AN ACTION SPECTRUM OF *NITROBACTER AGILIS*

by

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B.Sc. (Hons.), University of Toronto, 1966

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

PHYSICS

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September, 1968

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ABSTRACT

The physiological literature on Nitrobacter is reviewed and a list of the unsolved problems presented. Modifications to the action spectrum apparatus built by Brooks (1967) are described. The apparatus was then used with Nitrobacter agilis, ATCC no. 14123, to obtain an action spectrum of the relief of carbon monoxide inhibition by light. The results of this study indicate that cytochrome a_1 is active as a terminal oxidase. The possibility of other cytochromes, principally cytochrome o, acting as oxidases has not been proven or ruled out. The results of a study on the rate of oxygen uptake versus oxygen concentration are also reported; the $K_m(O_2)$ values range from 0.021 to 0.055 mM oxygen.

The action spectrum reported here is the first one to be determined on a chemolithotropic bacterium.

I would like to thank Dr. C. P. S. Taylor for his guidance, advice and encouragement in the course of this investigation and for his helpful criticisms in the preparation of this thesis.

Financial support from the National Research Council and the University of British Columbia is gratefully acknowledged.

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INTRODUCTION

The work of Brooks (1967) has le d to an instrument based on that of Castor and Chance (1955) which measures the reversal by light of the inhibition of respiration by carbon monoxide. He suggested several improvements for the apparatus, some of which have now been carried out. The light chopper was replaced by a tuning fork and the path of the reference beam was changed. These changes have resulted in the elimination of the instrument artifacts that plagued Brooks. They also allow easier manipulation of the apparatus. The changes and others associated with these are outlined in Chapter II. The improved apparatus was then used in a study of the terminal oxidase of Nitrobacter agilis.

Along with this the literature on the physiology of Nitrobacter was critically surveyed. (Chapter I)

Oxygen uptake as a function of the oxygen concentration was studied using a polarographic technique and the results were compared to a similar manometric study performed by Butt and Lees (1964).

CHAPTER I

THE PHYSIOLOGY OF NITROBACTER

I-1. Nitrobacter

Nitrobacter is a micro-organism of the family Nitrobacteriaceae of the order Pseudomonales. It is prevalent in nature and is usually isolated from soil by repeated plating on silica gel or agar plates. The cells are gram-negative short rods 0.5 x 1.0 microns. Flagella have been seen on Nitrobacter in the electron microscope.

I-2. Conditions for Growth

2.1 Growth Factors

Nitrobacter functions in the control of the nitrogen balance of the soil by the oxidation of nitrite to nitrate. This oxidation is its primary and probably only source of energy. It is autotrophic in that it uses carbon dioxide as its source of carbon. Nitrobacter is grown on a mineral medium usually containing K, Ca, Mg, Mn, and Fe (Lees and Simpson, 1957). Mg, Fe, and phosphate are all essential but the requirement for other growth factors has not been thoroughly investigated. Aleem and Alexander (1958) report a requirement for calcium. Pure cultures tend to die out indicating that there may be a requirement for other growth factors or trace elements. It has been reported that biotin

in catalytic amounts stimulates the growth of Nitrobacter. (Krulwich and Funk, 1965)

2.2 Effect of Light

Bock (1965) has shown that light produces an inhibition of nitrite oxidation in Nitrobacter winogradski. Müller-Neuglück and Engel (1961) showed that light inactivation proceeds to the point where no oxidation occurs. Nitrobacter are reactivated in the dark but it is slower the longer the light has shone and the greater its intensity. Blue light 366-436 nanometers (nm) is most effective, red light has no effect. Müller-Neuglück and Engel feel that photo-oxidation is the cause of this inactiviation. Nitrobacter winogradski possesses no carotenoids. In other bacteria, carotenoids have been found to have a protective mechanism and thus prevent destruction by photo-oxidation.

2.3 Nitrite Requirement

Gould and Lees (1960) have studied the optimal nitrite concentration for the growth of Nitrobacter. They found that an initial concentration of 100 µg nitrite-N/ml with subsequent additions to bring the concentration to 200µg nitrite-N/ml when the first had been used up resulted in the fastest rate of growth. Initial additions of more than 300 µg nitrite-N/ml led to a depression of the oxidation rate and initial concentrations of 600 µg nitrite-N/ml stopped all cell growth for one week. Subsequent studies (Butt and

Lees, 1960, Boon and Laudelout, 1962) have confirmed this and have shown that at atmospheric conditions--20% oxygen-- maximal oxygen uptake occurs at a nitrite concentration of 16 mM (200 μg nitrite-N/ml). At lower oxygen tensions the maximal rate occurs at lower nitrite concentrations. Gould and Lees (1960) have shown that the rate of nitrite oxidation is logarithmic until 200 μg nitrite-N/ml have been oxidized, i.e. the log of the rate versus nitrite concentration is a straight line. All growth ceased when 2200 μg nitrite - N/ml had been consumed. The maximal rate under these conditions was 6 μg nitrite-N/ml per hour. Dialysis of nitrate from the solution each day resulted in the absence of the stationary phase even up to the oxidation of 13,000 μg nitrite-N/ml. As a function of nitrite used the dry weight of cells was slightly larger in the latter case. The maximal oxidation rate was 200 μg nitrite-N/ml/day. Aeration of the cultures increased the rate, and use of both techniques simultaneously gave an oxidation rate of 100 μg nitrite-N/ml/hr. After the logarithmic phase the rate of oxidation was linear at a rate dependent on the air flow.

2.3.1 Oxygen Effect on Nitrite Requirement

Butt and Lees (1964) calculated the concentrations of nitrite needed for maximal rate at different partial pressures of oxygen. The theoretical calculations are made assuming that a ternary complex of nitrite, the oxidizing enzyme, and an oxidized carrier is formed which breaks down, after reaction, into nitrate, the enzyme, and reduced carrier.

The carrier is then oxidized by oxygen. An inactive complex can be formed if the enzyme reacts with two molecules of nitrite. On the basis of this he obtains the following results:

% O_2 in gas phase	Nitrite Concentrations for Maximal Rate	
	Theoretical	Experimental
20	9 mM	15 mM
10	8 mM	9 mM
2.5	5 mM	6 mM

Boon and Laudelout calculated the K_m of the terminal oxidase of the respiratory chain with respect to the substrate oxygen by plotting v vs v/S. At 32°C, K_m(oxygen) was 16μM or a partial pressure of 1.5%. When the oxygen concentration is much greater than K_m the reaction proceeds at a constant rate but when it is less than K_m it follows first order kinetics. Below the K_m(oxygen) the oxidation proceeds much more slowly with incresing temperature due to the temperature characteristics of K_m and the maximal rate V_{max}.

Aleem, Hoch and Varner (1965) have shown that it is not the oxygen from O_2 but rather from water that serves in the conversion of NO_2^- to NO_3^-.

I-3 Inhibitor Studies on Whole Cells and Cell-Free Extracts

All authors are in essential agreement on the subject matter presented to this point. At this point however theories begin to disagree and even some observations are contradictory.

3.1 Inhibition by Nitrite

Boon and Laudelout have investigated the effects of nitrite concentration, pH, and nitrate concentration on the oxidation of nitrite, both in whole cells and in cell-free extracts. At first they suggest that at higher concentrations, nitrite inhibits its own oxidation by substrate inhibition. However they then go on to suggest that the effect is actually due to undissociated nitrous acid which on the basis of kinetics is a non-competitive inhibitor. Maximal Nitrobacter growth occurs at a pH of 7.8. On the acid side of this, inhibition by nitrous acid explains most of the decline in growth rate. On the alkaline side, Boon and Laudelout suggest that inhibition is due to the absorption of hydroxide ions on the enzyme site.

These authors find that the K_m values for nitrite as the substrate are almost identical for intact cells and cell-free extracts, being 1.6 and 2.2 mM respectively. The similarity of K_m values points to an oxidizing system located on the outer cell membrane as do unpublished electron microscope observations on fractionated cell-free extracts reported by Boon et al. However, Aleem and Alexander (1958) using cell-free extracts containing nitrite oxidase activity found no toxicity up to concentrations of 50 mM--a concentration at which whole cells have a rate close to zero. Their data indicate a high Michaelis constant for nitrite as substrate, probably of the order of 20 mM. This does not agree with that found by Boon.

3.2 Inhibition by Inorganic Ions

Butt and Lees (1960) investigated the effects of several inorganic ions on the oxidation of nitrite by whole cells. They found that nitrate, cyanate, and arsenite all acted similarly.

3.2.1 Inhibition by Nitrate

A certain concentration of the ion, e.g. 33 mM nitrate was inhibitory at normal oxygen concentrations but this same concentration of ion at a lower oxygen concentration stimulated the oxidation rate. Boon and Laudelout's investigation showed that Nitrobacter in a growth medium containing precipitated salts exhibited this phenomenon but in a medium free from precipitate, nitrate acted as a simple non-competitive inhibitor, showing no stimulation at low oxygen concentrations. Boon and Laudelout attribute the stimulatory effect of nitrate at low oxygen to an ion exchange effect. Nitrate added to the washed suspension of cells and mineral precipitate might release trace elements absorbed onto the mineral particles. Their values for the inhibition of nitrate (K_i = 180 mM) did not explain the inhibition observed by Lees and Simpson at 100 mM.

3.2.2 Inhibition by Cyanate

Using the same medium as they had done in 1960, Butt and Lees in 1964 investigated the effect of cyanate on whole cells and cell-free extracts. They found that cyanate

a powerful inhibitor of nitrite oxidation by whole cells at normal oxygen tensions, had no effect whatever on the cell-free extracts. The theory that they propose to account for this is the following: there is a transport system which brings nitrite from the medium to the enzyme and that cyanate interferes with this enzyme, although not with the nitrite oxidase. In this way, at normal oxygen tension, all the nitrite that would reach the enzyme normally would be oxidized. The interference of the cyanate prevents nitrite from reaching the enzyme and slows down the rate. At lower oxygen concentration, too much nitrite would normally reach the enzyme resulting in substrate inhibition. Addition of cyanate prevents the inhibition by lowering the nitrite concentration at the enzyme, thereby stimulating nitrite oxidation. There are two possible problems with this theory. The one is the possibility as stated by Boon that the enzyme is already on the outer surface of the cell and that nitrite does not have to diffuse through the membrane. The second is that if these ions are sufficiently like nitrite to affect the carrier, then it would seem possible that they would also affect the nitrite oxidase. Van Gool and Laudelout (1965), in contrast, have evidence that cyanate has approximately the same effect on whole cells and cell-free extracts as the concentration for 50% inhibition in the two cases is 2.5 and 7 mM.

3.2.3 Inhibition by Chlorate

The effects of chlorate have been investigated

by Lees and Simpson (1957) and by Van Gool and Laudelout (1965). The 50% inhibition after 60 min at 7 mM ClO_3^- found by Lees is comparable with that of Van Gool. The incubation period must be stated since decomposition products of the inhibitor destroy the cytochrome activity according to a first-order rate law (Lees and Simpson).

Of all the inhibitors studied by Van Gool and Laudelout, only chlorate does not give similar values for whole cells and cell-free extracts. The similarity of the values would seem to accord with a peripheral location of the enzyme system causing nitrite oxidation.

I-4 Oxidation of Nitrite by Cell-Free Extracts

I have previously mentioned the use of cell-free extracts in studying Nitrobacter (c.f. Sec. I-3). Aleem and Alexander in 1958 were the first to disrupt cells by sonification and they found that the extracts contained nitrite oxidase activity, that this was coupled to oxygen uptake and that the oxidized nitrite could at all times be recovered as nitrate indicating that there are no intermediates. As mentioned previously there is no nitrite toxicity observed to a concentration of 50 mM. Optimum conditions for oxidation require the presence of iron and a pH of 7.5 to 8.0. The nitrite oxidase is inhibited by low cyanide concentrations. Along with the iron requirement this latter fact suggests a similarity between the nitrite oxidizing enzyme and the cytochrome oxidase. Further centrifugation shows that the nitrite oxidase is

situated in the particulate fraction at 144,000 x g.

I-5 Spectroscopy of Nitrobacter

5.1 Absorption Spectra

Lees and Simpson (1957) reported cytochrome absorption peaks at 589, 551, and 520-525 nm on addition of nitrite or dithionite. Aleem and Nason (1959) show that nitrite oxidizing activity resides solely in a cytochrome containing particle. Addition of nitrite to this particle resulted in absorption peaks appearing at 550 and 520 nm representative of the α and β peaks of a c type cytochrome and in the 585-590 and 438 nm regions indicative of the α and γ peaks of and a type cytochrome, probably a1. Added dithionite gave essentially similar peaks but of several fold greater magnitude, and also produced an absorption maximum at 415 nm corresponding to the γ peak of cytochrome c. Production of the peaks was specific for nitrite as substrate, succinate, DPNH, or lactate failing to produce them. Hemoglobin, catalase and peroxidase were shown to be absent from both the particulate and supernatant fractions. (Different copper and iron effects have been observed. Aleem and Alexander (1958) showed that copper was inhibitory, but in 1959 Aleem and Nason say that it has no effect but that it enhances the non-enzymatic disappearance of nitrite. The iron requirement was shown but iron also reduces the cytochrome c like component non-enzymatically.

5.2 Difference Spectra

A more recent spectroscopic study was performed by Van Gool and Laudelout in 1965 on Nitrobacter winogradski. Difference spectra for intact cell suspensions showed maxima at 523, 554, and 597 nm with a shoulder at 609 on the last. In the zone of 450 to 490 nm the reduced system had a higher transmittance than the oxidized. The minimum occurred at 465 nm. This is possibly indicative of the bleaching of flavin components. In the Soret region there were two peaks, at 419 and 439 nm, which were fused in a turbid cell suspension to 440 nm. Slight differences were noticed with cell-free extracts. The peak at 597 for example, occurred in this system at 594 nm. At low temperature on cell-free extracts the highest peak in the visible was split into three peaks, at 604, 587, and 579 nm. The peaks at 609 and 450, 594 and 439 are indicative of a type cytochromes. Those at 554, 523 and 419 indicate a cytochrome c with the high wavelength of the α peak, 554, indicating a bacterial type cytochrome c. The following table is a summary of this and other spectroscopic data taken from the paper by Van Gool and Laudelout.

Author	Cytochromes a				Cytochromes c		
	a_α	a_γ	$a1_\alpha$	$a1_\gamma$	c_α	c_β	c_γ
VanGool and Laudelout	609	450	594	439	554	523	419
Lees and Simpson	-	-	589	-	551	520-525	-
Aleem and Nason	-	-	586-590	438	550	520	415
Zavarzin	-	-	592	-	552	-	-

5.3 Carbon Monoxide Difference Spectra

A carbon monoxide difference spectrum is recorded as the absorption of the carbon monoxide-reduced compound minus the absorption of the reduced compound. This spectrum indicates what modifications of the cytochromes in the organism or extracts have been caused by the addition of carbon monoxide. It has been shown in many organisms that carbon monoxide complexes only with the terminal compound in the cytochrome chain, in competition with oxygen. It thus prevents the oxidation of this oxidase and of the other cytochromes in the chain with the result that these compounds become reduced, or more reduced as the case may be, in the presence of a reducing agent, i.e. supply of electrons. On addition of carbon monoxide one would thus expect to observe an increased reduction of the cytochromes generally plus changes in the absorption of the oxidase due to its forming a complex with carbon monoxide.

A comparison of the reduced and carbon monoxide-reduced difference spectra often enables one to determine which of the cytochromes are present and which are reacting with carbon monoxide since the cytochromes are defined on the basis of their absorption spectra. To date, four types of cytochromes have been found to act as oxidases. They are cytochromes $\underline{a}_1$, $\underline{a}_2$, $\underline{a}_3$, and $\underline{o}$.

5.3.1 Results with Nitrobacter

Van Gool and Laudelout have reported a

CO-reduced minus reduced difference spectrum of Nitrobacter. Peaks at 523, 554, and 608 nm in the visible and 419 in the Soret region occur at the same positions as those in the reduced difference spectrum. These peaks are due to increased reduction of the compounds--namely cytochrome a and c--which do not combine with carbon monoxide. Troughs were recorded in the carbon monoxide difference spectrum at 439 and 594 nm, the positions of peaks in the reduced difference spectrum. These troughs are indicative of reduced absorption in the carbon monoxide sample with respect to the reduced sample. Since these wavelengths do correspond to the peaks of cytochrome a_1 in the reduced difference spectrum, their disappearance indicates that cytochrome a_1 has complexed with carbon monoxide. Other indications of carbon monoxide binding were a peak at 450 nm in the carbon monoxide difference spectrum of the cell-free extract and a peak at 426 nm and trough at 440 in the cell suspensions.

The peak and trough of the cytochrome a_1 reported at 426 and 440 for intact cells and 439 for cell-free extracts correspond well with data reported by Chance (1953). His figures for these positions in A. pasteurianum are 427 and 442 nm. Chance also reports a band at 590 while Van Gool and Laudelout mention a trough corresponding to the 594 peak in the reduced difference spectrum.

I-6 Flavin Involvement in the Cytochrome Chain

Inhibitor and difference spectra studies by Van Gool

and Laudelout point definitely to the involvement of a flavin component, as there was strong inhibition by quinacrine and reversal of this by FAD. Zavarsin (Lees, 1960) has reported that iron along with either molybdate or tungstate stimulate nitrite oxidation. This simultaneous requirement for iron and molybdate led Zavarzin to postulate that a molybdeno-flavoprotein and cytochrome act sequentially. Van Gool and Laudelout say that the involvement of a flavin would indicate that the process of nitrite oxidation must be pulled over a thermodynamically unfavourable step.

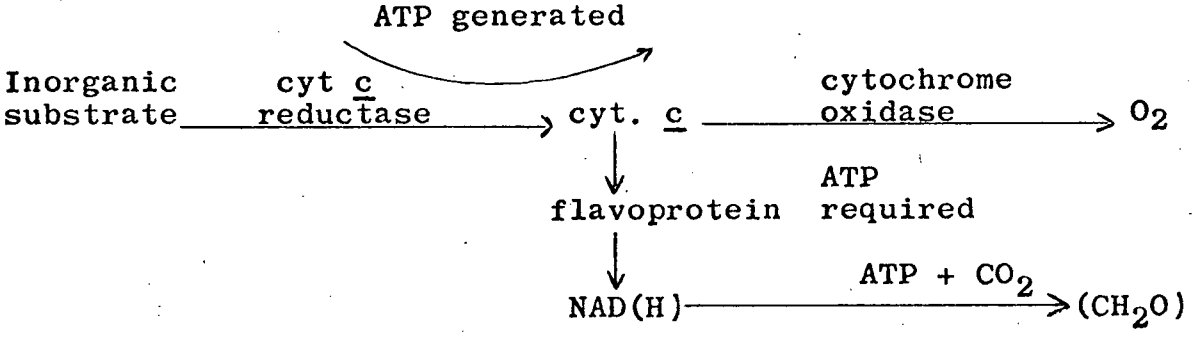
I-7 Energy Production and Efficiency

The driving force for the reduction of carbon is the oxidation of the inorganic ion by oxygen. The efficiency of these organisms is thus best expressed by the ratio of inorganic nitrogen oxidized to CO_2 carbon assimilated. In Nitrobacter the N:C ratio varies from 76:1 to 135:1 (Alexander 1961). The microorganisms do not get all the energy potentially available but only a small proportion determined by their efficiency of energy utilization. The free energy data of Baas-Becking and the assumption that Nitrobacter contains 50% carbon on a dry weight basis is used to show that the free energy efficiency is 30% in the presence of 200µg nitrate-N/ml and 15% in the presence of 1000 µg nitrate-N/ml. (Gould and Lees, 1960)

Aleem and Nason in 1960 have shown that phosphorylation is coupled to nitrite oxidation. Partially purified nitrite

oxidase particles catalyzed the formation of high energy phosphate bonds concomitant with the oxidation of nitrite. ATP is formed when ADP is supplied as the acceptor. Several other nucleotides were also effective. All their attempts to uncouple phosphorylation from oxidation proved unsuccessful although aged nitrite oxidase particles displayed decreased rates of phosphorylation while their rates of oxidation remained unchanged. Magnesium ions were found to stimulate phosphorylation.

In 1963, Aleem, Lees, and Nicholas demonstrated that there was a reduction of NAD concomitant with the oxidation of added mammalian ferrocytochrome c. Aleem (1965) showed that the hydrogen for NAD reduction was supplied by water. Aleem et al (1963) state that the reduction of NAD is dependent on ATP and that the theoretical requirement is 4 ATP/NAD. In cell free systems the experimental value was 10-20:1. These authors have proposed the following scheme for the overall metabolism of Nitrobacter.



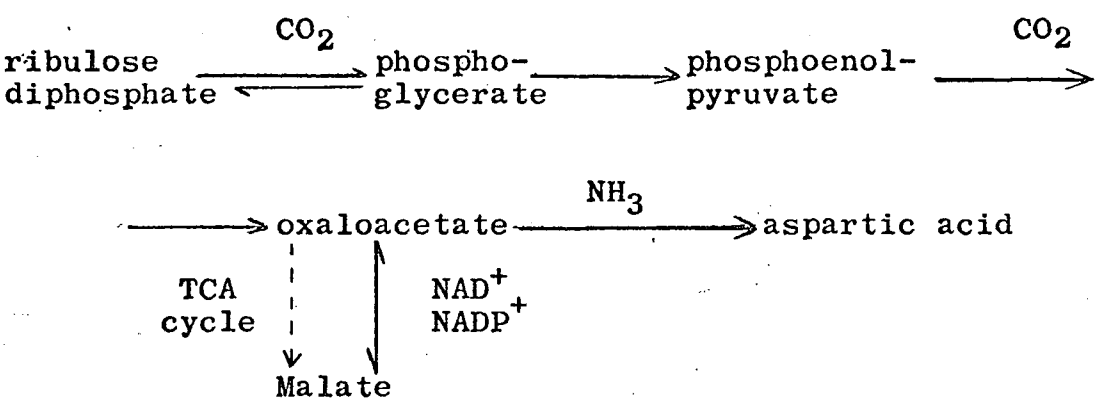
Such a modified scheme seems to be necessary in order to produce NADH (although I do not agree that Aleem et al have proven this in their experiments). The reason for this

modified scheme is that the E_o' for the nitrate-nitrite system is +0.35 while that for the NAD/NADH couple is -0.32. Thus energy would be required to couple these systems and result in the oxidation of nitrite. It would seem probable that there might also be a modification in the first step of the proposed sequence since the E_o' of mammalian cytochrome c Fe^{+++}/Fe^{++} is +0.25. It could perhaps be as Lees (1962) suggests, namely that Nitrobacter may be compelled to synthesize some such compound as adenyl nitrite so as to lower the redox potential of the nitrite couple. (See however Sec. I-4) In considering the values of these redox potentials I felt that some reservations should be borne in mind: especially that these values are obtained in "in vitro" situations at pH 7and the cyt. c couple E_o' value used is that for mammalian cytochrome c. It is possible and quite probable that the values may not apply to the situation in intact Nitrobacter cells. In intact cells, the oxidation of nitrite might proceed much more favourably to produce energy due to a change in redox potential resulting from the physical configurations or distortions of the molecules involved and from local pH and concentration changes.

I-8 The Fixation of Carbon Dioxide

The energy obtained from the oxidation of nitrite, in the form of ATP and NADH is used to fix and reduce carbon dioxide. Aleem in 1965 performed an extensive study on the pathway of carbon fixation using radioisotopes. The first stable compounds to be labelled were phosphoglyceric acid

and aspartic acid. After 10 sec exposure to $C^{14}O_2$, 60% of the radioactivity appeared in phosphoglycerate, 20% in each aspartic acid and sugar phosphates, 4-6 % in each malic acid, glutamic acid and phosphoenolpyruvate. In dealing with cell-free extracts the carboxylating enzymes were localized in the supernatant after 144,000 x g centrifugation. The supernatant catalyzed rapid fixation of CO_2 in the presence of added ribulose-1,5-diphosphate or ribose monophosphate and ATP. The label appeared in phosphoglycerate. Without added CO_2-acceptor the soluble fraction catalyzed rapid assimilation when supplied with ATP and NADH. The carboxylating enzymes required magnesium ions. Carboxydismutase (D-ribulose-1,5-diphosphate decarboxylase) and other enzymes of the Calvin-Benson, Kreb's and reductive pentose phosphate cycles were localized in the supernatant. Aleem states that the above observations point to the standard autotrophic pathway of CO_2 assimilation, namely:



Aleem also gives the possible pathways of carbon metabolism.

Bock and Engel (1966) have shown that if Nitrobacter is deprived of carbon dioxide, oxygen uptake slows down. Under

aerobic as well as under anaerobic conditions, the nitrifiers are able to fix carbon dioxide after oxidation has occurred. The strongest activity in this respect is seen in cells which have carried out oxidation shortly before being allowed to fix CO_2.

I-9 Unsolved Problems and Criticisms

There are still many problems with regard to the physiology of Nitrobacter. Some of these are listed below.

(i) First there is the problem of a calcium requirement. Aleem and Alexander (1958) mention that calcium is required for growth of Nitrobacter. Lees and Simpson (1957) make no mention of a calcium requirement yet the medium used by these authors contains calcium ions. Boon and Laudelout (1962) on the other hand, used a medium which contained no calcium.

(ii) A second point arising out of the paper by Boon and Laudelout concerns their suggestion that the inhibition of Nitrobacter at high pH is due to OH^- ions. That the concentration of hydroxyl ions, when placed in the kinetic equation yields a curve close to the experimental proves only that an ionization is involved, not that OH^- is the active inhibitor.

(iii) The effects of nitrate on nitrite oxidation are still not clear. The inhibition observed by Lees and Simpson, and Butt and Lees seems to be very different from that described by Boon and Laudelout (cf. I-3.2.1)

(iv) The differences in the effects of nitrate by these authors could be dismissed as just a difference in degree except that cyanate, a second inhibitor is reported as behaving in a contradictory manner by the different authors (cf. Sec. I-3.2.2). Thus Butt's and Lees's observations on the effects of nitrate and cyanate and their difference in whole cells and cell-free extracts is in accord with their proposal that a carrier is required to bring nitrite into the cell. All of the observations of Boon and Laudelout on inhibitors and their unpublished electron microscope studies on the other hand, point to the particulate oxidase fraction being located on the outer membrane of the cell.

(v) The final problem to be mentioned here is concerned with ATP generation. As stated in Sec. I-7, I do not agree that Aleem has proven that NADH is produced in the manner indicated since loading of a cytochrome chain with ATP will drive it backwards. A scheme such as he has proposed does seem to be required however, in order to oxidize nitrite and produce energy from this oxidation. A decisive experiment to perform at this point would be to utilize Chance's rapid flow technique with inhibitors to determine the order of reduction of the compounds in the cytochrome chain.

I-10 Summary

There is much contradictory evidence concerning Nitrobacter especially in relation to inhibitor studies and the location of the nitrite oxidase particle (on the outer membrane or inside the cell). This could be due to the use of different strains, to different growth conditions-especially media, or to different experimental technique. It seems well established however that Nitrobacter oxidizes nitrite to nitrate and that this oxidation supplies energy in the form of ATP and NADH for the fixation of carbon dioxide and the production of material for growth. A cytochrome chain and flavoprotein are seen to take part in the electron transport system. Water is the source of hydrogen for the reduction of pyridine nucleotide and of oxygen in the oxidation of nitrite. Molecular oxygen is only involved in the oxidation of the terminal oxidase in the electron transport chain. Carbon dioxide fixation is similar to that of other chemoautotrophic bacteria in that it makes use of the Calvin-Benson and reductive pentose phosphate pathways.

CHAPTER II

GROWTH METHODS

II-1 Medium and External Conditions

Nitrobacter agilis from the American Type Culture Collection No. 14123 were grown in a medium identical to that of Gould and Lees (1960). To 100 l. of water were added 30 gm KH_2PO_4, 1 gm $CaSO_4$, 0.5 gm $MgCl_2$, 0.01 gm $MnCl_2$, and 0.2 ml of dialyzed iron. This was filtered and the pH adjusted to 7.8, Sodium nitrite ($NaNO_2$) was added to make a concentration of 100 μg nitrite-N/ml (8mM). 50-100 ml of medium plus sodium nitrite were placed in 250 ml Erlenmeyer flasks and sterilized after which approximately 0.5 ml of a dilute Nitrobacter suspension were added. The cultures were grown in the dark (cf. I-2.2). Nitrite was added when required by the addition of a sterilized 0.8 M $NaNO_2$ solution to bring the concentration of nitrite in the culture to 10 mM.

At the onset of a decreased requirement for nitrite, or just prior to this as estimated by the turbidity of the solutions, the cultures were diluted two to ten times, depending on the immediate requirement for concentrated cultures. Or alternately, new cultures were made by adding 2-5 ml of a concentrated solution to 100 ml of fresh medium. In this manner a type of continuous culture method was employed providing steadily growing and fairly concentrated cultures at all times. Once cultures reached the point where they required no more nitrite (3-8 wk), dilution usually proved

unsuccessful in producing viable cultures.

Aeration and nitrate removal were not employed although such methods lead to a prolonged growth phase (Gould and Lees, 1960). The method of diluting cultures as mentioned above ensured that the nitrate concentration did not become high enough to prevent further growth. The problem of adequate aeration was dealt with by keeping the cultures relatively shallow (about 2 cm) and allowing air to circulate through cotton plugs in the tops of the flasks.

The cultures used in the experiments were oxidizing nitrite at a rate of 25-50 µg nitrite-N/ml/day (2-4 mM nitrite/ml/day).

II-2 Test Methods

2.1 Nitrite

The presence of nitrite was determined using a method employing sulfanilic acid and N,N dimethyl-1-naphthylamine (Snell and Snell, 1949). Two stock solutions were first made up. Solution A was made by dissolving 3.3 gm of sulfanilic acid in 750 ml water by heating. 250 ml glacial acetic acid were then added to this. Solution B contained 5.25 gm of N,N dimethyl-1-naphthylamine in 1 litre of 1:3 glacial acetic acid: methanol.

For a qualitative test of the presence of nitrite equal volumes of solution A and B were mixed. To about 1 ml of this mixture a sample of less than 0.5 ml of medium was added. Development of colour (pink to purple) indicated the presence of nitrite. A colourless solution was indicative of the

absence of nitrite. This method was employed to determine the nitrite requirement of the cultures.

The best conditions for a quantitative test were found to be as follows. 1 ml of the solution to be tested was diluted to 100 ml with distilled water. 5 ml of solution A was added followed by 5 ml of solution B. Ten minutes after the addition of solution B a reading on the Klett colorimeter was taken using a green filter. The timing here is quite crucial as each additional 30 seconds results in a change of 6-10% in the reading.

The development of colour is also very sensitive to temperature and the age of solution B. Thus it is necessary to run one standard each time a determination is performed. Since a plot of Klett reading versus concentration is proportional in the concentration range encountered one standard is usually sufficient.

Solution B is unstable over periods of time greater than two weeks. This is not of any consequence for qualitative tests as colour develops in the presence of nitrite and does not develop in its absence. In both tests older solutions give a deeper colour but too old a solution results in the appearance of a precipitate in less than ten minutes so that a quantitative test has no validity.

2.2 Nitrate

Nitrobacter converts nitrite to nitrate so that it is desirable to test for the production of nitrate to

ensure that the Nitrobacter are the agents causing the disappearance of nitrite. This is done using the following method employing a phenoldisulfonic acid reagent (Snell and Snell, 1949).

The reagent was prepared by dissolving 25 gm of colourless phenol in 150 ml of concentrated H_2SO_4. Traces of nitric acid in the sulphuric acid were removed by agitating with mercury. 75 ml of fuming sulphuric acid containing 13% free sulphur troxide were then added. This was stirred and heated two hours on a boiling water bath.

10 ml of sample were evaporated to dryness. 2 ml of the phenoldisulfonic acid reagent were added rapidly to the centre and the dish rotated to ensure contact of the reagent with all of the residue. This was allowed to stand for 10 minutes at which time 15 ml of cold water were added to dissolve the residue. 1:2 ammonium hydroxide:water was added until the solution was slightly alkaline. This was accompanied by the production of a deep yellow colour. The solution was then made up to 50 ml and a reading taken on the Klett colorimeter using a blue filter. Calibration was only required once as the colour developed is unaffected by external conditions.

CHAPTER III

INSTRUMENTATION AND SAMPLE PREPARATION

III-1 The Oxygen Electrode

1.1 Instrumentation

The rate of oxygen uptake and hence of nitrite oxidation as a function of oxygen concentration can be obtained using an oxygen electrode. This consists of a platinum electrode polarized at -0.6 to -0.9 volts with respect to a Ag/AgCl electrode placed in the same solution. At this potential in the solutions used, oxygen diffusing to the platinum electrode is selectively reduced. The amount diffusing to the electrode is a function of the concentration and results in a current in the external circuit proportional to the concentration. The current is measured by passing it through a resistor and monitoring the voltage drop across the resistor with a Heathkit Servo Recorder model EUW-20A.

In the experiments with Nitrobacter a small cylindrical chamber holding 8 ml of fluid was employed for measuring oxygen uptake (Fig. 1). A magnetic stirrer was used to prevent the Nitrobacter from settling to the bottom and to keep the medium mixed with respect to the oxygen concentration. This resulted in a lower diffusion distance and a higher current than would be found in a stationary case. Equilibration of the oxygen concentration in the medium with that in air was prevented by closing the chamber with a greased cover slip.

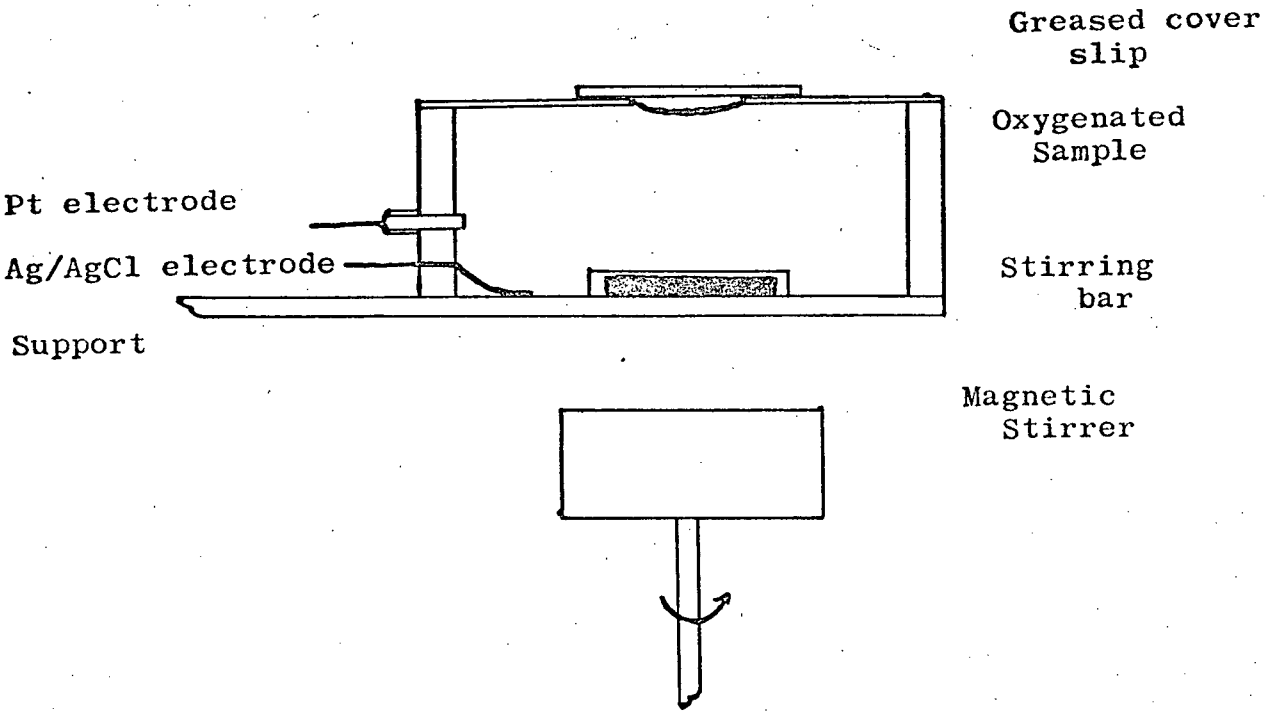


Figure 1 The Oxygen Electrode

This allowed the use of oxygen concentrations higher than that in air.

1.2 Calibration

The oxygen electrode was calibrated using a 0.1 M sodium chloride solution. 100% oxygen concentration was determined by bubbling the solution with oxygen for about two minutes before placing in the apparatus. The sensitivity was then set to ensure that this concentration occurred at the appropriate point. 0% oxygen was determined by bubbling the solution with nitrogen and 20% oxygen could be determined by allowing the solution to equilibrate with air.

1.3 Preparation of the Sample

10 ml of culture were removed and bubbled with oxygen for 1 min. Enough Nitrobacter were then placed in the apparatus to prevent the formation of an air space when the opening was closed with the cover slip. For more concentrated samples the Nitrobacter in 50 ml of culture were centrifuged at the fastest speed on a clinical centrifuge for 15 min and resuspended in 10 ml of 20 mM phosphate buffer (18 mM Na_2HPO_4 plus 2 mM KH_2PO_4) or in 10 ml of their own medium with the appropriate nitrite concentration. These samples were also bubbled with oxygen for 1 min before being placed in the apparatus.

III-2 The Action Spectrum Apparatus

The action spectrum apparatus developed by Brooks (1967)

and modified here is based on an instrument described by Castor and Chance (1955). It is a sensitive instrument for measuring the photochemically reversible inhibition of oxygen uptake by carbon monoxide. The essential features of this equipment are 1) a light source and monochromator, 2) an intensity measuring device, and 3) a chamber and respiration rate sensor. These features are described by Brooks while modifications to the reference beam and intensity measuring apparatus are described in later sections of this thesis.

In short, the light source provides both the monochromatic beam and the reference beam required to obtain the action spectrum. To measure the intensity of either beam, the light, chopped by a mechanical device, falls on a lead sulphide photodetector and the signal from this is passed through a phase sensitive detector (PSD, Fig. 2). A Keithley model 150 A microvoltmeter ammeter is used as a null device with a bucking voltage supplied from an external circuit. A closed chamber provides for the use of a controlled atmosphere of carbon monoxide and oxygen in the experiments. The rate sensor consists of a platinum electrode polarized at -0.6 to -0.9 volts with respect to a Ag/AgCl electrode placed in a drop of cell suspension. This drop is held between the two electrodes and a cover slip forming the bottom of the chamber. The current measurements are made by bucking out the electrode current using the Keithley microvoltmeter ammeter and an Esterline Angus recording milliammeter as null detectors.

Unlike the oxygen electrode, the purpose in the action

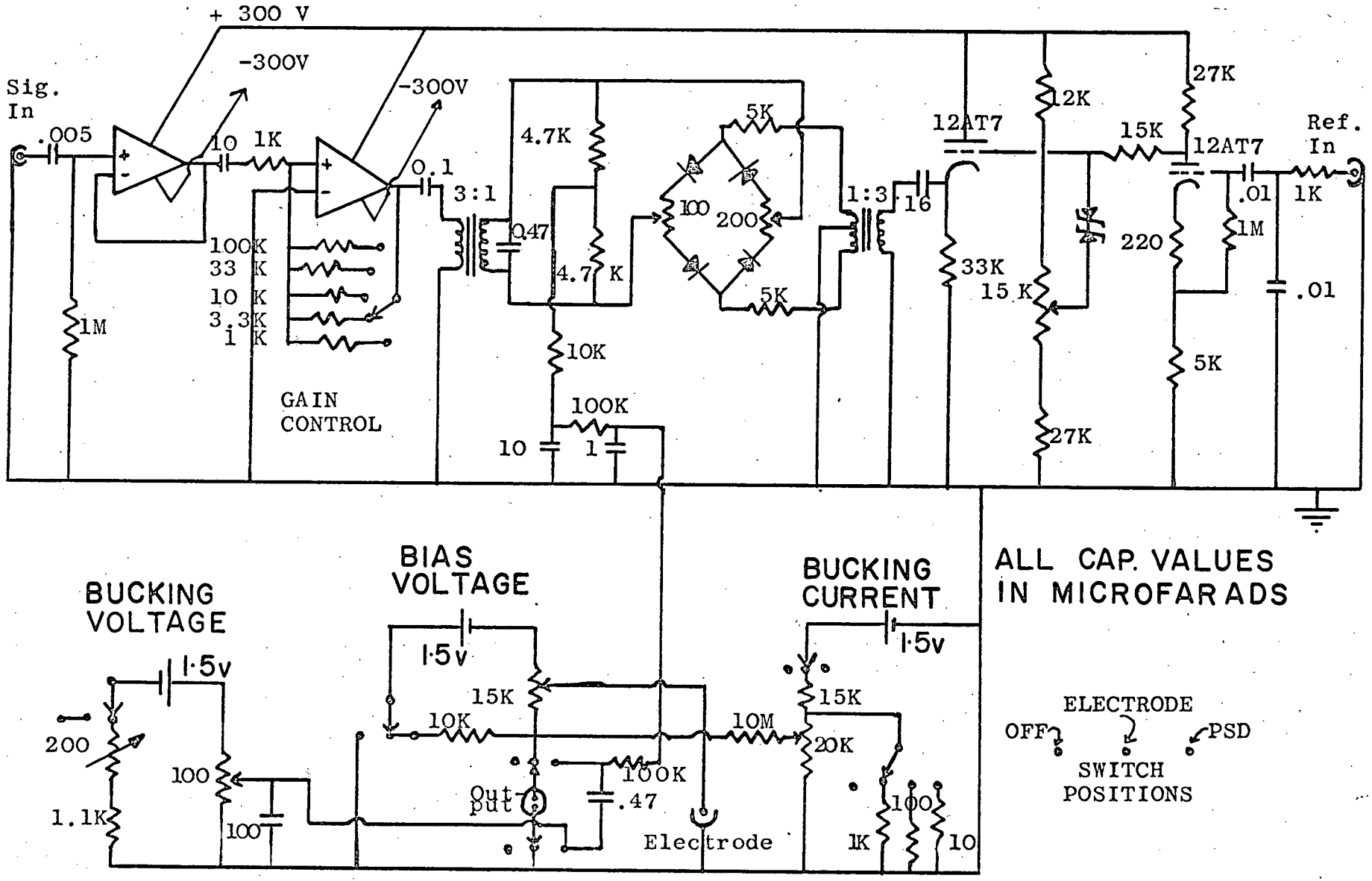


FIGURE 2 Circuit Diagram of Phase Sensitive Detector

spectrum apparatus is to set up a steady state produced by the opposing effects of diffusion of oxygen from the surface of the drop to the platinum microelectrode and respiration by the organism in the drop. Small changes in respiration rate will alter the steady state with a consequent change in current in the external circuit.

2.1 Modifications to the Reference Beam

Brooks (1967) suggested several improvements to reduce error and extend the range of the action spectrum apparatus. Several of these have been carried out.

The method of bringing the comparison beam from the lamp to the sample was altered. To do this the light source used, was changed from the 8v-50w Ace bulb to a 24v-150w Phillips quartz iodine lamp (model no. 7158). The latter bulb has a quartz envelope but has no reflective coating as the former. This makes provision for taking a beam of light from the back of the bulb as well as the front but at the same time reduces the intensity of both beams. Some increase in intensity was then provided by using the higher power lamp.

The current and voltage are provided by a Sorenson and an Eico power supply placed in series. Stability is achieved by placing two twelve volt batteries in parallel with the power supply. (Fig. 3) The power units are adjusted to ensure that there is no current passing through the batteries.

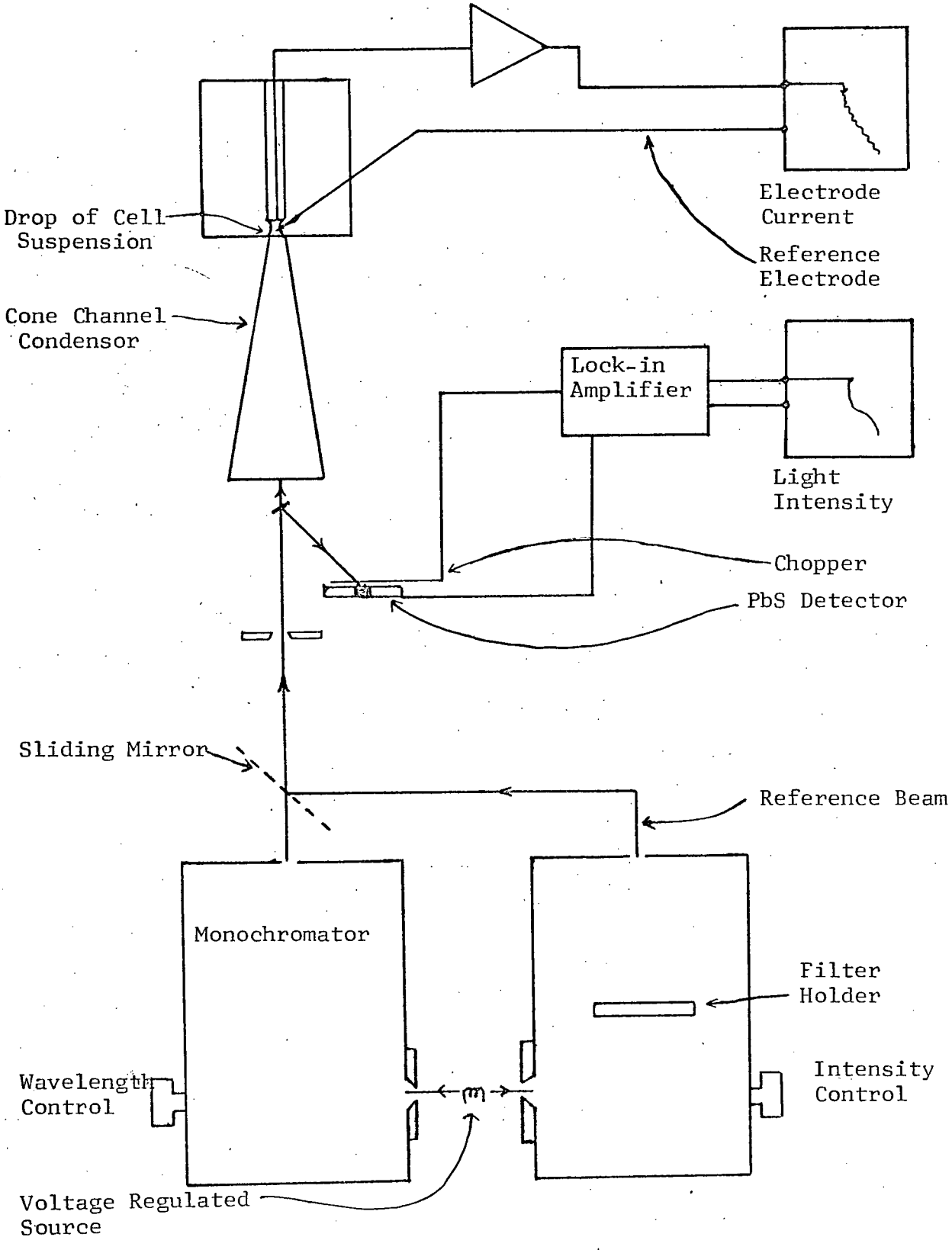


Figure 4
Block Diagram of Apparatus

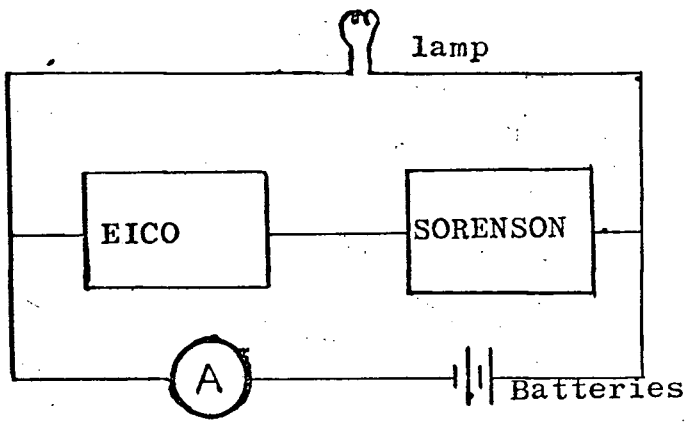


Fig. 3 Power Supply for the Light Source

The beam taken from the one side of the lamp passes through the monochromator as described by Brooks. The second beam follows a path optically equivalent to that of the monochromatic beam. Condensing lenses image the light from the back of the lamp onto a slit which is in the focal plane of a convex lens. The collimated beam is condensed by a second convex lens and imaged at the exit slit of the monochromator. The lenses are housed in a tube 2" in diameter which carries the light out the back of the lamp, around the monochromator box and allows it to enter the monochromator at a position perpendicular to, but just below the exit slit. A small mirror placed on a track can be slid into position to reflect the reference beam onto the exit slit. (Fig. 4) The track consists of two small rods such that when the mirror is moved out of the path of the monochromatic beam, this beam passes between these rods and is focused on the slit. The result is that the exit slit is at all times filled with light, having the advantage as outlined by Brooks, that no transient effects

are produced in the sample.

The louvers were moved from the monochromator housing to an equivalent position in the collimated portion of the reference beam thereby reducing the amount of scattered light included in the monochromatic beam. Any filters required in the reference beam could be placed in the louver housing.

2.2 Modification of the Chopper

The above changes necessitated the introduction of a further improvement. The only physically identical path followed by the two beams is that between the sliding mirror and the sample. Since the intensity of both beams is required for determination of the action spectrum, the chopper and detecting system must be so placed that measurements can be made on both beams. Because of space limitations the chopper must be small. A magnetically driven tuning fork suggested by Brooks meets this requirement. The light chopping wheel was thus discarded and replaced by a Bulova light chopper operated on 28 volts, 20 milliamps from an Eico DC power source. The natural frequency of the tuning fork is 400 Hz.

Fig. 4 depicts the arrangement used for obtaining intensity measurements. A small spherical mirror intercepts a portion, about 10%, of the light entering the cone and partly focuses the louvers or the grating on the PbS, lead sulphide, detector. Thus a cross section of the beam is sampled by the PbS detector and although this does not remove inhomogeneities in the beam it does allow a 1:1 correspondence of PbS readings

with intensity at the top of the cone. The inhomogeneity of the beam is removed by the scrambling effect of the cone and consequently is not transferred to the sample.

2.3 Modifications to the Phase Sensitive Detector

In order to measure the 400 Hz signal, the phase sensitive detector, PSD, required modification. (Fig. 2) The rejection filter in the feedback loop of the first amplifier was removed. The purpose of this filter was to act as a band pass so that the second amplifier would not become overloaded with noise signals. The overloading of the second amplifier was not a problem and the rejection filter introduced unwanted phase shifts. The feedback loop of the second amplifier was changed to introduce a more uniform gain control.

A 0.01 microfarad capacitor was added across the reference input in order to round off the square wave supplied by the tuning fork. A 0.047 microfarad capacitor was placed across the secondary of the signal transformer to correct the phase of the signal.

2.4 Sample Preparation for the Action Spectrum Apparatus

For use in the action spectrum apparatus 50 ml of culture were centrifuged on the highest speed of a clinical centrifuge for 15-20 min. All but 2-3 ml of medium was then removed and the Nitrobacter resuspended by shaking. Best results were obtained if the sample was allowed to stand for 30 min before use. At the end of this time 0.2-0.5 ml of the

concentrated suspension were placed in the syringe and a drop inserted between the electrodes. A mixture of $CO:O_2$ in the ratio 4:1 saturated with water vapour was sucked into the chamber by a 50 ml syringe. Some samples did not recover from centrifugation and had to be discarded. Those that showed activity remained alive in the apparatus a maximum of 90 min. In the air, the concentrated suspensions lasted up to 3 hours with the result that several samples could be obtained from a single centrifugation.

CHAPTER IV

CALIBRATION AND PERFORMANCE

IV-1 Relative Intensity at the Top of the Cone

Since the intensity of light is measured at the bottom of the cone a calibration is required to determine the actual intensity of light falling on the sample. This is most important for the monochromatic beam as the transmission of the cone with wavelength is variable. The composition of the reference beam is always constant, only the intensity varies. Thus in determining the action spectrum the factor relating the intensity of light at the top of the cone to that at the bottom for the reference light is not required as it cancels from the equation determining the relative extinction coefficent.

The method of carrying out this calibration is as follows. An RCA 5819, S-9 response, photomultiplier tube was placed at the top of the cone to gather all of the light. The circuit diagram for the arrangement is shown in Fig. 5. A Keithley 240 A High Voltage Power Supply was used to bias the tube at 600 volts. The output current was read from an Avometer. At no time was it possible to distinguish a dark current on the 50 μamp scale of the Avometer indicating a dark current much less than 1 μamp.

At each wavelength the anode current from the photomultiplier and the voltage from the phase sensitive detector

C1,C2,C3, 16μf

R11, 100K

R1-R10, 47K

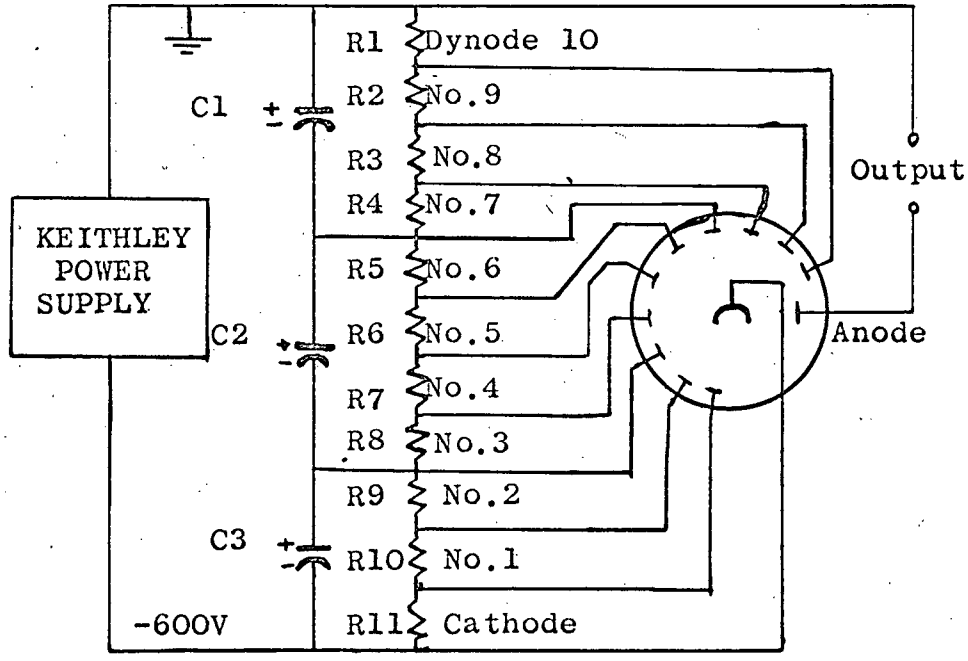


Figure 5 Circuit Diagram for Photomultiplier Tube

were recorded. The latter was corrected for temperature deviation from 24°C and to 10 times gain as described by Brooks. Temperature measurements were taken from a calibrated thermocouple attached to the PbS detector. The results of this calibration are tabulated in Table I.

It was then necessary to calibrate the photomultiplier for its wavelength sensitivity. For this a calibrated Eppley Thermopile serial no. 5094 was used. The calibration for this is 0.054 microvolts/microwatt/ square cm. At each wavelength the thermopile and photomultiplier were placed in the monochromatic light emerging from the slit with the cone removed. Their sensitive elements were placed at the same distance from the slit and in the same mean position. The exposed area of the photomultiplier was 0.0576 cm^2, the same order of magnitude as the output area at the top of the cone (0.0256 cm^2). Repeated (7-10) measurements of the change in voltage from the thermopile were taken and the average of these is reported in Table II. The current from the photomultiplier is also an average over several readings. I suspect that most of the 1% variation in photomultiplier readings is due to fluctuations in the output of the lamp.

A similar calibration to determine the wavelength sensitivity of the PbS detector was made. Since readings for this calibration were not taken at the same time it is possible only to compare ratios of intensities. Thus on different days, the output of the lamp may vary as much as 20%, yet the ratio of intensity at a wavelength, λ, to that

TABLE I Calibration Values at the Top of the Cone

Wavelength	Photomultiplier Current	PSD Voltage 24°C, 10x gain
nm	μA	mv
400	60	6.70
410	86	8.07
420	113	9.38
430	144	7.82
440	178	12.1
450	215	13.6
460	261	15.7
470	305	17.5
480	334	18.5
490	358	19.6
500	371	20.6
510	380	22.5
520	405	24.9
530	400	25.7
540	373	27.6
550	339	27.7
560	304	28.7
570	260	31.2
580	215	31.5
590	162	33.6
600	108	33.7
610	72	34.5
620	48	34.6
630	32.8	40.5
640	24.5	42.8

TABLE II Calibration of Wavelength Sensitivity of Photomultiplier

Wavelength nm	Photomultiplier Current μA	Thermopile Voltage μV	Spectral * Sensitivity, Y μw/μA
400	60	1.21±.04	.0215
410	78	1.44±.02	.0197
420	99	1.65±.02	.0168
430	122	1.85±.02	.0162
440	143	2.09±.09	.0156
450	165	2.30±.04	.0149
460	194	2.69±.04	.0148
470	212	2.91±.05	.0146
480	222	3.05±.10	.0147
490	227	3.16±.04	.0149
500	22	3.37±.15	.0158
510	226	3.49±.13	.0165
520	235	4.01±.13	.0182
530	218	4.09±.09	.0200
540	195	4.22±.18	.0231
550	175	4.30±.08	.0268
560	149	4.38±.20	.0313
570	125	4.54±.08	.0387
580	100	4.66±.20	.0497
590	74	4.81±.11	.0694
600	50.5	4.84±.17	.102
610	33.2	4.87±.17	.1565
620	22.2	4.96±.17	.238
630	15.1	4.95±.13	.350
640	11.2	5.16±.20	.491

* See Appendix

at 550 nm varies less than 10% and usually not more than 4%. Absolute and relative correction factors for the PbS readings are tabulated in Table III. The absolute calibration is only accurate to ± 20%. the relative calibrations to ± 5%.

From the calibrations performed it is possible to arrive at a curve for the relative intensity at the top of the cone to that at the bottom, I_T/I_B (Fig. 6). A second curve shown in the same diagram was calculated from the relative intensity at the top of the cone divided by the relative intensity as measured by the PSD, I_T/I_{PSD}. Since the only theoretical differences in the numbers used in these calculations should be constants relating areas, the curves should by identical, such constants cancelling in the calculation. Within experimental error these curves are identical. (See Appendix for the calculations leading to these curves)

IV-2 Intensity Response of PSD

The response of the PSD to increasing intensity of the reference beam is shown in Fig. 7. This calibration was performed by placing an interference filter in the reference beam and adding calibrated neutral density filters. The response of the PSD was found to mirror that of the PbS detector as measured by an oscilloscope. Thus the non-linearity of response with intensity is produced by the PbS detector, not the electronics connected with the PSD.

TABLE III Phase Sensitive Detector Wavelength Sensitivity

Wavelength, nm	Spectral Sensitivity, Q* μw/mv
400	0.134
410	0.132
420	0.131
430	0.175
440	0.128
450	0.126
460	0.127
470	0.123
480	0.122
490	0.119
500	0.121
510	0.115
520	0.119
530	0.118
540	0.113
550	0.115
560	0.113
570	0.108
580	0.110
590	0.106
600	0.107
610	0.108
620	0.106
630	0.092
640	0.089

* See Appendix

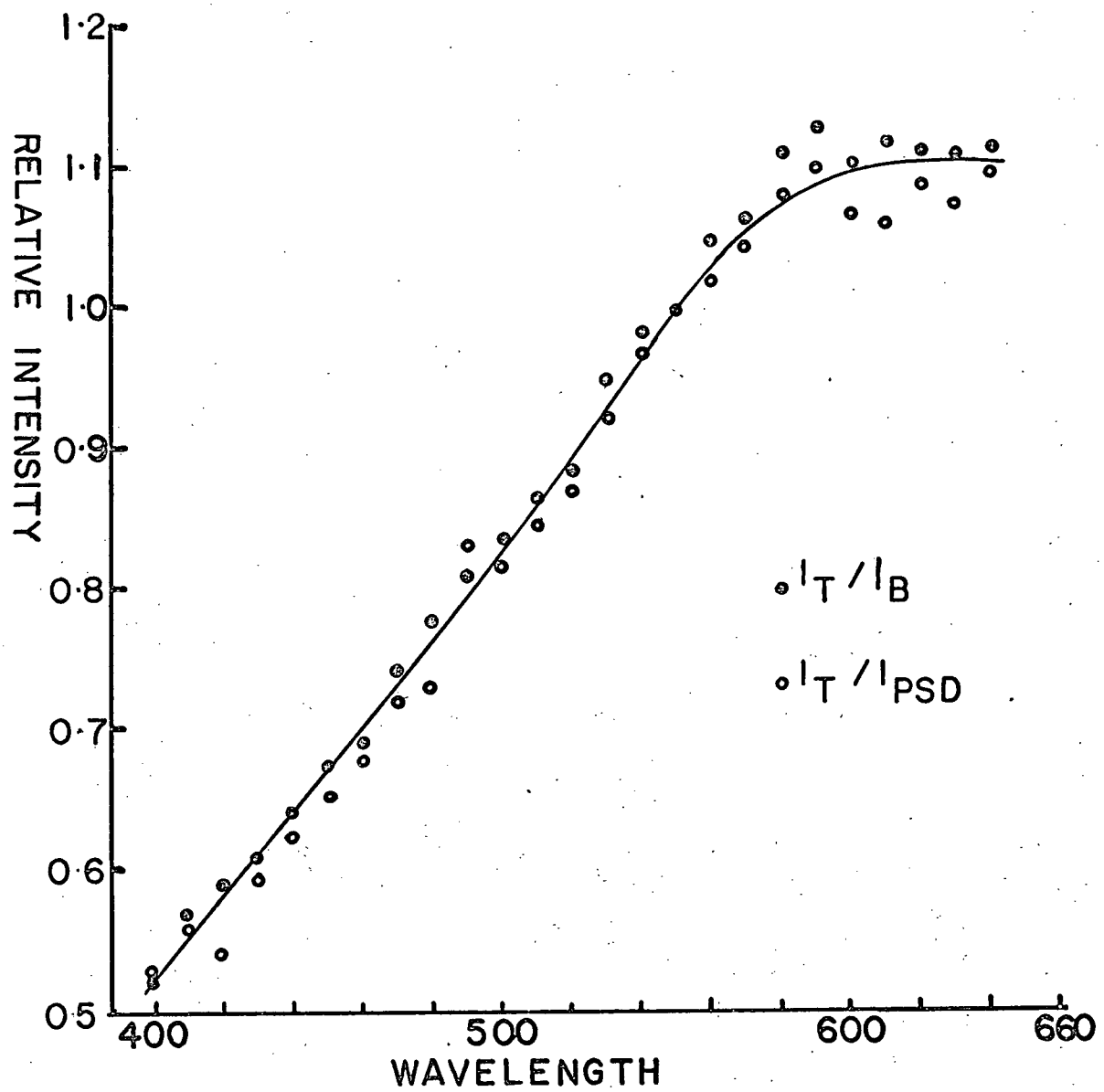
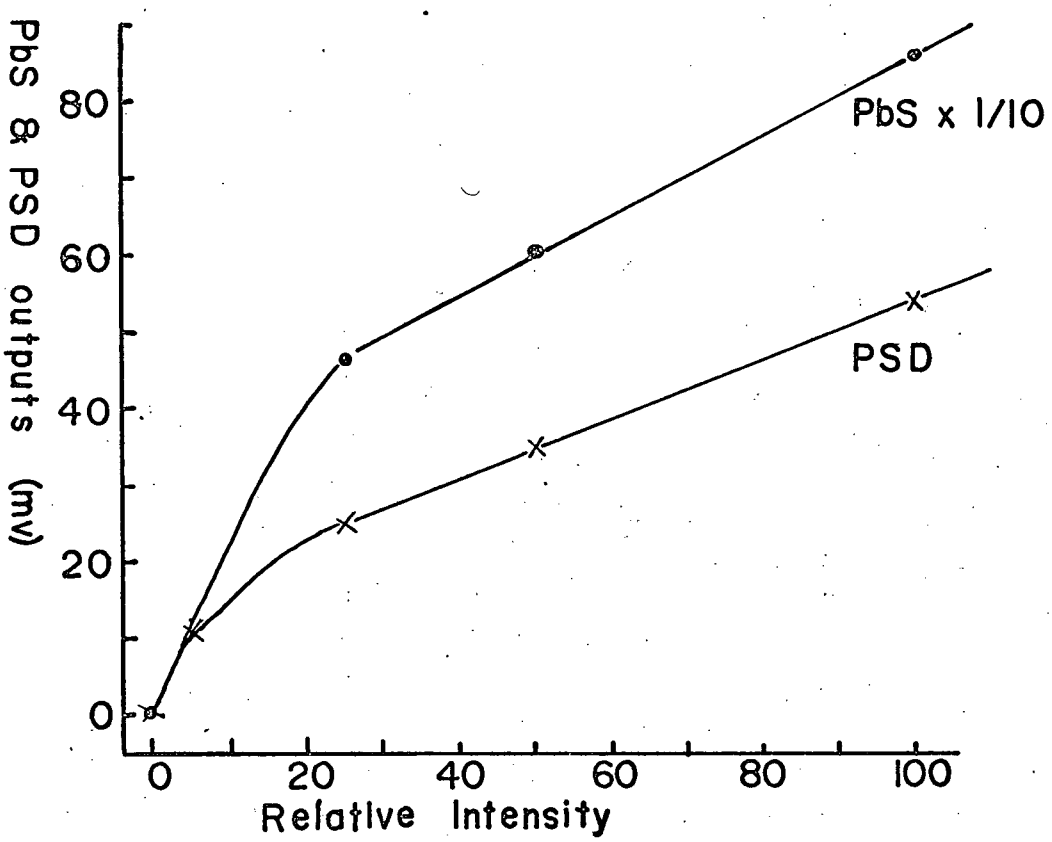


Figure 6 Relative Intensity at the Top of the Cone to that at the Bottom

Figure 7 Intensity Response of Lead Sulphide and Phase Sensitive Detectors



IV-3 Actinometer Calibrations

An attempt was made to perform a more accurate calibration of the intensity at the top of the cone using a potassium ferrioxalate actinometer. The technique as described by Hatchard and Parker (1956) is good for wavelengths up to 450 nm if a 0.15 M solution is used. In these experiments a solution thick enough to absorb all of the light emerging from the cone was used. Irradiation of the solution was carried out for periods up to three hours. Following this phenanthroline monohydrate and buffer were added and optical density difference readings taken between the sample and a blank. The optical density of the sample treated in this manner seldom reached 0.1 and the difference between the two was usually less than 0.05. The low values resulted because of the low output of the tungsten lamp in that spectral region. With such low values it was impossible to get reproducible results, in part because of the scattered light and also likely due to fluctuations in the lamp output over the long intervals of time required for the exposure. Thus it was found that in this situation this chemical actinometer could not be used.

CHAPTER V

RESULTS AND ERRORS

V-1 Oxygen Electrode Results

Samples for these experiments were prepared as in Sec. III-1.3. A recording of oxygen concentration versus time was made over the period required for the Nitrobacter to consume all of the oxygen in solution. From the calibrations and the recorded curves of oxygen concentration versus time the rate of oxygen uptake versus oxygen concentration was calculated. Plots of velocity, v, versus substrate concentration, $[O_2]$, and of 1/v vs $1/[O_2]$ are shown in figures 8 and 9 respectively. From these curves the Michaelis constant, K_m, for oxygen as substrate ranges from 0.021 to 0.055 mM O_2 depending on the concentration of nitrite. The results are recorded in Table IV.

Table IV K_m Values for Nitrobacter with Oxygen as Substrate

Preparation Procedure	K_m Values, mM O_2 v vs s	1/v vs 1/s	Fig. 8 and 9 Curve
2x concentration	0.032	0.032	3
5x concentration	0.040	0.043	1
5x concentration	0.021	0.022	2
5x concentration	0.055	0.055	

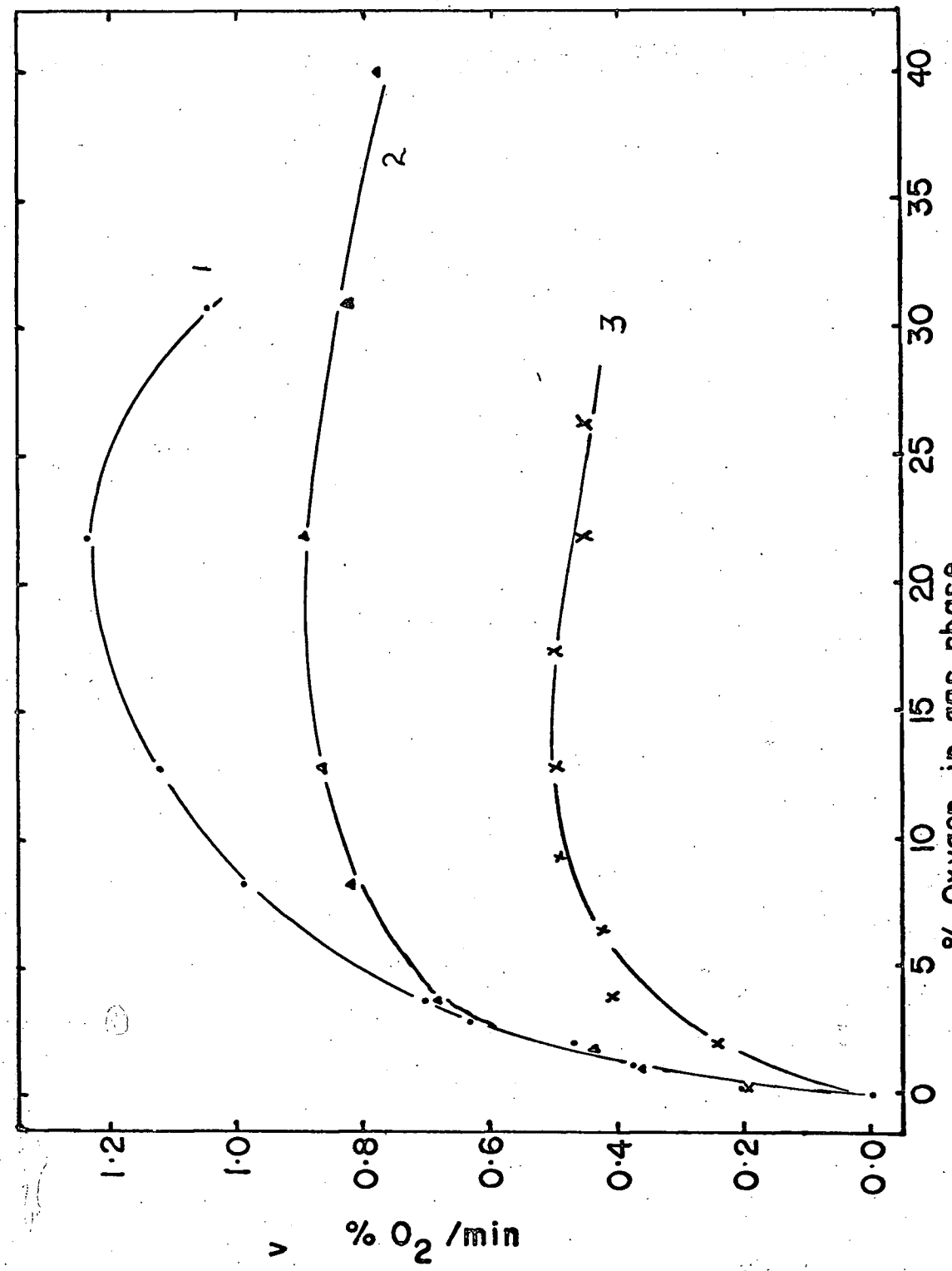


Figure 8 Effect of Oxygen Concentration on Rate of Nitrite Oxidation

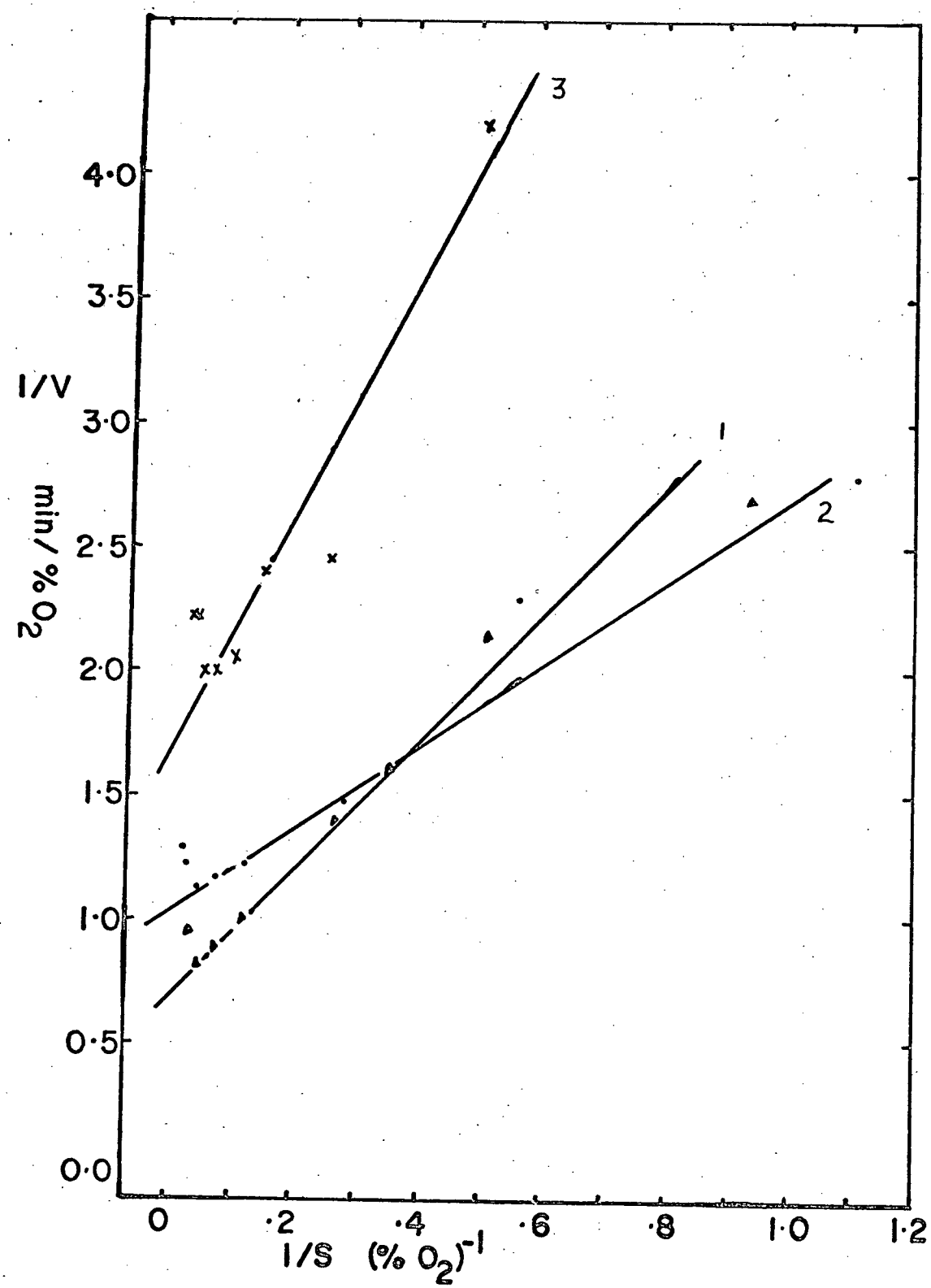


Figure 9 Michaelis-Menten Plots for Data of Fig. 8

V-2 Results from the Action Spectrum Apparatus

2.1 Procedure for Obtaining Results

The prepared sample was placed in the chamber as described in Sec. III-2.4. The electrodes were polarized and after a period of about 15 min the current, recorded on an Esterline Angus recording milliammeter, reached its equilibrium value. At this time when light was switched onto the sample, the current throught the electrodes decreased to a new equilibrium value. The reason for this change is that the light breaks up the carbon monoxide-oxidase complex, thus allowing the oxidase to react with oxygen. This results in a lower oxygen concentration in the drop and a lower electrode current. The dark current values ranged from 20-40 nA. A change from dark to the reference beam (with a heat filter, Corning aklo no. 97, and a green band pass filter to limit the spectral range) decreased the current 3-5 nA. A greater sensitivity in the form of larger absolute current changes was obtained with lower values of the dark current. All slit widths were 1 mm and the 1nA scale was used for the current readings with the remainder of the current bucked out by and external circuit. A typical change from dark to light and vice versa is shown in figure 10.

The CO action spectrum is a plot of the relative extinction coefficient $\varepsilon_{\lambda 1}/\varepsilon_{\lambda 2}$ of the enzyme-CO compound with wavelength. The reference wavelength is usually taken as 550 nm. As shown by Brooks, the ratio is given by

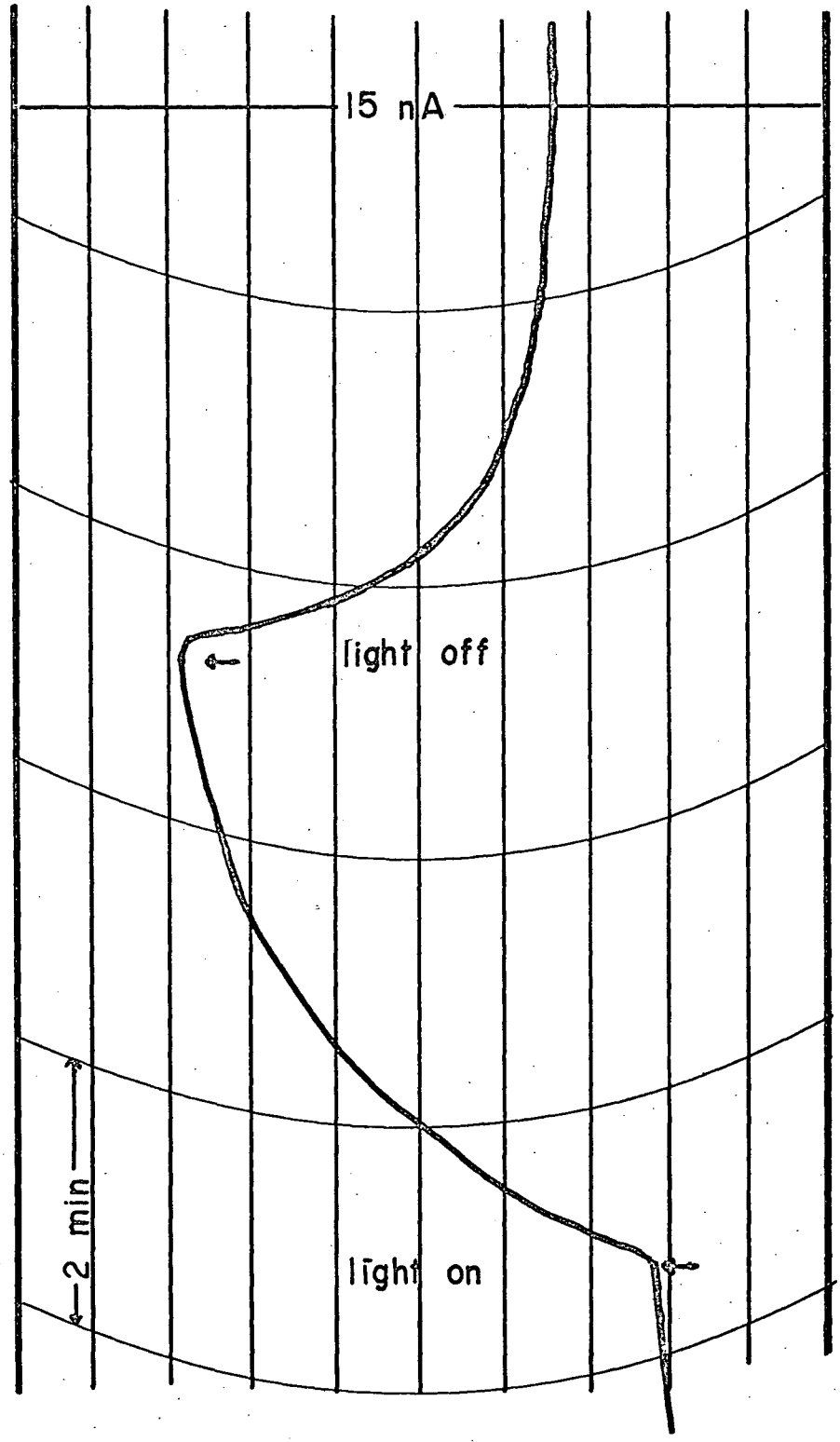


Figure 10 The Effect of Light on the Electrode Current of a Carbon Monoxide Inhibited Sample of Nitrobacter

the formula:

$$\frac{\varepsilon_\lambda}{\varepsilon_{550}} = \frac{C_\lambda}{W_\lambda} \frac{W_{550}}{C_{550}} \frac{550}{\lambda}$$

C_λ is the intensity of the comparison light which gives the same photochemical effects as the monochromatic light of wavelength, λ.

C_{550} is the intensity of comparison light which gives the same photochemical effect as 550 nm.

W_λ is the intensity of λ

W_{550} is the intensity of 550 nm light.

In practical terms this means that switching from the reference beam with intensity C_{550} to the 550 nm monochromatic beam produces no change in the electrode current.

The intensities which cause equal effects were determined by first shining the light of wavelength, λ, on the sample and after equilibration switching to the reference beam. If the current decreased, the reference light was too intense and the louvers were adjusted. This operation was repeated until no change was observed on switching. Since there was usually a drift in the current, a decrease or increase in current was indicated by a change in the slope of the drift. The resulting trace of current with time is shown in Fig. 11. The estimation of the balance point could only be made within 1 millivolt of the intensity of the reference light since the smallest turn of the louvers during the operation corresponded

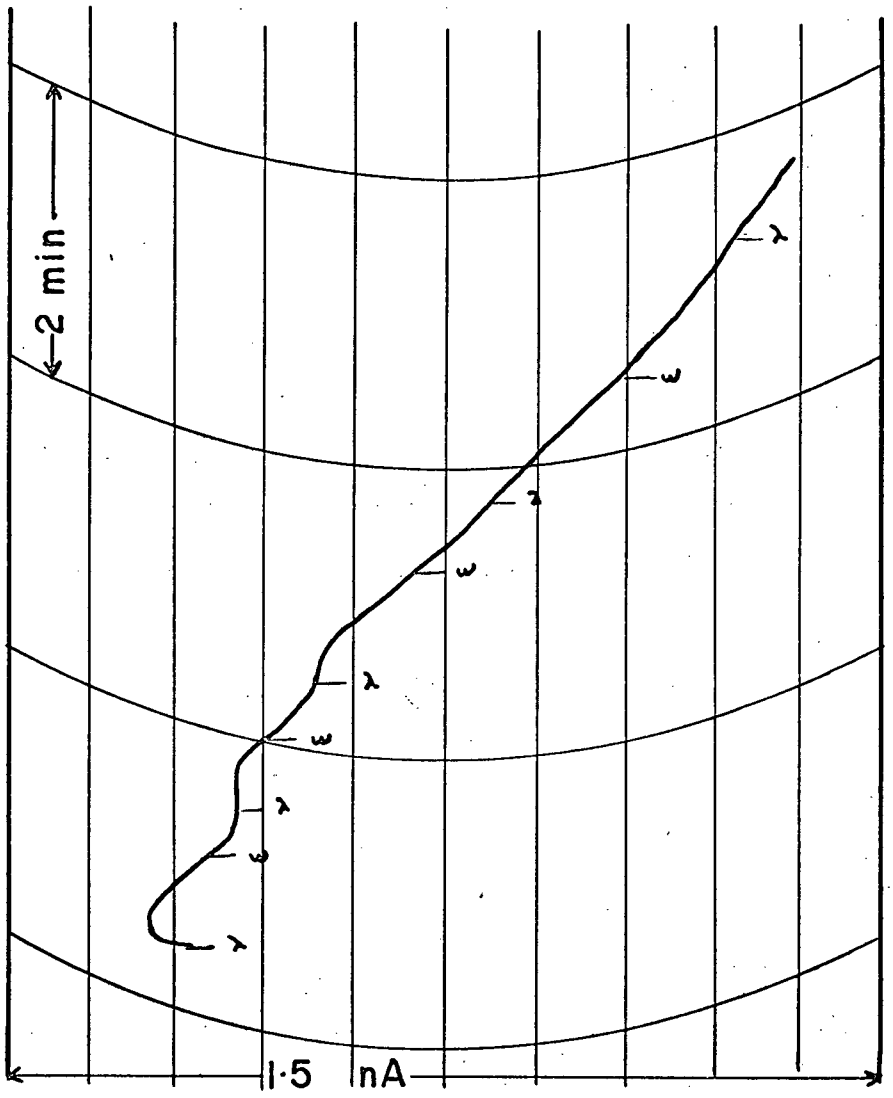


Figure 11 A Typical Determination of the Balance Point for Light of Wavelength, λ, and The Reference Light, ω.

to a 1 mv intensity change. The average intensity of the reference beam in these experiments was 67 mv. Thus the uncertainty in the balance point is $1\frac{1}{2}$ %.

Once the balance point was determined the intensities of the reference and monochromatic beams were measured and the temperature of the PbS detector taken. The balance for the intensity measurements was made on the 10 mv scale of the Keithley microvoltmeter-ammeter with the bucking voltage supplied by an external circuit (cf. Fig. 2). The PbS readings were corrected to 24°C and 10 x gain on the PSD. The gain factors were 2.57 for the three times scale and 0.324 for the 30 times scale. The intensity of the monochromatic beam was corrected to give the intensity at the top of the cone, i.e. corrected for PbS wavelength sensitivity and cone transmission. Since the response of the PbS detector is not proportional to the intensity falling on it a correction to the intensity of the comparison beam is also required. A sample calculation of $\varepsilon_\lambda / \varepsilon_{550}$ is outlined below for λ=600 nm.

DATA	W_{550}	C_{550}	W_{600}	C_{600}
PSD Readings (mv)	31.7	84.4	36.2	85.75
Temp. (mv)	0.96	0.96	1.00	1.00
Temp. corrected PSD readings *	30.9	82.3	37.2	88.1

CALCULATION

$$\frac{\varepsilon_{600}}{\varepsilon_{550}} = \frac{88.1 \times 30.9 \times 550}{37.2 \times 82.3 \times 600}$$

$= 0.815$

From Fig. 7, the correction for the linearity of the PbS detector is 1.02 and from Fig. 6, I_T / I_{PSD} for $\lambda = 600$ nm is 1.067. Therefore,*

$$\frac{\varepsilon_{600}}{\varepsilon_{550}} = 0.815 \times 1.02 \times 1.067$$
$$= 0.890$$

* See Appendix.

2.2 Results

The resulting values of $\varepsilon_\lambda / \varepsilon_{550}$ are displayed in figures 12 and 13. The first, Fig. 12, shows the results obtained with two preparations of Nitrobacter. These results were picked to be shown in this manner because of their self-consistency. Results obtained were usually more consistent within preparations than between preparations. The second graph, Fig. 13, is a graph of all those points which I felt were credible. The reasons for including these points and not others that I took are the following:

i) Once the Nitrobacter in the chamber started to die the current increased at an accelerated rate. As this happened, the determination of the balance point became more and more uncertain. Points obtained with a fast drift were thus usually discarded.

ii) Erratic response of some preparations meant that balance could only be obtained on the 3 nA

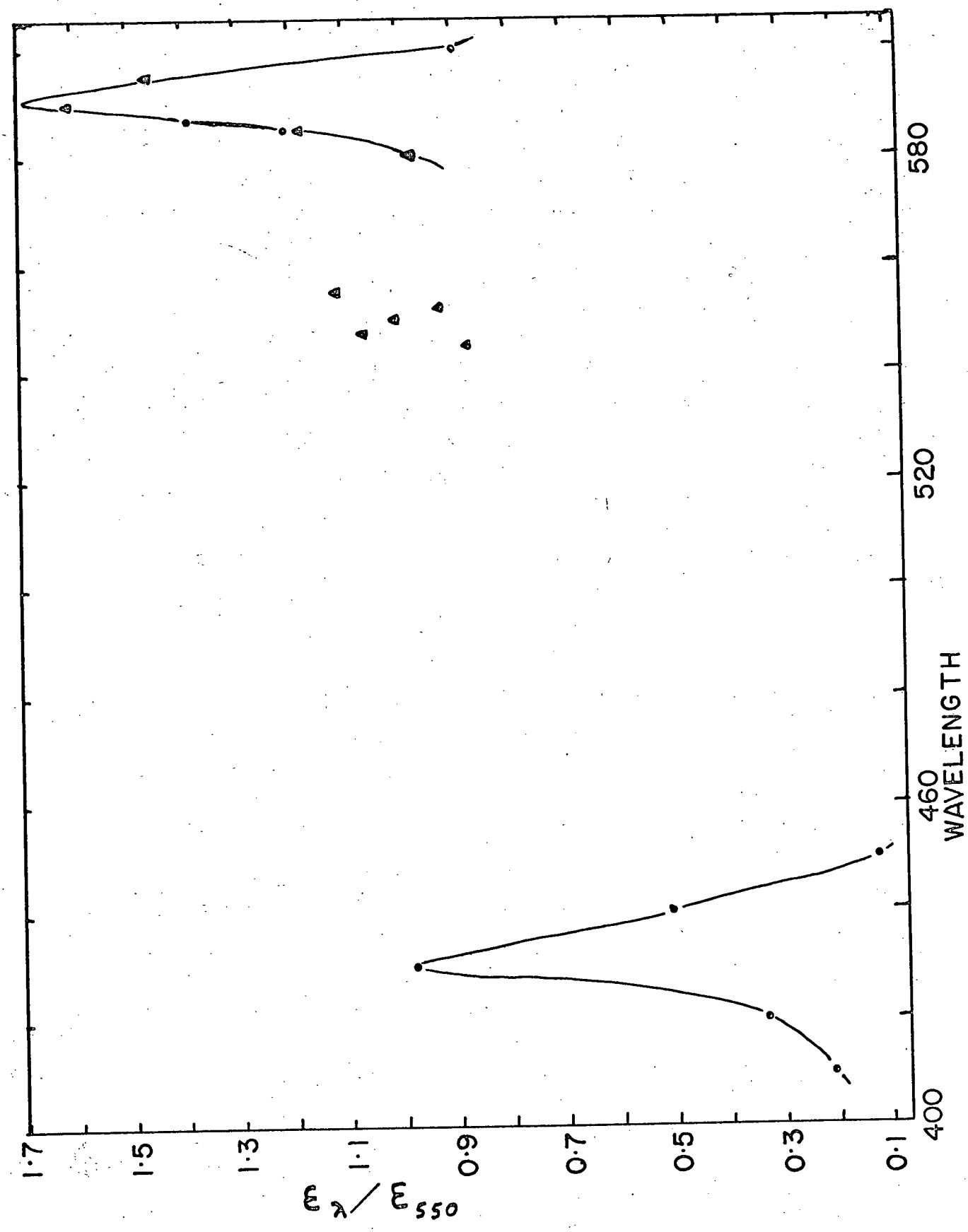


Figure 12 Relative Extinction Coefficients Obtained with two Preparations of Nitrobacter

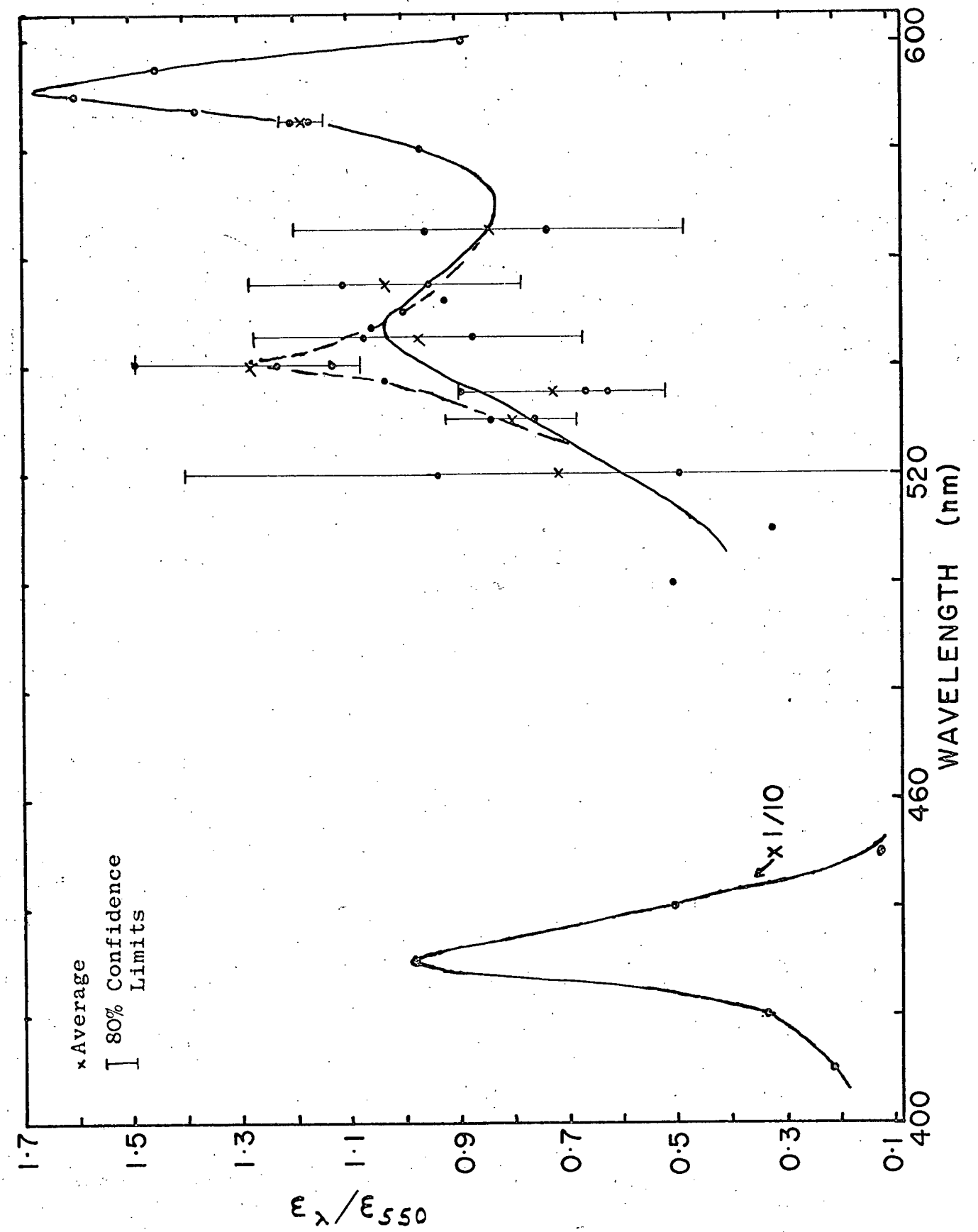


Figure 13 Action Spectrum of Nitrobacter agilis

scale. This was later discovered not to be a sensitive enough determination and the points were disregarded.

iii) Some preparations gave rise to only one point on the graph of $\epsilon_\lambda / \epsilon_{550}$ vs λ. If, coincident with this the total change from dark to light was less than 2 nA, these points were also discarded.

2.3 Errors in the Relative Extinction Coefficient

The error in $\epsilon_\lambda / \epsilon_{550}$ is the sum of the inherent errors and uncertainties in each of the terms in the equation.

i) The determination of the balance point is accurate to $1\frac{1}{2}$ % (cf. Sec. V-2.1). Since there are two such balances required for determination of a point the error introduced is 3%.

ii) Brooks shows that the calculation of the temperature coefficient and its application introduces a 1% uncertainty in the monochromatic intensities with much less than 1% error in the reference beam.

iii) Variation in output of the lamp and in the gain levels of the PSD introduce a 5% uncertainty in the values of the relative intensity at the top of the cone divided by those measured by the PbS and phase sensitive detectors.

The accuracy of $\varepsilon_{\lambda} / \varepsilon_{550}$ is thus

$$\frac{\varepsilon_{\lambda}}{\varepsilon_{550}} = \frac{C_{\lambda}}{W_{\lambda} \pm 6\%} \frac{W_{550} \pm 6\%}{C_{550}} \frac{550}{\lambda} \pm 3\%$$

making the limit of accuracy $\pm$ 15%.

On the graph, Fig. 13, the error bars are the 80% confidence limits calculated according to Wilson (1952, pg.239). They are larger than the 15% calculated above, in some instances. The reason for this discrepancy lies in the necessity of using different samples of Nitrobacter. Thus while each point is accurate to within 15%, the points at the same wavelength in different samples of bacteria may not have relative extinction coefficients within 15% of one another. The discussion in Sec. VI-2 is relevant to this apparent discrepancy.

2.4 Errors in the Positions of the Peaks

The peaks at 430 and 591 nm can be more accurately determined because of their steepness. (See Castor and Chance, 1955) Thus the error in these peaks is $\pm$ 2 nm of which 1 nm error is introduced in the determination of the wavelength delivered at the exit slit by the monochromator. The peak at 540-550 nm is much broader and therefore harder to outline. Added to this the line of average points is not continuous introducing further estimation errors. Thus the error limit on the two apparent peaks at 541 and 550 nm is $\pm$ 5 nm.

CHAPTER VI

DISCUSSION

VI-1 Oxygen Electrode Results

The variation of V_{max} and $K_m(O_2)$ has been reported and well documented by Butt and Lees (1964). The maximal rates reported by these authors were found to increase and then decrease with increasing nitrite concentration. The same was true of the $K_m(O_2)$. They reported K_m values between 2 and 7.5 % O_2 in the gas phase. Comparable results were obtained in these experiments with K_m values between 0.021 and 0.055 mM (1.75 and 4.6 % O_2 in the gas phase).

Samples for which rates were obtained above 20% O_2 showed a decrease in rate with increasing O_2. This is not due to the establishment of the polarization voltage which takes several minutes to establish in the sample chamber. This process results in an apparent increase in the rate. Thus the effects are possibly one of two: 1) a substrate inhibition, or 2) that the cells had not recovered from centrifugation and exhibited a lower rate until complete recovery was reached. (Note the 30 min wait required in the action spectrum experiments, Sec. III-2.4) This latter effect is very difficult to deal with since the effects of centrifugation vary from sample to sample even though the previous history appears to have been identical.

Maximal rates reported by Butt and Lees ranged from

1.9 to 2.7 micromoles oxygen/vessel/hr where each vessel contained 3 ml of suspension. Values obtained for 7 ml of suspension in these experiments ranged from 0.7 to 3 μmoles/vessel/hr. These rates are lower but of the same order of magnitude as those of Butt and Lees, the discrepancy due probably to a difference in the concentration of cells in the suspension but perhaps also due to the method of measurement.

VI-2 Action Spectrum Results

Comparison of the peak positions obtained here at 430, 540-50, and 591 nm with those obtained by the same methods on other organisms (Castor and Chance, 1959) show that these peaks indicate the presence of either cytochrome $\underline{a}_1$ or $\underline{a}_3$, peaks being reported at 427-8, 548 and 591-2 nm for cytochrome $\underline{a}_1$ and at 430-2, 547-50 and 585-91 for cytochrome $\underline{a}_3$. Absorption spectra of Nitrobacter report peaks at 586-94 and 438 showing an $\underline{a}_1$ rather than an $\underline{a}_3$ cytochrome. (cf. Sec I-5.1 and I-5.2) Carbon monoxide reduced minus reduced difference spectra (Sec. I-5.3.1) implicated cytochrome $\underline{a}_1$ in the binding of CO with peaks at 450 and 426 and troughs at 439 and 594 nm. Although Van Gool and Laudelout report no peak around 590, Castor and Chance (1953) reported a peak at 590 nm for cytochrome $\underline{a}_1$ in a CO difference spectrum of A. pasteurianum.

None of the peaks shown in this action spectrum is as well defined as those shown by Castor and Chance (1959)

They were able to determine the points on their spectra consecutively and "efforts to return later to a given wavelength showed a reproducibility of about ± 5%". This was with responsive organisms such as E. coli. Nitrobacter is however a much more sensitive organism with a short life span in the apparatus. Both of these effects result in poorer reproducibility and necessitate trying to obtain an outline of a peak with one set of Nitrobacter rather than trying to define the peak in detail using many sets of Nitrobacter. This latter approach usually resulted in a poorly defined peak.

Comparison of the relative extinction coefficients of the peaks for cytochrome a_1 bands of the action spectrum of A. pasteurianum (Castor and Chance, 1953) gives values of 2.35 and 21 for the 592 and 428 peaks. Values of $\epsilon_\lambda/\epsilon_{550}$ for the same peaks in this work were 1.68 and 9.8. The ratio of these values is 0.112 for Castor and Chance and 0.168 for this work. The error in the 0.112 determination is of the order of 10%, that in the 0.168 is 30%. Thus the two values are the same within experimental error. The cause of the difference if it is significant most likely lies in the role of cytochrome o. No diagram of the β, 548 band for A. pasteurianum was given nor are there any other values of the relative extinction coefficients for cytochrome a_1 in other organisms. Thus it is not possible to make an extensive comparison of these values for significant differences.

Castor and Chance (1959) showed that when two oxidases are present in a cell, either pigment appears capable of

catalyzing most of the respiration. This experiment was performed by illuminating the cells with light which would only be absorbed by one pigment. With this light most of the CO inhibition was relieved. Thus (Horio and Taylor, 1965) when two or more oxidases operate in parallel the action spectrum may or may not reflect this fact, depending on both the relative activities and the light sensitivities of the CO complexes. Thus the possible presence of other cytochromes can not be ruled out. This is especially evident in the peak at 540-550 nm. The displacement of this peak to lower wavelengths could be due to the presence of a second cytochrome with a peak at or below 540 nm. The major candidate is a cytochrome o peak at 535-7 nm. The presence of cytochrome o would also require an indication of peaks at 416-7 and 566-7. These are not evident in the spectrum displayed. If cyt. o is present, the reason for the absence of these two peaks could be one of two effects. The first is that the peaks are masked by the cytochrome a_1. The effect on the ß peak would seem to indicate the presence of cytochrome o in large enough quantities to affect the spectrum more drastically in the region of 415-430 nm. Thus masking does not seem to be the answer. The second possible explanation lies in a difference in the cells used to determine the points. Castor and Chance (1959) showed different cytochrome oxidase activities in stationary and log phase cells. Thus it is possible that cells containing no cytochrome o or containing a cytochrome o with a very low activity were used to determine the α and γ

peaks but that the ß peak was partially determined with cells containing and active cytochrome *o*. Although absorption spectra give no hints of cytochrome *o*, there is no reason to rule it out because the present method is much more sensitive.

Thus it can be stated that cytochrome a_1 is acting as a terminal oxidase in *Nitrobacter agilis* and that there is a possibility that other cytochromes may also act as oxidases in certain cells. If cytochrome *o* does act as a terminal oxidase, a study of log phase cells is very likely to show this as all of the types of organisms studied by Castor and Chance (1955 and 1959) which showed some cytochrome *o* activity in the stationary phase showed only cytochrome *o* activity in the log phase of growth.

Action spectra results tabled by Castor and Chance (1959) implicate cytochrome a_1 as an oxidase in four bacteria, all heterotrophs. They are *Acetobacter pasteurianum*, *Acetobacter peroxydans*, *Azotobacter vinelandii*, and stationary phase *Proteus vulgaris*. *A. vinelandii* also showed cytochrome *o* activity, *P. vulgaris* also showed cytochrome *o* and a_2 activity in the stationary phase and showed only cytochrome *o* activity in the log phase. To date, only cytochrome a_3 has not been shown to be active in cells with active cytochrome a_1. A generalized statement about the associations of oxidases can not be made, however, until more organisms have been studied in their different phases of growth. The spectrum reported here is the first recorded action spectrum of a chemolithotropic bacterium.

VI-3 Summary

(i) A survey of the literature on Nitrobacter was performed and the unsolved problems pointed out.

(ii) Modifications to the action spectrum apparatus are described and the performance of the apparatus assessed.

(iii) The $K_m(O_2)$ Of Nitrobacter agilis varies from 0.021 to 0.055 mM O_2 depending on the nitrite concentration in agreement with the data of Butt and Lees (1964).

(iv) Cytochrome a_1 acts as a terminal oxidase in Nitrobacter agilis.

(v) Parallel participation of other cytochromes as oxidases is not ruled out. A distorted β band suggests the presence of cytochrome o in some cultures.

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APPENDIX

Calculations Performed in Calibrating the Action Spectrum Apparatus

In this section a sample calculation of the relative intensity at the top of the cone is performed for the wavelength 600 nm.

Abbreviations:

Pm, Photomultiplier reading, μA

Tp, Thermopile reading, μV

PSD, Phase Sensitive Detector Reading, mV

$(Pm)_{Top,\lambda}$ Photomultiplier reading at the top of the cone at a wavelength, λ

$(Pm)_{Bott,\lambda}$ Photomultiplier reading at the bottom of the cone at a wavelength, λ

$(Tp)_{Bott,\lambda}$ Thermopile reading at the bottom of the cone at a wavelength, λ

TABLE V Data Required for the Calculation of the Relative Extinction Coefficient

λ nm	$(Pm)_{Top}$ μA	PSD mv	$Temp_{PSD}$ mv	$(Pm)_{Bott}$ μA	$(Tp)_{Bott}$ μV
550	339	28.4	.962	175	4.30
600	108	32.8	.990	50.5	4.84
Area of measurement (sq. cm.)	.0256	.04		.0576	

Thermopile Calibration: 0.054 μV/ μw/ cm^2

Temperature Correction:

$$\log S = -2.4 \times 10^{-2} \quad T$$

At 24°C the thermocouple reading is 0.98 mv and the slope of the calibration graph is 24.4 C° per mv. Thus,

$$\log S_2 - \log S_1 = -2.4 \times 10^{-2} \times 24.4 \ (0.98 - X)$$

where, X = thermocouple reading at T_1
S_1 = PSD signal at T_1
S_2 = PSD signal at 24°C

Application of this formula leads to a change in the PSD readings, from 28.4 to 27.7 and from 32.8 to 33.7 mv.

Photomultiplier Correction for Wavelength Sensitivity

The correction factor for the photomultiplier readings at a wavelength, λ, are given by Y below:

$$Y = \frac{(T_p)_{Bott,\lambda} \ \mu V}{.054 \ \mu V/\mu w/ \ cm^2} \times \frac{.0576 \ cm^2}{(P_m)_{Bott,\lambda} \ \mu A}$$

$$= .0268 \ \mu w/\mu A \text{ at } 550 \text{ nm}$$
$$= .102 \ \mu w/\mu A \text{ at } 600 \text{ nm} \quad \text{(cf Table II and Fig. 14)}$$

PSD Correction for Wavelength Sensitivity

The correction factor, Q, for the wavelength sensitivity of the phase sensitive detector is given by:

$$Q = \frac{(Tp)_{Bott,\lambda} \ \mu V}{.054 \ \mu V/\mu W/cm^2} \times \frac{.04 \ cm^2}{PSD_\lambda \ mv}$$

$$= 0.115 \ \mu w/mv \text{ at } 550 \text{ nm}$$
$$= 0.107 \ \mu w/mv \text{ at } 600 \text{ nm} \quad \text{(cf Table III and Fig. 15)}$$

Since the phase sensitive detector and thermopile

Figure 14 Spectral Sensitivity of Photomultiplier Tube

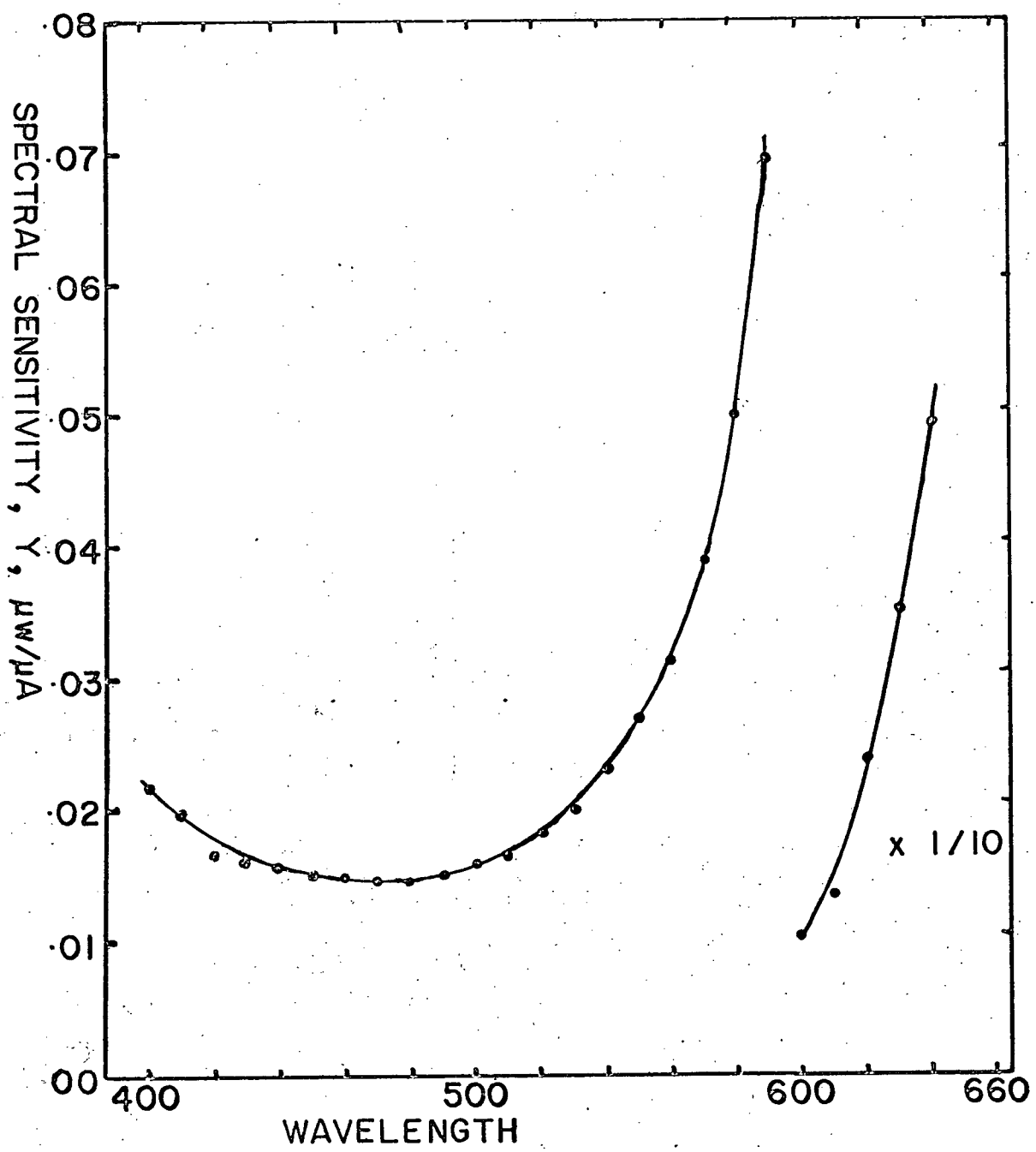
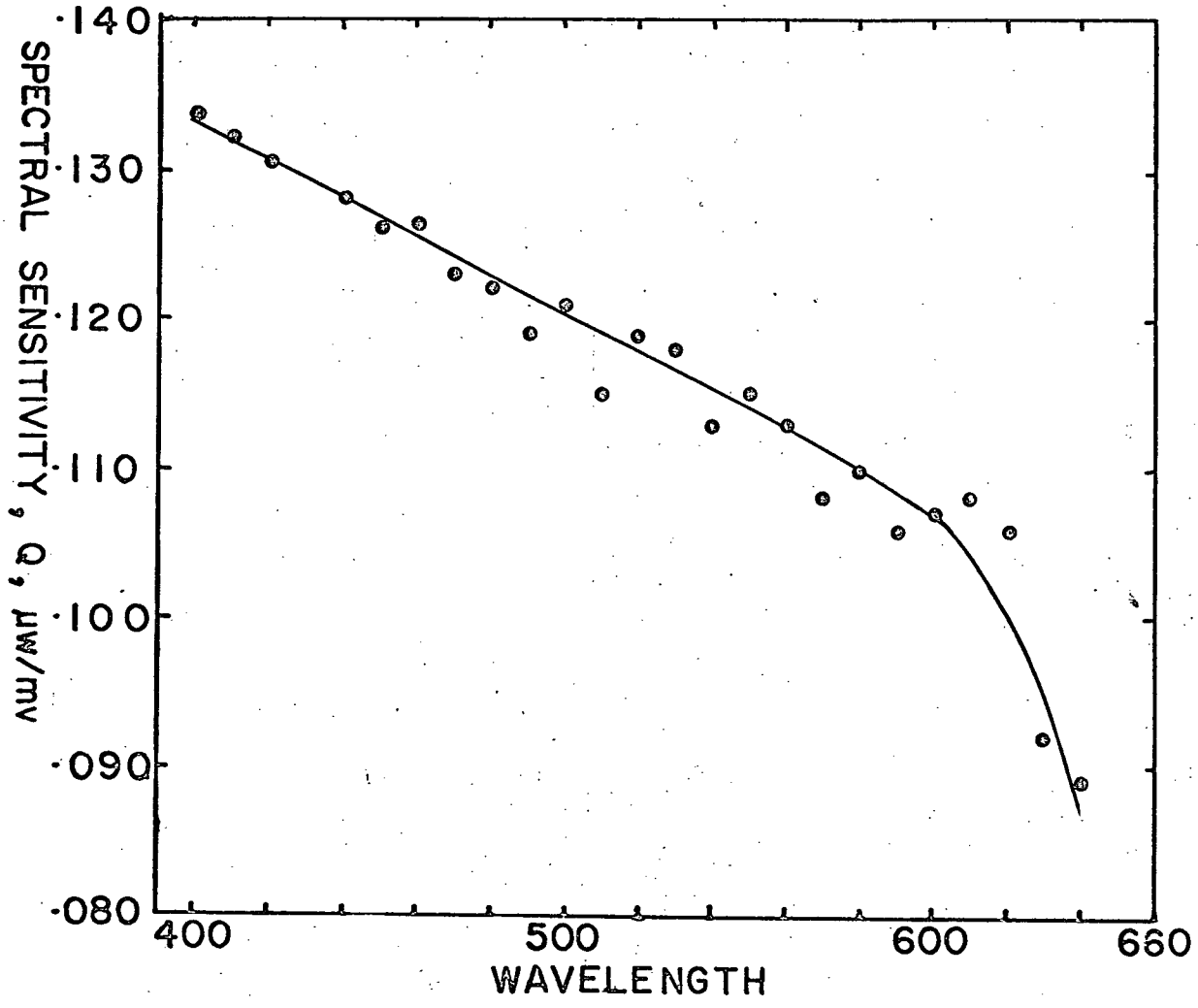


Figure 15 Spectral Sensitivity of Phase Sensitive Detector



readings are not taken at the same position there are factors relating the differences in areas, position and effects of the spherical mirror and tuning fork which are necessary if these numbers, by themselves are to mean anything. However, since all these factors are constant with wavelength, a relative calibration with reference to a common wavelength, 550 nm, is meaningful.

Calculation of I_T / I_B (Fig. 6)

$$\frac{I_T}{I_B} = \frac{\text{Relative Intensity at the Top of the Cone}}{\text{Relative Intensity at the Bottom of the Cone}}$$

$$= \frac{(Pm)_{Top,\lambda} \times Y_\lambda \; / \; (Pm)_{Top,550} \times Y_{550}}{(Pm)_{Bott,\lambda} \times Y_7 \; / \; (Pm)_{Bott,550} \times Y_{550}}$$

For λ = 600 nm,

$$I_T/I_B = \frac{108/339}{50.5/17.5}$$

$$= 1.105$$

Calculation of I_T / I_{PSD} (Fig. 6)

$$\frac{I_T}{I_{PSD}} = \frac{\text{Relative Intensity at the Top of the Cone}}{\text{Relative Intensity at Bottom of Cone as measured by the PSD}}$$

$$= \frac{(Pm)_{Top,\lambda} \times Y_\lambda \; / \; (Pm)_{Top,550} \times Y_{550}}{(PSD)_{Bott,\lambda} \times Q_\lambda \; / \; (PSD)_{Bott,550} \times Q_{550}}$$

$$= 1.067 \text{ at } 600 \text{ nm}$$

Thus as noted in Sec. IV-1, the values of I_T/I_{PSD} and I_T/I_B differ by 4% and are, within experimental error,

identical. The number I_T/I_{PSD}= 1.067 means that if the intensities of the two beams at the bottom of the cone are identical, the 600 nm light will be 1.067 times as intense as the 550 nm light at the top of the cone. Thus to correct W_{550}/W_{600} it is necessary to multiply by 1.067.