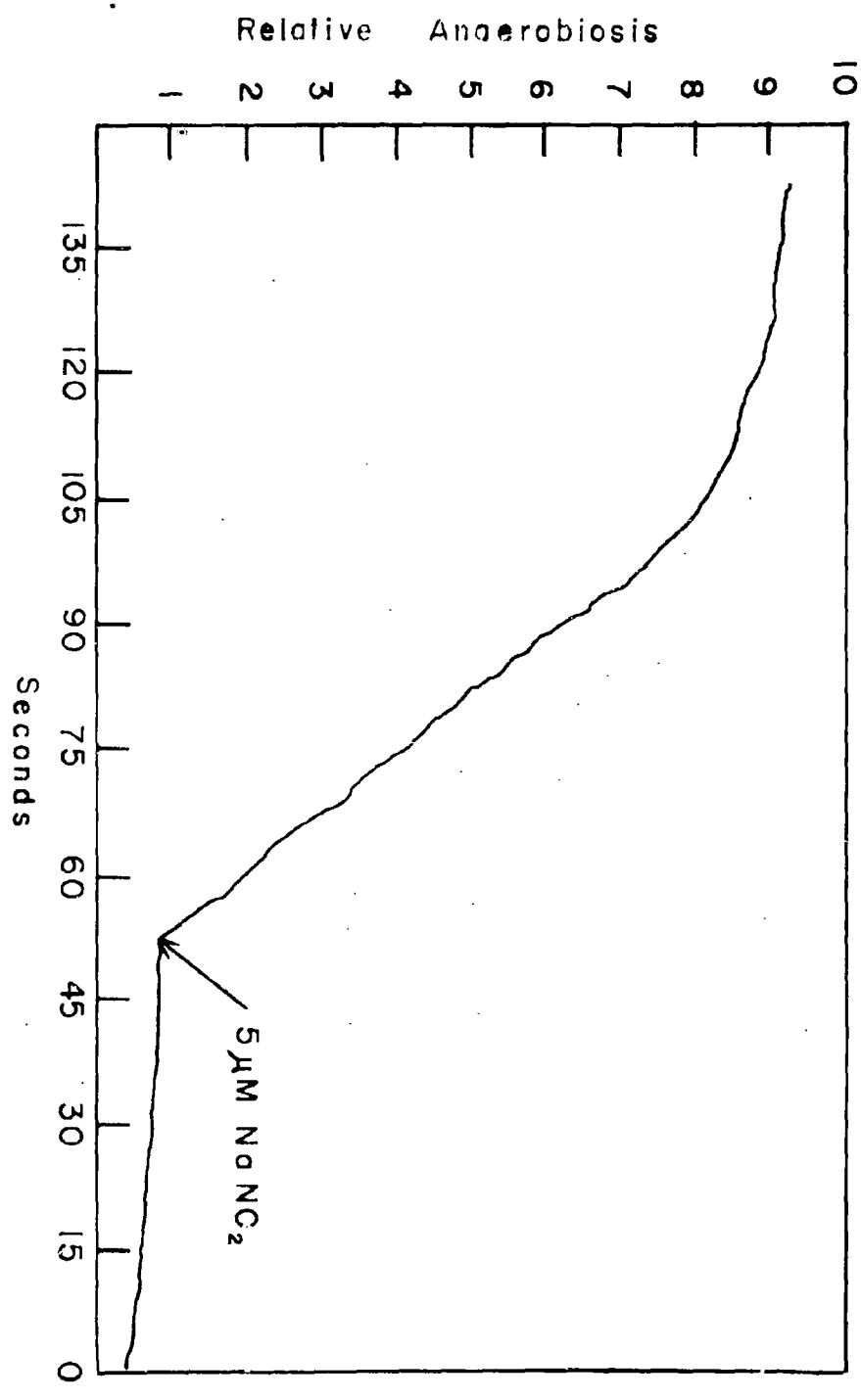

Relative Anaerobiosis
1
2
3
4
5
6
7
8
9
10
Seconds
0
15
30
45
60
75
90
105
120
135
5μM Na NC2

Figure 5

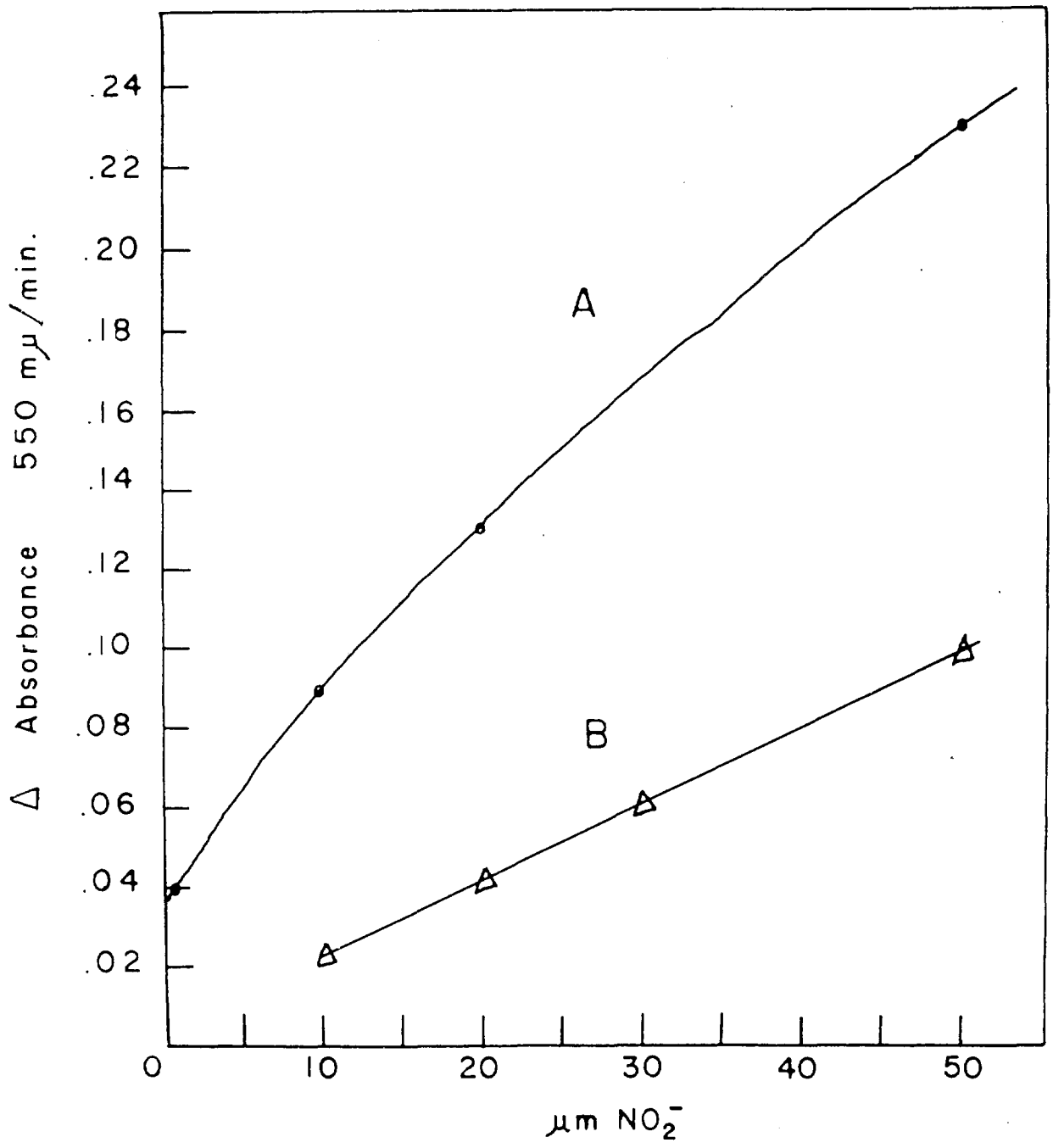
EFFECT OF NITRITE CONCENTRATION ON FERROCYTOCHROME C OXIDATION AT PH 5.6

Protocol

Curve A Ferrocytochrome c 0.13 μm
Acetate buffer, pH 5.6 , 200 μm
$NaNO_2$: varied 1 to 50 μm

Curve B Ferrocytochrome c 0.08 μm
Phosphate buffer pH 6.0 , 200 μm
$NaNO_2$: varied 10 to 50 μm
Water to total volume of 3.0 ml.

Data obtained with a Bausch and Lomb recording spectrophotometer, model 505 against a water-buffer blank.



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