POLAROGRAPHIC STUDIES

ON

THE HYDATID CYST SYSTEM

BY

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TO

MY DEAR WIFE, FAITHFUL PARTNER

IN LIFE

I DEDICATE THIS WORK

ACKNOWLEDGMENT

The author wishes to express his deep indebtedness and gratitude to Dr. C. Richard Zobel under whose supervision and wise guidance this research was done.

For his great contributions in the biological aspects of this study, Dr. C. Schwabe deserves sincere thanks.

ABSTRACT

In this thesis the results of some studies on the respiratory rate of hydatid cyst parasites in cyst fluid are presented and discussed. By utilizing polarographic techniques it was possible to determine the time rate of change of dissolved oxygen in the system under investigation. The relative results, obtained in terms of diffusion current from the Polarograph, were converted to absolute values through the use of a calibration curve. The calibration curve was prepared by making absolute oxygen determinations with an "Ascorbic Acid Oxidase" chemical method. The use of Winkler's method for absolute oxygen determinations was also investigated but it was found to be inapplicable to the cyst fluid.

Graphical presentation of the variation in dissolved oxygen content of the cyst fluid (due to oxygen consumption by the parasites) with time shows that after a limiting concentration is reached no more oxygen is consumed. When the rate of oxygen consumption is plotted as a function of the oxygen concentration sigmoidal shaped curves are usually obtained.

By determining the oxygen tension of the cyst fluid in vivo and comparing this value with the previous data, it is possible to estimate the relative, in vivo, respiratory activity of the parasites.

The significance of the results obtained is discussed

and where possible these results are compared with those of other investigations on similar systems.

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INTRODUCTION

Echinococcosis or hydatid disease is a disease of significant incidence in Lebanon as well as other temperate or subtropical areas in countries where sheep-raising is a major occupation. Tests on a series of animals (camels, cattle, dogs and sheep) in Lebanon revealed that the incidence of infection varied from about 65 - 6%, which is significant. In the 12 year period from 1937 - 1949, 86 cases of human infection were reported at the Hospital of the American University of Beirut. (24)

Although the prevalence of the disease and some of its pathological aspects have been known since the nineteenth Century, little fundamental information concerning its basis is currently available. For this reason a program of investigation of the host-parasite relationships and possible therapy of the disease has been initiated by Dr. C. Schwabe (Chairman, Department of Tropical Health) at the American University of Beirut.

One aspect of these investigations is an attempt to discern the respiratory mechanism of the hydated cyst in vivo. This requires a quantitative knowledge of the oxygen exchange between cyst scolices and the cyst fluid and may be conveniently studied by polarographic analysis - the determination of this data is the subject matter of this Thesis. It should, perhaps, be pointed out that a more

common method of investigation of respiratory mechanisms is the manometric Warburg technique. However, this method suffers from the disadvantage that the rate of oxygen exchange may be determined only at given fixed pressures in any individual experiment. If the rate of exchange is wanted at several different pressures, several different experiments must be carried out. The polarographic method has the advantage of determining the rate continuously as the oxygen pressure varies continuously. It should be noted, however, that CO_2 exchange can not be determined polarographically.

Before discussing the details of the current investigations it might be worthwhile to briefly sketch the biology and history of the hydated disease. The adult Echinococcus granulosus lives in the small bowel of the dog and other definitive hosts. Its eggs are discharged with the feces of the host. Injestion of these eggs through the mouths of cattle, sheep etc and humans results on their becoming infected. The eggs frequently become dispersed throughout the body but are usually found to the greatest extent in the lungs, liver and spleen. The growth of the egg results in the formation of a cyst which may eventually attain a diameter of several inches. The cyst consists of an external wall and is filled with fluid and scolices, which are capable of further development. (It is the respiratory behavior of these scolices which were investigated here.) If the cyst is later injested by a dog (or other

host) the parasites will mature the adults and the cycle may repeat itself again. (12)

Although a moderate number of publications concerning the hydated cyst have appeard during the past half Century, very little data is available concerning its metabolic processes. Most of the papers published have been concerned with the composition of the cyst fluid (both inorganic and organic constituents) (14), (19) the structure of the membrane wall (8), (10), (25) and the physiology of the parasite. (11)

T. Von Brand and coworkers have published a report (4) of some preliminary investigations of the metabolic process of E. granlosus. By manometric techniques they determined the rate of oxygen consumption of cyst scolices in ringer's solution at various temperatures, as well as in the cyst fluid. They also investigated the effect of various inhibitors and variations in the salt content of the solutions on this rate.

The experiments reported below are, to a certain extent, related to those of Von Brand et al, and when possible the results obtained here will be compared with theirs. Several significant limitations are apparent in Von Brand's work:

1) the rate was determined over a rather short time interval (one hour).

2) most of the experiments were carried out in an

artificial medium rather than in the cyst fluid.

3) the oxygen tension existing in the cyst fluid in vivo was not determined.

4) exceedingly small samples were used which makes possible the introduction of rather significant errors.

5) the rate was determined only at a one oxygen tension rather than determining it at various oxygen tensions.

The experiments carried out here were briefly as follows:

1) the oxygen tension of the cyst fluid in vivo was determined.

2) the rate of decrease of the concentration of dissolved oxygen in the fluid containing scolices, was determined over an extended period of time.

By comparing the variation in the rate of oxygen consumption by the parasites with the oxygen concentration of the cyst fluid, it was hoped to gain some insight into the relative importance of anerobic and aerobic respiration mechanisms in the scolices. It is possible to compare our results somewhat indirectly with Von Brand's work (4), but it must be kept in mind that due to the different procedures used, direct comparison is not possible.

Since this is the first time a polarographic procedure has been applied to this system, it was found necessary to make a great many trials to determine the applicability and limitations of this procedure. Consequently these trial experiments constitute a significant portion of these investigations.

POLAROGRAPHY

Under certain controlled conditions a precise relationship exists between the applied voltage and the current flowing through an electrolysis cell. Furthermore the required voltage and the current flow are directly related to the nature and quantity of the reacting substance. This makes possible not only the qualitative, but also the quantitative analysis of an unknown solution from a knowledge of the current-voltage relations under specified, controlled conditions.

The apparatus employed for this type of analysis (polarography) is called the polarograph and the current-voltage curve obtained is a polarogram.

As is well known from the electrochemical series, every substance has a characteristic oxidation-reduction potential at which it may be electrolyzed (oxidized or reduced as the case may be). This then is the feature which allows the qualitative analysis of various substances by a knowledge of the potential required for their electrolysis. Secondly, in a diffusion process the rate of diffusion is controlled by the concentration gradient involved. Thus in the case of the diffusion of ions, the diffusion current is a measure of the concentration gradient and by standardization can be made a direct measure of the concentration of ions existing in the bulk of a solution.

The essential elements of a polarograph are: a small polarizable electrode at which electrolysis is carried out (the dropping mercury electrode or d.m.e.); a non-polarizable reference electrode (which may be either a large mercury pool or a saturated calomel electrode (S.C.E.) as used in these experiments); and the associated apparatus needed to yield the current - voltage relations at the d.m.e.

Since the electrolysis current is small, the quantity of substance electrolyzed is subsequently extremely small, so that the solution concentration remains essentially unchanged. This makes it possible to repeat the process several times on a given sample without the introduction of a measurable degree of error. Although the accuracy of the usual method of analysis is limited to $\pm$ 1% in solutions in the concentration range 10^{-2} down to 10^{-4} molar, and of the order $\pm$ 5% between 10^{-4} and 10^{-5} molar concentrations, greater accuracy can be obtained if needed. (16)

Polarography has been applied successfully in the analysis and study of almost all inorganic substances and to many types of organic substances.

It has also been applied to a rather large number of biological systems in the study of respiratory mechanisms. In 1938 and 1939 Petering and Daniels et al. (22), (23) investigated the applicability of the polarograph (actually only the d.m.e.) in determining the dissolved oxygen in biological fluids. They then determined the respiratory rate of yeast sample. Since that time a large number of

papers, on similar applications have appeared (see particularly the papers by Baumberger (1), (2), Bronk (5), (6), (7), Longmuir (18) and Skerman (29). But this method does not seem to have been applied to parasitological problems.

Definitions, Abbreviations and Fundamental Concepts

Polarogram -

the current-voltage curve (fig. 1) obtained during the electrolysis of the unknown substance at the d.m.e.

Half-Wave Potentials (Volts) -

the potential at the midpoint of the current-voltage curve, where the current is one half of its limiting value. It is designated by the symbol $E_{\frac{1}{2}}$. In these investigation the symbols $E^1_{\frac{1}{2}}$ and $E^2_{\frac{1}{2}}$ are used to denote the first and second half-wave potentials for oxygen, and are given in volts. "Half-wave potentials are usually independent of the concentration and have exact thermodynamic significance. They are related to saturated standard electrode potentials, and are reported with reference to the saturated or normal calomel electrode, which in turn are referred to the standard hydrogen electrode". (15)

Every element has a characteristic half-wave potential, which may be shifted by varying the pH of the solution, because pH variations may affect the concentration of the molecular form of the reducible or oxidizable material on

which the half-wave potentials are dependent.

Range of Reduction of Wave: (Volts) -

the potential range over which a substance is reduced. (from point A to B or A' to B' on figure I.)

i_d (mm.) -

the diffusion current, measured in millimeters of galvanometer deflection.

Limiting Current -

the maximum current obtained at any voltage and is a function of six known factors: the residual current, the diffusion current, the absorption current, the migration current, the kinetic current and the catalytic current.

Only the diffusion current is proportional to the concentration of the substance under test, the others are not; hence, the necessity of working with a controlled diffusion process by eliminating all other factors. Since the catalytic and kinetic currents do not appear in the reduction of oxygen they will not be discussed.

The Diffusion Current -

The reducible (or oxidizable) ion concentration is the same throughout the solution, before its decomposition potential is reached. Once the decomposition potential is reached, the ions in contact with the mercury drop begin to plate out, decreasing the concentration of ions at the interface of the Hg drop while the concentration of the ions in the body of the solution remains constant. Thus a

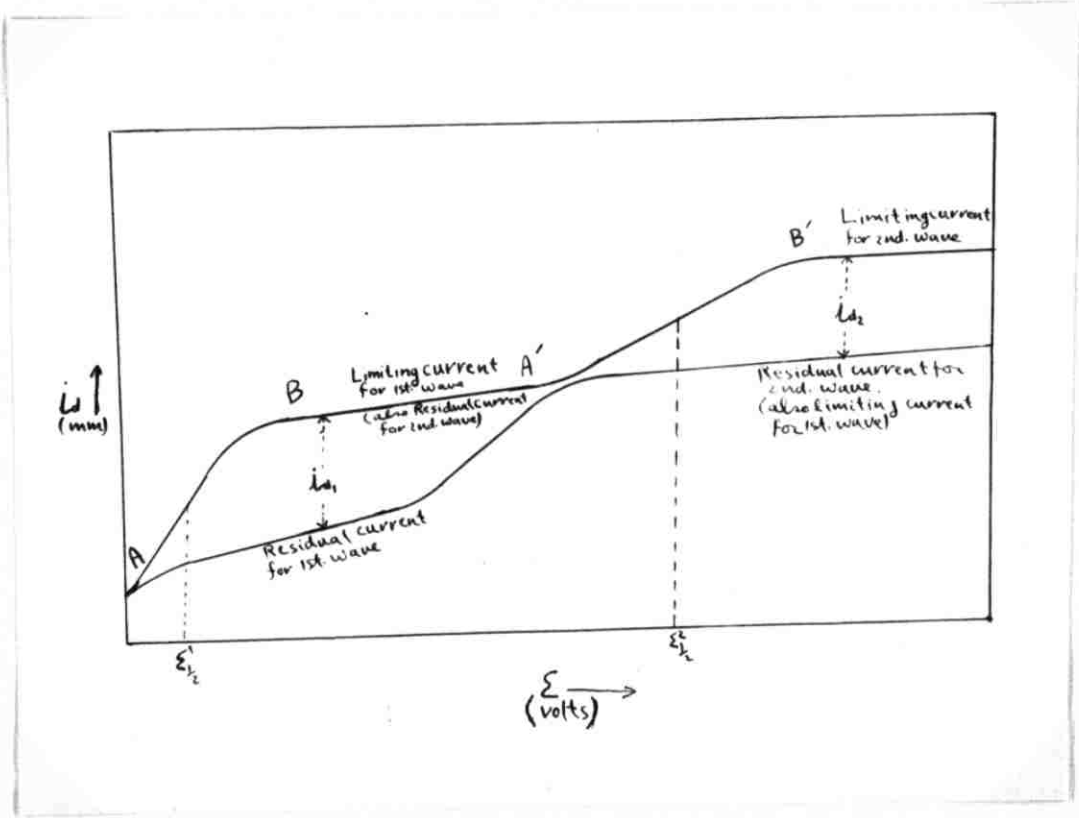


Fig. I

A Polarogram drawing of oxygen wave potentials

concentration gradient is formed and ions travel by diffusion from the body of the s olution to the interface of the mercury drop where they plate out; their rate of diffusion is proportional to the concentration gradient established. However, as the voltage increases the rate of plating out increases and the diffusion current increases accordingly until the concentration of ions at the interface of the d rop becomes zero, i.e., the ions are plated out as soon as they reach the Hg drop. The diffusion current reaches its limiting value and levels off, it does not increase further no matter how much we raise the voltage because at that stage it is proportional to the concentration of ions in the body of the solution, which is constant.

The temperature coefficient of the diffusion current is about 2%/1°C. (3). Thermostating temperature control is therefore important.

On applying polarographic analysis to oxygen, a negative increasing potential is applied to the d.m.e. so that every drop of Hg is charged negatively with respect to the mercury pool (or S.C.E.) anode. The oxygen reduction takes place on the interface of the Hg drop followi ng the same process mentioned above.

The Residual Current - the rather small current that flows before the decomposition potential of the substance under test is reached. It increases slightly but almost proportionally with the applied voltage.

By subtraction of the residual current from the

limiting current the diffusion current is obtained.

Migration Current - In general the d.m.e. is negatively charged, and the substance (or ion) to be determined should be positively charged or easily reducible even if it is in the molecular form such as oxygen.

The positively charged particles migrate to the Hg drop, not only by a simple diffusion process but also by electrical coulombic attraction. The resulting current is thus due to diffusion plus migration. The diffusion current is proportional to the concentration of the substance while the migration current is not, so it is a source of error and should be eliminated. However, since oxygen is in the molecular form there is no coulombic attraction and the migration current is not serious. This migration current is eliminated by adding an indifferent electrolyte in excess relative to the concentration of ions under test so that the electrical current is carried by it, and the test ions travel by diffusion only. Since the "inert electrolyte" has supported in controlling the diffusion process, it is called the "supporting electrolyte".

Absorption Current - This current appears in the polarogram in the form of a maximum super imposed on the normal diffusion current. Its exact origin is not clear and need not be known for the purpose at hand as long as it can be eliminated.

The adsorption current is eliminated, (i.e., the maximum is suppressed) by adding a small concentration of

surface active substances, such as gelatine, to the solution being tested.

Ilkovic Equation - There exists a definite relationship between the diffusion current and the concentration of the electrode active substance, the rate of its diffusion towards the electrode and the characteristics of the electrode.

Ilkovic (1934) derived an equation which relates all these variables for diffusion controlled processes:

$$I_d = 605\ n\ D^{1/2}\ Cm^{2/3}\ t^{1/6} \quad \ldots\ldots\ldots\ldots\ldots\ 1$$

Where "I_d is the mean diffusion current in micro-amperes", "m" is the weight (in mg) of Hg flowing from the capillary per second. "t" is the time necessary for the formation of one drop of Hg, "c" the millimoles of reacting material per liter, "D" the diffusion coeffident of the reducible substance (in $cm^2\ sec^{-1}$) and "n" the number of electrons involved in the reduction of one molecule of the reducible substance. (21)

When concentration is being determined all of the factors in the Ilkovic equation except the concentration must be maintained constant. By always using the same capillary, the same height mercury column and maintaining the same temperature, the variables will be constant for any one given substance. Under these conditions the Ilkovic equation is reduced to a linear relationship between i_d and C.

$I_d = KC$ 2

From equation 2 it is clear that the polarograph measures only relative concentrations in terms of i_d rather than absolute units; hence, the necessity of a calibration curve to change the measured relative concentrations to absolute concentrations.

Determination of oxygen by Polarography -

The reduction of dissolved oxygen at the dropping mercury electrode produces two waves of equal height. The mechanism responsible for these two waves is believed to be the following (17):

The first wave results from reduction of oxygen to hydrogen peroxide:

$O_2 + 2H^+ + 2e$ ------ H_2O_2 (acid medium)

$O_2 + 2H_2O + 2e$ ------ $H_2O_2 + 2OH^-$ (neutral or alkaline medium)

The second wave results from reduction of the hydrogen peroxide either to water or hydroxyl ion:

$H_2O_2 + 2H^+ + 2e$ ----- $2H_2O$ (acid medium)

$H_2O_2 + 2e$ ----- $2OH^-$ (alkaline medium)

All the above reactions are irreversible (17) at the dropping mercury electrode. The first wave of oxygen has a maximum, which can be easily suppressed by gelatine.

EXPERIMENTAL PROCEDURE

A Sargent Model XII was used with an H type cell which was slightly modified.

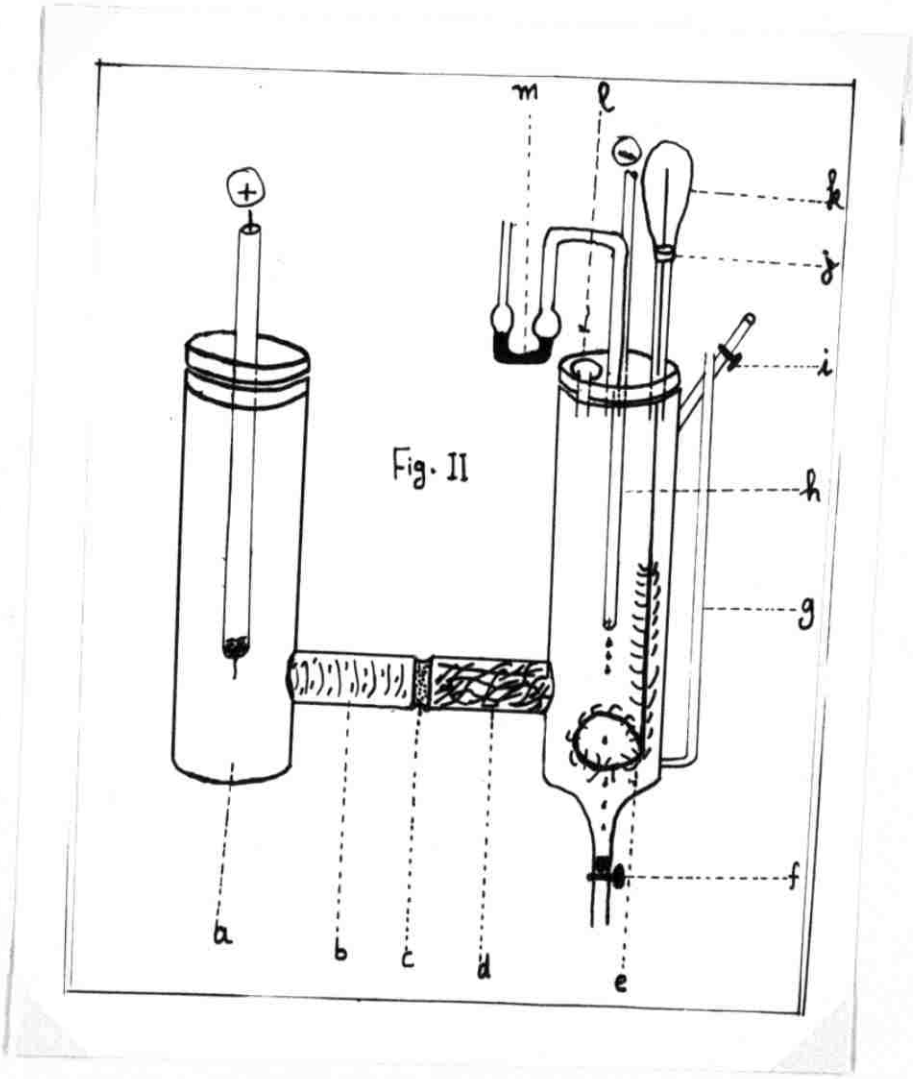


Fig. II

(Fig. II)

An H type polarographic cell was modified as shown in the diagram for use in the present study.

a: a saturated calomel electrode.

b: agar-agar bridge saturated with KCl at 25°C, to prevent the contamination of the solution with the calomel constituents.

c: a thin sintered glass disc.

d: glass wool, to prevent the accumulation of scolices in the arm thus preventing their ready access to disolved oxygen.

e: a glass rod stirrer wrapped with cotton which can be used without exposing the solution to the atmosphere by handling it by means of ballon k, which is tightly mounted over a rubber stopper j.

f: a stopcock to remove Hg as soon as it accumulates in moderate quantities, or at the end of the experiment, thus minimizing the effect of Hg on parasites.

g: a capillary side arm for bubbling oxygen and nitrogen as desired. After the bubbling the capillary was sealed with paraffin wax.

h: the capillary d.m.e. electrode which is connected to the cathode.

i: another slanting arm with a stopcock; the fluid was usually filled up to this arm and then a layer of paraffin oil (which was previously boiled and cooled) was put above it. The paraffin oil layer could be removed through arm i by suction without any agitation of the sample.

l: a penicillin rubber stopper through which the parasites

may be introduced by means of a syringe needle without exposing the sample to air when desired.

m: a U tube, which contains a few milliliters of paraffin oil. Its function is to let excess bubbled gas escape thus keeping the apparatus sealed to air.

Mercury was washed with dilute nitric acid twice, then vacuum distilled, and finally was filtered through pinholed filter paper to remove any dust particles.

The following conditions were kept constant in all experiments (unless specified otherwise):

1. The temperature was maintained at 37°C. (To within 0.1°C by a Fenwal Thermoregulator).

2. The height of mercury column was kept constant at 67.05 cm.

3. The same capillary electrode was used throughout.

All rubber stoppers and tubes were boiled with concentrated NaOH to remove traces of sulfur or other contaminates, then rinsed with distilled water and dried.

The polarograms were recorded on bromine film papers and developed with Kodak D72. The tangent method (33) was used to determine the half-wave potentials.

Various studies to determine the applicability of the Polarographic Method.

I.- The first and second half-waves of oxygen and their range of reduction were studied in 0.1, 0.25, 0.5, 1.0 N KCl.

Table I

The first and second half-wave of oxygen in different concentrations of KCl - H_2O solutions.

Sample No.	conc. of KCl	$E_{\frac{1}{2}}^{1}$ & its reduction range (volts)	$E_{\frac{1}{2}}^{2}$ & its reduction range (volts)
1	0.1N	(0.09)±0.03 0.05 - 0.17	(0.81)±0.03 0.54 - 1.17
2	0.25N	(0.15) 0.08 - 0.22	(1.01) 0.74 - 1.30
3	0.50N	(0.14) 0.07 - 0.20	(0.97) 0.75 - 1.18
4	1.00N	(0.15) 0.09 - 0.21	(1.02) 0.79 - 1.28

From the above results (Table I) the following conclusions are drawn:

a: the first and the second half-wave potentials of oxygen are lower in a 0.1N KCl solutions (0.09 and 0.81 volts respectively) than in higher concentrations of electrolyte and they seem not to be affected by the electrolyte concentrations when it is above 0.1N.

b: the range of reduction of the first half-wave is very

short (from 0.08 - 0.22 volts) compared to the wide range of reduction of the second half-wave (range from 0.74 - 1.30)

II. The first and second half-wave potentials $E^{1}_{\frac{1}{2}}$ and $E^{2}_{\frac{1}{2}}$ of oxygen were studied in different samples of the hydated cyst fluid of different electrolyte compositions (measured as Cl^{-} by Volhard Method. (13).

Table II

The $E^{1}_{\frac{1}{2}}$ and $E^{2}_{\frac{1}{2}}$ of oxygen and their reduction ranges in various hydated cyst fluid samples.

Sample No.	pH	Conc. of Cl^{-}	$E^{1}_{\frac{1}{2}}$ & range of reduction (volts)	$E^{2}_{\frac{1}{2}}$ & range of reduction (volts)
1	7.8	0.10N	(0.14)±0.03 0.08 - 0.20	(1.00)±0.03 0.76 - 1.27
2	8.2	0.16N	(0.17)±0.03 0.10 - 0.24	(1.05)±0.03 0.74 - 1.29
3	8.2	0.16N	(0.18)±0.03 0.10 - 0.24	(1.00)±0.03 0.74 - 1.22
4	8.0	0.06N	(0.12)±0.03 0.06 - 0.21	(0.95)±0.03 0.70 - 1.23

From the above study it is noticed that the variation of $E^{1}_{\frac{1}{2}}$ and $E^{2}_{\frac{1}{2}}$ of oxygen in the various samples of hydated cysts is not affected to any appreciable extent by small variations of electrolyte concentrations or by the nature of organic matter present which is of a similar type in all hydated cysts.

III. The first and second half-waves of oxygen as well as the concentration of oxygen were studied vs. pH variations.

Table III

The effect of pH variations of $E^1_{\frac{1}{2}}$ and $E^2_{\frac{1}{2}}$ and oxygen concentrations.

Sample No.	pH	$E^1_{\frac{1}{2}}$ and range of reduction (volts)	$E^2_{\frac{1}{2}}$ and range of reduction (volts)	O_2, i_d mm.
1	8.2	(0.14)±0.03 0.07 - 0.15	(1.01)±0.03 0.78 - 1.27	380
2	7.5	(0.14)±0.03 0.07 - 0.21.	(0.94)±0.03 0.70 - 0.21	370
3	6.5	(0.13)±0.03 0.06 - 0.20	(0.98)±0.03 0.77 - 1.20	360
4	2.2		(0.92)±0.03 0.74 - 1.10	290
5	1.4		(0.87)+0.03 0.66 - 1.05	290

The following conclusions are drawn from the above study:

a) The oxygen content is not affected appreciably by variations of 1 to 2 pH units. However, a decrease of 5 pH units results in a 20% decrease in i_d which is significant.

b) $E^1_{\frac{1}{2}}$ was found to be pH independent, while $E^2_{\frac{1}{2}}$ is slightly affected with pH variation. It tends to decrease with decreases in pH. but this variation is still slightly lower than

the experimental error and thus in the range studied is negligible. This is especially true when only small variations of pH are considered. Since the variation in pH of the cyst fluid is less than one pH unit no significant error results from using $E_{\frac{1}{2}}^{2}$ in i_d measurement.

IV. The concentration of oxygen was studied in various concentrations of KCl solutions. All experimental conditions except for the concentrations of KCl electrolyte being kept constant. The results are tabulated below.

Table IV

The variation of oxygen content with concentration of electrolyte. (15°C).

Sample No.	Conc. of Cl^-	i_d (conc. of O_2	% decrease
1	0.1N	530	-
2	0.25N	450	$\frac{530-450}{530} \times 100 = 15\%$
3	0.50N	435	$\frac{530-435}{530} \times 100 = 18\%$
4	1.0N	400	$\frac{530-400}{530} \times 100 = 25\%$

From the above results it can be seen that the oxygen concentration decreases as the electrolyte concentration increases.

An increase of 2.5 times in electrolyte concentration decreases the oxygen content by 15%, an increase of 10 times

in electrolyte concentration causes a decrease in oxygen content of 25%.

V. The decomposition of the cyst fluid, and the efficiency of freezing in preventing cyst decomposition were studied. (Table V). Column 2 in Table V shows the concentration of Cl^- (estimated as NaCl) determined when the sample was received. Column 3, shows the concentration of Cl^- for the same sample when kept frozen for more than two weeks. The 4th column shows the chloride concentration in the sample when kept unfrozen for one to four days. The samples kept unfrozen developed a very disagreable odor.

Table V

The composition of Cl^- of the frozen and unfrozen cyst fluid.

Sample No.	Cl^- conc. (cyst as received)	Cl^- conc. (cyst kept frozen)	Cl^- conc. cyst kept unfrozen	Notes
1	0.05N	0.05N	0.10N	Kept unfrozen for four days. When kept for one more day no detectable increase in Cl^- conc. was observed
2	0.20N	0.20N	0.30N	Unfrozen for four days
3	0.10N	0.10N		
4	0.16N	0.16N	0.20N	Unfrozen for one day
5	0.06N	0.06N	0.07N	Unfrozen for one day

The following conclusions are drawn from this experiment:

a) The hydated cyst varies in its electrolyte concentration from sample to sample.

b) When the samples were kept frozen no detectable decrease or increase in Cl^- concentrations were observed, so most probably no decomposition in the samples had taken place. When they were kept unfrozen for four days a considerable change in chloride concentration was observed, probably due to the decomposition of the sample. When the samples were kept unfrozen for one day a slight decomposition was observed.

In view of these results the hydated cyst was kept frozen in the refrigerator whenever not in use, to prevent decomposition.

VI. The effect of gelatine on suppressing the maximum of the first reduction wave of oxygen was studied by adding 0.05, 0.10, 0.15, 0.20, 0.30, 0.5, 0.6, 1, $1\frac{1}{2}$, 2 mls. of gelatine, to 0.1N KCl solutions.

It was found that the minimum amount of gelatine required to suppress the maximum of the first wave of oxygen in electrolyte solutions was 0.15 mls. of 0.2% gelatine, and the maximum amount that could be used was 0.5 ml. of 0.2% gelatine beyond which a detectable effect due to the gelatine on $E_{\frac{1}{2}}$ and i_d was observed.

It was found also that the cyst fluid contains enough organic substances to suppress the maximum, so there was no need for adding gelatine.

Since the migration current which arise from the electrostatic attraction of ions to the d.m.e., is minimized in solutions being analyzed for molecular constituents (i.e. oxygen in this case). Consequently only very small amounts of supporting electrolyte must be added to completely eliminate it and the cyst fluid, perse, contains sufficient concentrations of electrolytes for this purpose.

Control Experiments:

Although utmost care was exercised in carrying out the various experiments for determining the dissolved oxygen content in the cyst fluid, there always existed the possibility of exchange of oxygen between the air and the experimental system.

To determine the efficiency of the precautionary measures employed to prevent this exchange and the limitations of these measures, the following series of control experiments was run (all experiments werecarried out at 37°C. and a cotton stirrer was used).

1) Determination of the reproducibility of the diffusion current readings on the polarograph.

A series of readings were made on a 0.1N KCl solution that was covered with paraffin oil. Gelatine was added to the solution to suppress current maximum. The oxygen content of the solution was in equilibrium with air at 37°C.

Table VI

$t_{min.}$	$i_{d(mm.)}$
0	325
45	320
75	320
120	320

A second series of readings were made on the cyst fluid at 37°C. As before the oxygen content was that determined by equilibrium with air. The sample was also covered with paraffin oil. This sample was filtered to remove all parasites.

Table VII

$t_{min.}$	$i_{d(mm.)}$
0	255
75	260
120	255
150	260

DISCUSSION

The oxygen concentration in both the 0.1N KCl solution and the cyst fluid studied in the previous experiments was brought to equilibrium with atmospheric oxygen to minimize the oxygen concentration gradient and consequently minimize the rate of exchange of oxygen. Paraffin oil was added to further hold the oxygen concentration in the sample constant and to isolate it from atmospheric oxygen. The experiment was run over a period of time during which the oxygen concentration under the above conditions should be constant and any variations in oxygen concentration could be considered errors, arising from diffusion current measurements. If we give the same weight to the diffusion current values listed in Tables VI and VII the error would be equal to the shunt value used in measuring the diffusion current. In both cases a shunt of 5 was used. This corresponds to an deviation of less 2½%. The constancy of the current readings over a long period of 2½ hours time also indicates that filtering the fluid is sufficient to remove cyst scolices.

2) In the following series of experiments the initial oxygen levels were obtained by bubbling the sample with oxygen or nitrogen depending on whether a high or low level of oxygen was desired. Unless otherwise noted the sample was filtered cyst fluid at 37°C.

Table VIII

a) Sample not covered with paraffin oil.

$t_{min.}$	O_2, $i_{d(mm.)}$	error	
0	1020		
10	1020		
20	1020		average rate
30	980		of change of
40	900	± 10	i_d is 2.44
60	880		per minute.
70	900		
80	800		
90	800		

b) Sample covered with paraffin oil

0	960		
15	960		average rate
45	940		of change of
60	940	± 10	i_d is 0.48
90	880		mm/min.
210	860		

c) Sample covered with paraffin oil

0	125		
15	120		
30	115		average rate
36	120	± 5	of change of
75	115		i_d is practi-
90	115		cally zero.
105	120		

Table VIII

a) Sample not covered with paraffin oil.

$t_{min.}$	O_2, $i_{d(mm.)}$	error	
0	1020		
10	1020		
20	1020		average rate
30	980		of change of
40	900	± 10	i_d is 2.44
60	880		per minute.
70	900		
80	800		
90	800		

b) Sample covered with paraffin oil

0	960		
15	960		average rate
45	940		of change of
60	940	± 10	i_d is 0.48
90	880		mm/min.
210	860		

c) Sample covered with paraffin oil

0	125		
15	120		
30	115		average rate
36	120	± 5	of change of
75	115		i_d is practi-
90	115		cally zero.
105	120		

CONCLUSIONS

From Table VIII it can be seen that the use of a layer of paraffin oil to prevent oxygen exchange results in a five fold decrease in the rate of loss of oxygen at high concentrations. At low concentrations the exchange of oxygen is essentially prevented by the use of paraffin oil.

In all subsequent experiments the samples were covered with a layer of paraffin oil to prevent the exchange of oxygen. In the experiments to determine the oxygen tension of the cyst fluid in vivo it was assumed that no error results due to oxygen exchange because the concentration is low. In the only samples that contain a high concentration of oxygen the respiration of the parasites reduces the concentration to a low level rather rapidly and hence in this case also oxygen exchange will not be source of error.

CALIBRATION CURVES

Since the polarograph records only relative concentrations it is necessary to prepare a calibration curve to convert the diffusion currents into absolute values. This is done by determining the oxygen content of a sample with the polarograph and also on an absolute basis by chemical methods. Two chemical methods were chosen for this purpose. One, the Winkler method (27) and two a method employing the oxidation of ascorbic acid (28), (29).

Although the Winkler method is a standard chemical technique for the determination of dissolved oxygen in water and has also been used to determine the oxygen content of solutions containing organic material (23) in the present istance it was found not to be applicable to the cyst fluid. The cause for this, and its possible implications are discussed below. The Ascorbic acid method was found to be convenient and satisfactory.

The Winkler Method:

Since, this method was not found to be applicable to the cyst fluid, a brief description of the method and possible reasons for its inapplicability are presented.

The basis for the method is the following series of reactions:

1) $2MnSO_4 + O_2$ (dissolved) $+ 4NaOH \longrightarrow 2MnO_2 + 2Na_2SO_4 + 2H_2O$

2) $2MnO_2 + 4KI + 4H_2SO_4 \longrightarrow 4I^0 + 2MnSO_4 + 2K_2SO_4 + 4H_2O$

3) $4I^0 + 4Na_2S_2O_3 \longrightarrow 2Na_2S_4O_6 + 4I^- + 4Na^+$

In step (1) MnO_2 is the major product when there is an appreciable amount of dissolved oxygen. However, when the oxygen concentration is small and there is excess Mn^{++}, $Mn(OH)_2$ is found since the solution is alkaline. At this stage, if the solution is exposed to air, oxygen from the air dissolves in the solution and reacts with the Mn^{++} resulting in highly inaccurate results. Hence, the solution must be isolated from atmospheric oxygen at this stage.

In step (2) Mn^{+4} oxidizes I^- into free iodine in acid solution. H_2SO_4 will also dissolve the extra precipitate of $Mn(OH)_2$, if present, resulting in a clear iodine solution. At this stage there is no fear of exposing the solution to atmospheric oxygen because oxygen will not oxidize Mn^{+2} except in alkaline solution.

In step (3) the free iodine is titrated with standard $Na_2S_2O_3$ using starch solution as an indicator.

Calculations:

If the milliliters of sodium thiosulfate used to titrate a given volume of solution are multiplied by -

Normality of $Na_2S_2O_3 \times 0.008 \times \frac{22.4 \times 10^6}{32} \times \frac{291}{273}$

The result will be given in mm^3 of oxygen under the conditions employed (i.e. 18°C).

EXPERIMENTAL PROCEDURE

Reagents (27):

Manganous sulfate solution:

48 gms of $MnSO_4$ dissolved in 100 mls. of distilled water.

Iodine solution:

360 gms of NaOH and 100 gms of KI dissolved in one liter of distilled water.

Sulfuric acid:

Sp. gr. 1.4.

Sodium thiosulfate solution:

0.01N $Na_2S_2O_3$ solution.

Starch solution:

0.5% starch solution, used as an indicator.

Technique:

A special modified technique which serves two purposes was used in determining the dissolved oxygen by Winkler's method:

First: the solution is well isolated from atmospheric oxygen.

Second: the modified flask (Fig. III) was used to deliver a representative sample of solution to the polarographic cell whenever the oxygen concentration was to be determined by both chemical and polarographic methods.

The flask in Fig. III is 300 ml. flat bottomed flask with capillary side arm (h) used for bubbling oxygen through the solution. (g), is a magnetic stirrer. (e & f) are

outlets, to which pieces of rubber tubing were attached that could be closed by a pinchcock.

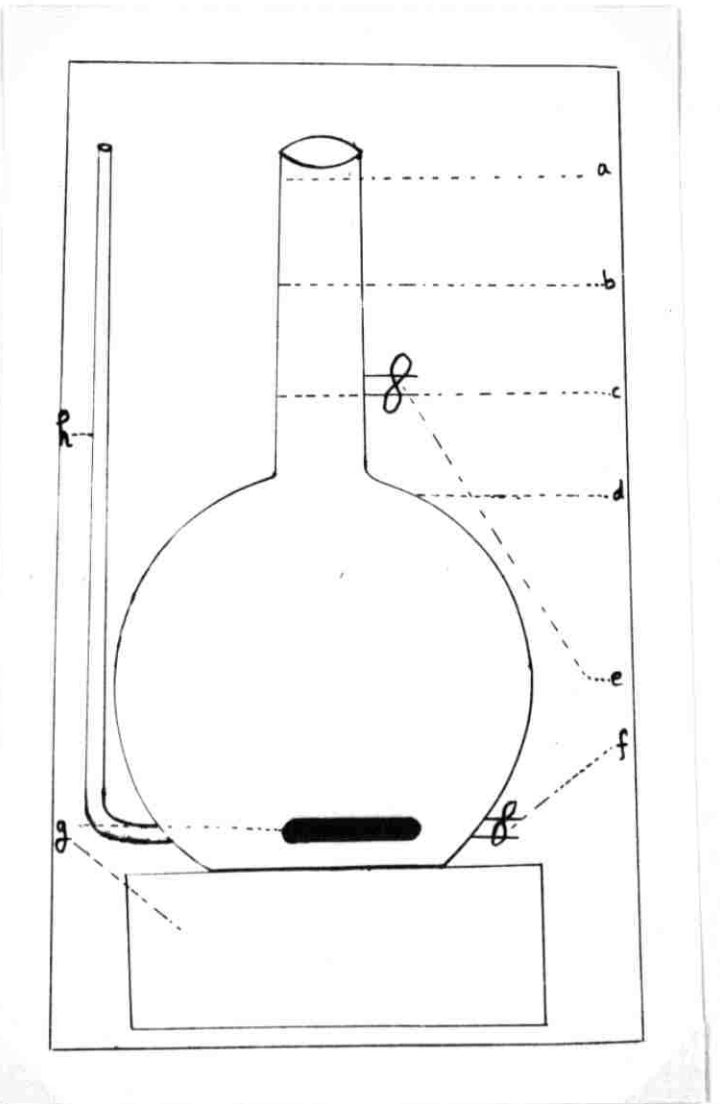


Figure III
A modified flask used in the determination of dissolved O_2

In determining the oxygen concentration without using a sample for the polarograph, the flask is filled to level (d) (250 ml.). A layer of previously boiled and cooled paraffin oil is then placed over the sample to prevent oxygen exchange with the atmosphere. The reagents are introduced through the paraffin layer and under the surface of the solution by means of 2 cc. pipettes. The mechanical stirrer (g) was used to mix the reagents; the paraffin oil being confined in the neck of the flask remains at the surface with a minimum of agitation, thus preventing oxygen exchange throughout the entire process.

The added reagents occupy the volume (d to c) and the paraffin from (c to b); thus by opening outlet (e) it is possible to remove the paraffin if so desired. A 100 cc. pipette is then introduced to take a sample for titration. Actually there is no need to remove the paraffin oil layer, the pipette could be introduced through the paraffin to take the sample. However, removal of paraffin prevents contamination of the sample with the oil.

The above flask is also very useful when samples are needed for determination of the calibration curve. By removing a sample for the polarographic cell from the same solution on which the chemical analysis is carried out. In that case the flask is filled to level (a). After bubbling oxygen through capillary (h), the top of the capillary is sealed with paraffin wax, and a few milliliters layer of

paraffin oil are added over the solution. After allowing a few milliliters of the solution to flush the outlet (f) clean, a 25 ml. sample is passed directly into the polarographic cell and covered immediately with a layer of paraffin oil. The oxygen concentration is then determined polarographically as discussed later. Then outlet (f) is closed, the chemical reagents are introduced through the paraffin layer and the chemical analysis is made as discussed above.

Nitrogen was bubbled through the reagents to remove any dissolved oxygen, that they might contain and that would result in erronous results. A correction factor was applied later to account for traces of dissolved oxygen in the added reagents. This correction factor was obtained by analyzing a sample of distilled water that had been boiled to remove oxygen and then allowed to cool after being covered with a layer of paraffin oil. If it is assumed that this sample contains no dissolved oxygen, then the results obtained must be due to oxygen dissolved in the reagents and / or introduced during the various manipulations. The correction factor thus found was 262 mm^3 of oxygen / liter of solution.

A comparision of the experimental results with the values reported in the literature (9) is shown in the following table:

Table IX

Sample No.	Time of bubbling O_2 in hrs.	Temp. °C.	Calculated value of O_2 in mm^3 O_2 per liter	Literature* value of O_2 in mm^3 of O_2 per liter	Deviation.
1	$2\frac{1}{2}$	30	28.0×10^3	28.1×10^3	$.1 \times 10^3$
2	$2\frac{1}{2}$	28.5	?27.3×10^3	28.5×10^3	1.2×10^3
3	$2\frac{1}{2}$	29	28.3×10^3	28.8×10^3	$.5 \times 10^3$
4	$2\frac{1}{2}$	25.5	29.6×10^3	29.6×10^3	0.0
5	$2\frac{1}{2}$	25.3	29.8×10^3	29.7×10^3	$.1 \times 10^3$

* Literature values have been converted from p.p.m. to mm^3 of oxygen per liter solution.

By analyzing samples of distilled water, in one case covering the sample with paraffin and in the other case leaving the sample uncovered, it is possible to show the necessity for the use of paraffin oil. The results of such an experiment are given in table X.

Table X

Sample No.	Temp. °C.	without paraffin O_2, mm^3/lit.	using paraffin O_2, mm^3/lit.	deviation mm^3 O_2/liter
1	19.8	6.77×10^3	5.86×10^3	0.91×10^3
2	19.8	6.83×10^3	5.91×10^3	0.92×10^3
3	19.8	6.80×10^3	5.91×10^3	0.89×10^3
4	19.8	6.83×10^3	5.80×10^3	1.03×10^3
5	19.8	6.77×10^3	5.91×10^3	0.86×10^3
6	19.8	6.83×10^3	5.89×10^3	0.94×10^3
				0.93×10^3 (average)

A calibration curve was obtained on 0.1N KCl solutions using Winkler's method, at 18°C to test the linear relationship between diffusion currents and the oxygen concentrations. The results are put in Table XI, the values are plotted in Figure IV.

Table XI

Temp. °C.	solution	O_2, mm^3 of O_2/liter	O_2, i_d (in mm.)
18	0.1N KCl	1.02×10^3	140
18	0.1N KCl	5.85×10^3	470
18	0.1N KCl	15.5×10^3	1340
18	0.1N KCl	27.8×10^3	2200

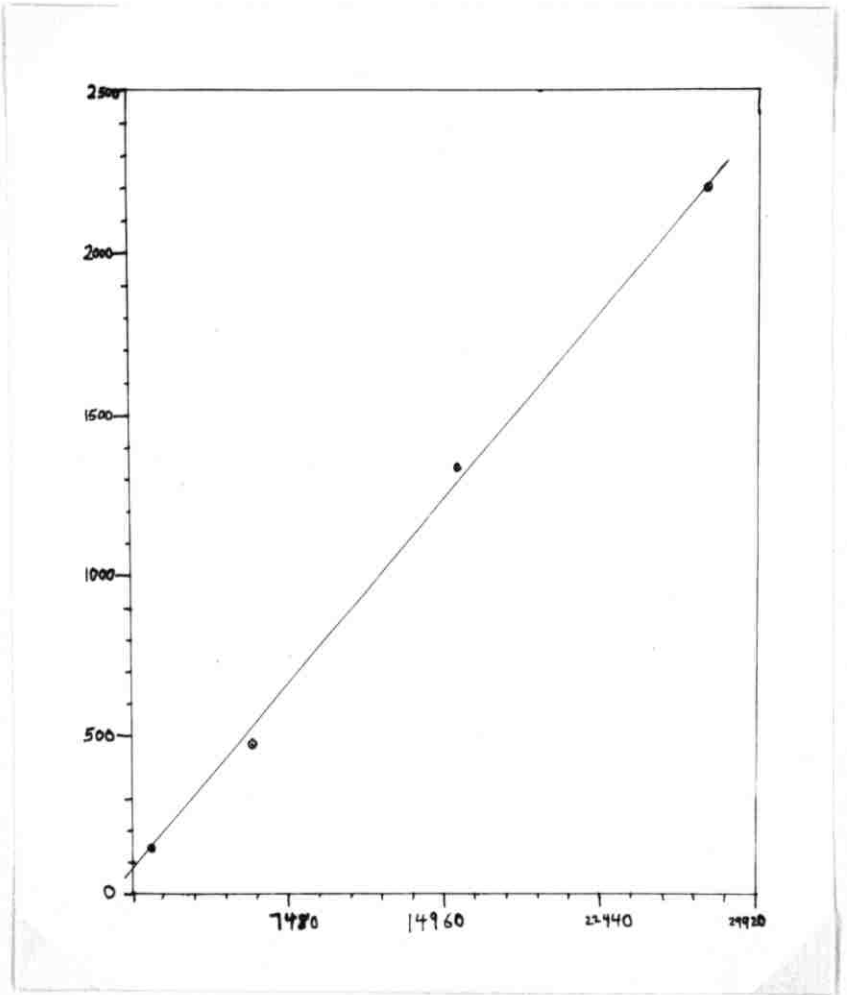


Fig. IV
(Data from Table XI)
Calibration curve obtained by using Winkler's method at 18°C.

DISCUSSION

The foregoing results show that Winkler's method is quite satisfactory when applied to the analysis of water or inorganic salt solutions. However, such satisfactory results were not obtained when the method was applied to the cyst fluid. In some respects this is not surprising since it has been reported that the method is not always applicable to solutions containing organic solutes, although a number of investigators have applied it to such solution (23). Because of this seeming ambiguity an effort was made to determine the source of the difficulty and to modify the method, if possible, so that it could be applied to the cyst fluid.

The initial analysis of the cyst fluid by this method always resulted in an apparent oxygen concentration of zero. This was true even in cases in which oxygen has been bubbled through the system. Determination of oxygen in a system by Winkler's method depends, in the final analysis, on the titration of iodine with sodium thiosulfate (see reactions listed page 29). Hence, a possible difficulty would be the loss of I_2 prior to titration. To test this, 25 ml. of 0.01N I_2 was added to 100 ml. of the cyst fluid. Subsequent titration of this sample showed that only 24 ml. of I_2 remained. Consequently, it seems apparent that some of the I_2 was reduced by oxidizing the organic matter present in the fluid. Equation 2 (page 29) shows that the iodide is oxidized by Mn^{+4} to iodine which is then titrated with

thiosulfate. However, if the fluid contains large amounts of organic matter some of the liberated iodine may react with the organic matter, as suggested above, but also the Mn^{+4} may also react with the organic matter (oxidizing it) and thus not react with the iodine added to the solution. That this actually occurred is suggested by the fact that the MnO_2 precipitate was observed to disappear without the subsequent formation of iodine (i.e., no color observed).

In some instances it has been suggested (30) that prior oxidation of the organic matter by $KMnO_4$ would alleviate this difficulty. However, since this is accompanied by two attendent difficulties, itself, it was not deemed to be satisfactory. Nine milliliters of saturated solution of $KMnO_4$ (64 gm./liter) were required to destroy the organic matter. However, with such a highly concentrated solution a slight excess would cause major errors due to oxidation of the iodide. To prevent this the excess was removed by the addition of oxalate solution, but here again an excess would allow the possibility of reduction of the Mn^{+4}. Dilute solutions could not be used due to the large quantities that would be needed and the subsequent quantities of oxygen that would be introduced dissolved in the reagents. Another difficulty that arises is that the solubility of dissolved oxygen is a function of the ionic strength of a solution; therefore, by adding electrolytes the concentration will be altered.

CONCLUSIONS

From the foregoing experiments it is possible to draw the following conclusions:-

1) Although Winkler's method is applicable to the analysis of dissolved oxygen in inorganic solutions it is not applicable to the cyst fluid.

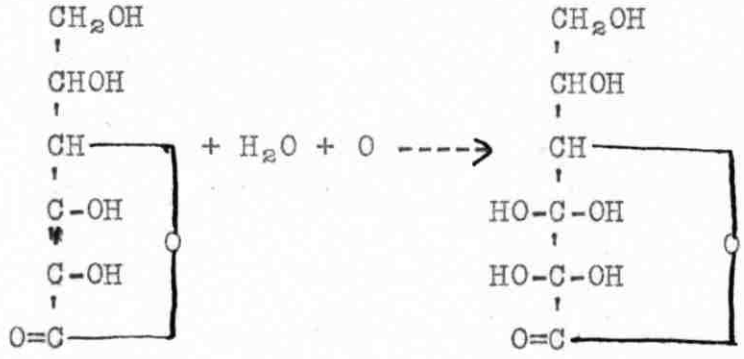
2) The method is precise.

3) Paraffin is necessary to prevent oxygen exchange with the atmosphere.

4) The relationship between the polarographic diffusion current and dissolved oxygen content of water samples in linear.

Ascorbic Acid Oxidase Method:

The principle of this method is based on the oxidation of reduced ascorbic acid by molecular oxygen (28), (31).



l-Ascorbic Acid (reduced form)

hydrate of dehydro Ascorbic Acid (oxidized form)

This reaction is quantitative, but extremely slow unless an enzyme (ascorbic acid oxidase) is used as a catalyst. In actual practice the procedure is to add a known quantity of the reduced form of ascorbic acid to the solution containing dissolved oxygen and then to determine the amount of reduced form remaining after the reaction has gone to completion. The oxygen concentration is then determined by difference.

EXPERIMENTAL PROCEDURE

Reagents:

Dye solution (2,6-dichlorophenol indophenol):

About 0.135 gms were dissolved in one liter of distilled water. The dye is standardized against l-ascorbic acid (reduced form), by weighing accurately, approximately 100 mgm of ascorbic acid, dissolving this sample in distilled water and diluting to one liter in a volumetric flask. 5 mls aliquots of this solution are then introduced into 25 mls of 0.1N H_2SO_4 and titrated at once with the dye solution to a light pink color (end point). A blank titration should be carried to the same end point and corrected for.

Sulfuric acid solution:

A 0.1N sulfuric acid solution was prepared. About 25 mls of this solution were used per titration, to deactivate the enzyme and stop its action.

Concentrated ascorbic acid solution:

3.0 gms of l-ascorbic acid (reduced form) and 10 gms of NaCl were dissolved in 50 mls of distilled water. A 0.1 ml of this solution was used per determination.

Ascorbic acid oxidase:

This enzyme could be prepared from squash (32), cucumber and some other vegetables. The method used by Sharp (28) to prepare this enzyme from the outer layer of cucumbers was adopted here, except that four fractions of

enzyme of different activity, instead of one, were prepared. Two kilograms of cucumbers were frozen, a few holes were made in the outer layer of each and they were left to drain at room temperature. Sample No. 1 was reserved from this solution; then the remaining solution was evaporated to 1/10 of its volume by dialysis through a cellophane bag. It was then filtered and samples No. 2 and 3, were reserved from the concentrate and the filtrate respectively: the cucumbers were expressed further manually and the liquid concentrated by evaporation, this constituted sample No. 4. The activity of the above enzyme fractions was determined. (See Table XII). A 0.1 ml. of sample No.2 is used per determination. Below pH 4 and above pH 8 the enzyme is deactivated (28) but in the optimum range of pH, 5 - 7.5, it has sufficient activity to complete the reaction in 15 minutes.

Table XII

Sample No.	Time of rx.	pH	mls of enzyme added	mls of ascorbic acid reacted in 15 min.
1	15 min.	6.5	0.1	1.55
2	15 min.	6.5	0.1	6.60
3	15 min.	6.5	0.1	3.30
4	15 min.	6.5	0.1	2.80

Since the volume of enzyme employed was the same in all cases, the volume of ascorbic acid oxidized is a measure of the enzymatic activity. Can be seen that sample No. 2 is the most active and that the relative activities of sample Nos. 1, 3 and 4 are 25, 50 and 40% respectively, of No. 1.

CONCLUSIONS CONCERNING THE APPLICABILITY OF THE ASCORBIC ACID OXIDASE METHOD:

1) By analyzing several samples of water by both the ascorbic acid oxidase method and Winkler's method it was found that the results deviated by less than 1½%, Hence, it is concluded that the ascorbic acid method is sufficiently precise.

Table XIII

Sample No.	Temp. °C.	Sample used	Winkler's Method mm^3 of O_2/liter	Ascorbic acid Method mm^3 of O_2/liter
1	19	water	6.23×10^3	6.30×10^3
2	19	water	6.20×10^3	6.30×10^3
3	19	water	6.15×10^3	6.37×10^3
4	19	water	6.30×10^3	6.23×10^3
5	19	water	6.25×10^3 (6.23×10^3) average	6.30×10^3 (6.30×10^3) average
1	19	cyst fluid	0.00	3.66×10^3
2	19	cyst fluid	0.00	3.75×10^3
3	19	cyst fluid	0.05×10^3	3.68×10^3

2) Repeated analysis on the same sample by both the ascorbic acid oxidase method and the polarographic method over an extended period of time showed that the oxidase reaction was complete (under the conditions employed) in approximately 15 minutes.

3) Since the reagents of the ascorbic acid method are specific for oxygen, where as the reagents employed in Winkler's method are not, it is applicable to the cyst fluid.

Calibration of the Polarograph:

Due to the inaplicability of the Winkler method in measuring dissolved oxygen in the cyst fluid, the polarograph was calibrated by making diffusion current measurements on samples of cyst fluid whose absolute oxygen content was then determined by the ascorbic acid oxidase method.

Procedure:

The chemical reaction and the polarographic determination were carried out in the same polarographic cell. After introducing the cyst fluid into the cell and covering it with a layer of paraffin oil, the capillary electrode was inserted in the cell and the diffusion current recorded. 0.1 ml of concentrated ascorbic acid solution was then introduced by a pipette and the solution was stirred well with a glass rod for about 1 minute. A 5 mls. aliquot was removed and titrated against the standard dye (2,6-dichlorophenol indophenol) to determine the initial concentration of

the reduced ascorbic acid. Then 0.1 ml of the enzyme (No. 2) was introduced into the polarographic cell by means of a graduated pipette and the reaction left to be completed within 15 minutes. Another 5 ml aliquot sample was removed and titrated to determine the final concentration of the reduced ascorbic acid. The difference between the initial and final volumes of dye required to titrate the ascorbic acid corresponds to the amount of ascorbic acid reacted with the dissolved oxygen present.

The error resulting from oxygen reacting with reduced ascorbic acid before the addition of the enzyme during the two minutes time of mixing was found to be negligible. This was determined by observing that the diffusion current was constant over a minute interval, indicating that the amount of oxygen is constant and this is an indirect way of saying that the reaction between oxygen and reduced ascorbic acid in the absence of enzyme is extremely slow.

25 milliliters of cyst fluid were used per determination in all the important experiments in the following section on the respiration of the parasites, hence it was found useful to obtain our results in mm^3 of oxygen/25 ml. of fluid. The conversion is performed by multiplying, the volume of dye equivalent to the ascorbic acid oxidized by the oxygen, by the factor -

$$\frac{\text{mgms ascorbic acid}}{\text{mls. dye}} \times \frac{1}{11} \times \frac{22.4 \times 10^{3}}{32} \times \frac{310}{273}$$

The first term in this factor is obtained from the standardization of the dye against an accurately weighed sample of ascorbic acid.

The calibration curve was repeated twice using more than ten samples of cyst fluid collected on different days, and most points were determined more than twice. (See Table XIV and Graph V).

Table XIV

O_2, i_d	O_2, mm^3/25 ml. cyst fluid	pH
25 (5 times)	0	6.5
115	30	6.5
120 (5 times)	30	6.5
140 (5 times)	40	6.5
150	50	6.5
210	52	6.5
250	78	6.5
320 (4 times)	108	6.5
420 (3 times)	138	6.5
630	222	6.5
640	218	6.5

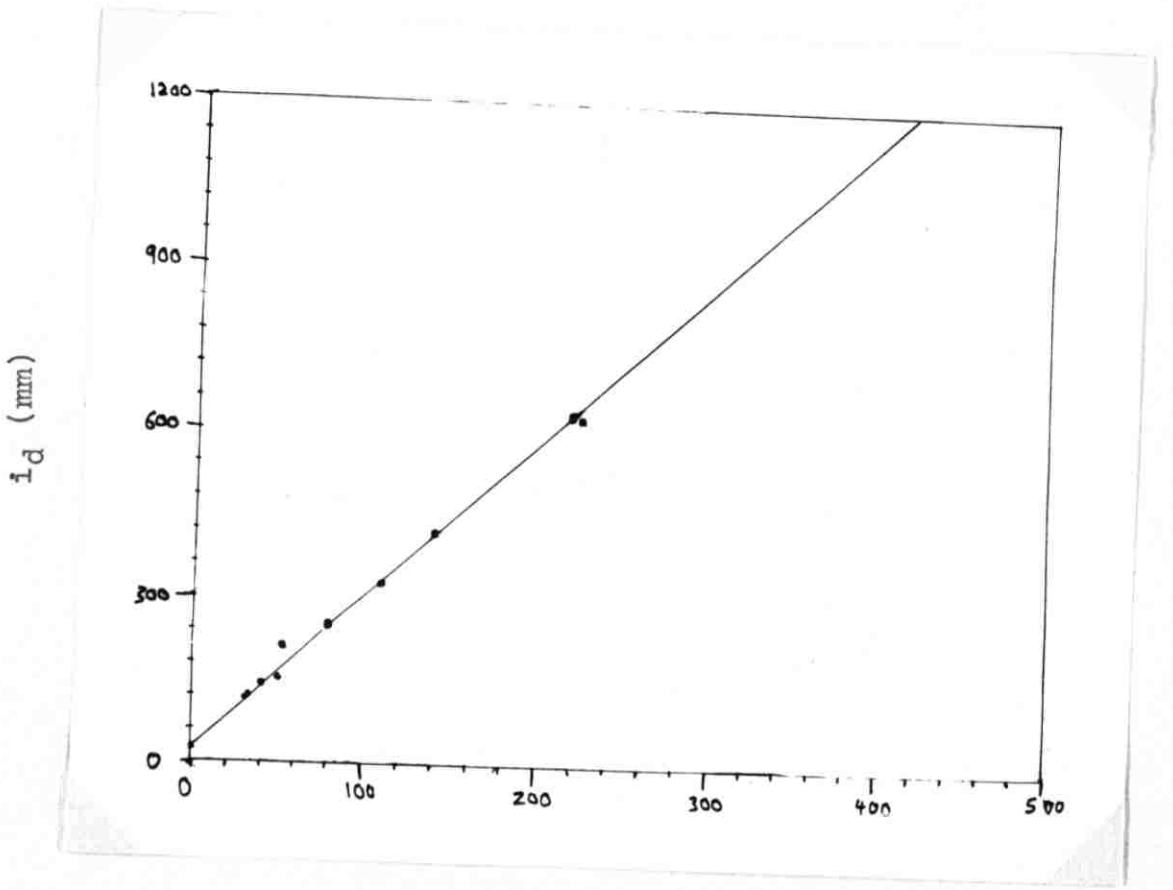


Figure V
(Data from Table XIV)

Calibration curve obtained by using ascorbic acid oxidase method, at 37°C.

RESPIRATION OF THE HYDATED CYST PARASITES IN CYST FLUID

The principal purpose of the present investigation was to study the respiration of "Echinoccocus granulosus" parasites as an aid to determining their metabolic mechanism. It was hoped that the information obtained would make it possible to determine whether the parasites respire aerobically, anerobically or both. In brief, the method employed was to determine the rate at which the oxygen tension of cyst fluid decreased over a considerable period to time. In fact the time interval chosen, although not constant, was usually sufficiently long for the rate of respiration to approach zero. A comparison of this rate as a function of the oxygen content of the fluid with the oxygen tension of the fluid in freshly killed animals (the experiments being carried out in such a manner that this latter value is presumed to be the oxygen tension in the cyst in living systems) makes it possible to estimate the relative contributions of aerobic and anerobic mechanisms in cyst respiration.

EXPERIMENTAL PROCEDURE

The oxygen content of the hydated cyst was measured by the polarograph in terms of diffusion current units using the two potential method. The first potential chosen just precedes the origin of the second half-wave of oxygen, and the second potential was chosen at the point where the diffusion current becomes constant. The two potentials used for oxygen measurements in all the experiments in this section were 0.75 and 1.30 volts respectively (unless specified otherwise).

The diffusion current values were converted to absolute concentrations using the calibration curve (Fig.V).

The exposure of the scolices to mercury in the cell was minimized by using the modified cell discussed on page 14, however, the effect of mercury under the experimental conditions was studied also.

The following conditions were maintained constant for all experiments:

1) a sample of 25 ml. of fluid containing scolices was used.

2) a 10 ml. aliquot of the above sample was dried at 100°C, cooled to room temperature and the dry weight of the sample determined (except where noted otherwise).

3) the experiments were carried out in a constant temperature water bath at 37°C ± 0.1°C. (except where

noted otherwise).

4) where noted, pure oxygen was bubbled through the sample to raise the oxygen tension of the cyst fluid to the desired value.

5) samples were agitated regularly with a cotton stirrer to permit maximum access of scolices to oxygen.

6) all samples were introduced into a polarographic cell which had been flushed and filled with N_2. Immediately after placing the sample in the cell it was covered with a layer of paraffin oil to prevent oxygen exchange with the atmosphere.

7) the Volhard method (13) was used to determine the chlorides in some of the cyst fluid samples.

Determination of the Oxygen Tension in Vivo:

From cysts in the lungs and livers of freshly killed animals 50 ml. samples of fluid were collected in sterilized syringes (a filter paper was used to prevent the entrance of scolices into the syringe). The syringes were then immediately sealed with vaseline to isolate the samples from the air. As soon as possible the oxygen tension in the samples was determined by the usual polarographic procedure. The sample was expelled into the polarographic cell (which had been flushed with nitrogen to remove all traces of atmospheric oxygen) by insertion of the needle of the syringe through a rubber cap. In this way it was possible to

effectively transfer the cyst fluid from the cyst in vivo to a sealed polarographic cell without permitting it to come into contact with the atmosphere and thus the oxygen tension may be presumed to be unchanged.

The results obtained are shown in Table XV. In both cases the samples were obtained from bovine lungs or livers, but the data refers to specimens obtained from two different animals.

Table XV

Sample No.	Source	conc. of Cl^- (Volhard)	pH	diffusion current (in mm.)	mms O_2 per 25 ml. fluid.
1	liver	0.110	7.4	190±2	57
2	liver	0.110	7.4	190±2	57
3	lung	0.098	7.4	235±5	70
4	lung	0.098	7.4	235±5	70
5	lung	0.098	7.4	250±5	78
1	liver	0.080	7.4	125±2	34
2	liver	0.080	7.4	120±2	32
3	liver	0.080	7.4	120±2	32
4	liver	0.080	7.4	120±2	32
5	liver	0.080	7.4	130±2	36

Presentation of Data:

In the following section the data from the various experiments is presented in both tabular and graphical form. The first graph for each experiment shows the variation in oxygen content of the cyst fluid, plotted as the ordinate (in mm^3 O_2/gm. dry weight/25 ml. fluid) with time, plotted as abcissa (in minutes). The second graph shows the variation of the dissolved oxygen content, plotted as ordinate (in mm^3 O_2/gm.dry weight/25 ml. fluid) with rate, plotted as abcissa (in mm^3 O_2/gm. dry weight/hr.). The rates were determined from the slopes of the graphs of oxygen content vs. time.

Experiment No. 1

Dry weight of sample: not determined.

Conditions A: Oxygen bubbled through system to an initial level of 334 mm^3/ 25 ml. of cyst fluid. Experiment was run at 31°C.

Table XVI

t (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml. fluid
0	960		334
5	940		326
10	880	± 20	306
15	840		290
20	820		284
25	820		284
31	800		276
41	720		248
50	680		232
60	670	± 10	230
70	640		218
82	590		200
94	560		190

(Date plotted in Fig. VI)

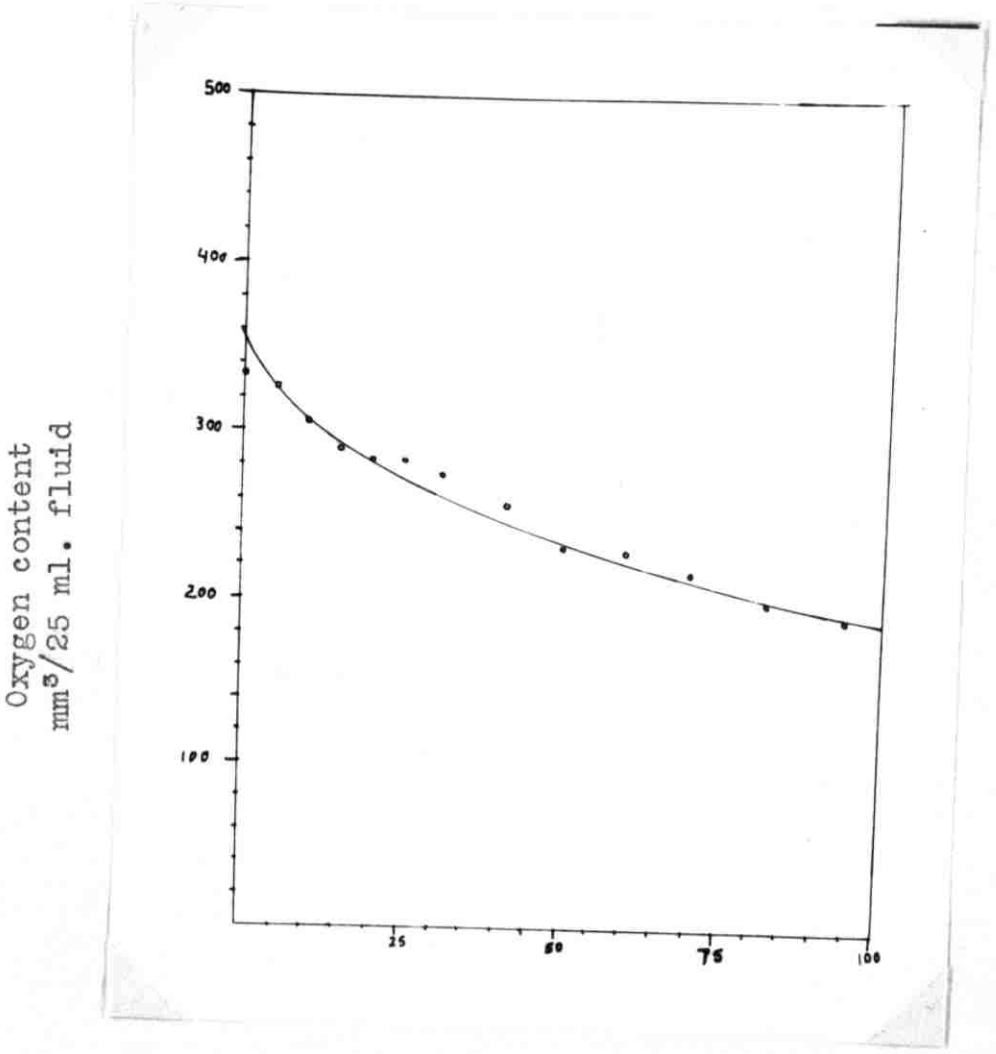


Fig. VI

Variation in dissolved oxygen content of the cyst fluid with time.

(Data from Table XVI)

Experiment No. 2

Dry weight of sample: not determined.

Conditions: Oxygen bubbled through system to an initial level of 414 mm^3/25 ml. fluid.

Table XVII

t (min.)	i_d (mm)	error (mm)	Oxygen mm^3/25 ml. fluid
0	1180		414
10	1140		400
20	1100	± 20	384
30	1040		362
40	980		342
50	940		326
60	900		312
75	820		284
90	780		270
105	730		250
120	670	± 10	230
135	660		226
150	570		194
165	560		190
180	530		180
240	470		158
255	430		144
270	390		128

(Data plotted in Fig. VII)

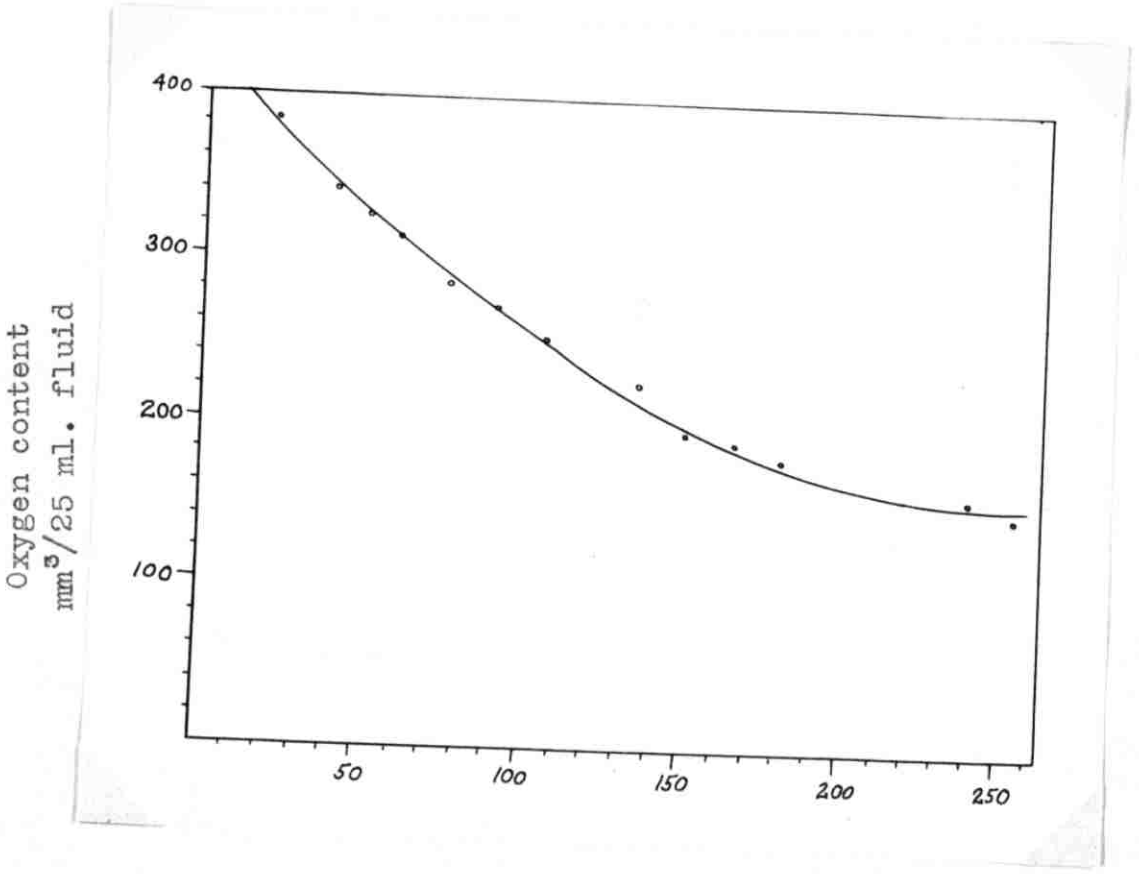


Fig. VII

Variation in dissolved oxygen content of the cyst fluid with time.

(Data from Table XVII)

Experiment No. 3

Dry weight of sample: 165 mgms.

Conditions A: Oxygen bubbled through sample to an initial level of 344 mm^3/25 ml. fluid.

Table XVIII

t (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml.	oxygen mm^3/gm./25 ml. fluid
0	990		344	2082
10	900		312	1890
20	820		284	1780
30	720		248	1500
40	680	→ ± 10	232	1405
50	650		222	1345
60	600		204	1235
70	550		186	1125
80	510		172	1042
90	460		154	935
100	430		144	875
110	395		130	789
120	370		122	740
130	345	→ ± 5	112	679
140	310		100	606
150	285		92	557

(Data plotted in Fig. VIII A)

Conditions B: previous sample rebubbled with oxygen to level of 248 mm^3/25 ml. fluid.

Table XIX

T (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml. fluid.	oxygen mm^3/25 ml. fluid/gm.	oxygen mm^3/gm/ hr.
0	720		248	1500	-
9	690		236	1430	551
19	620	± 10	212	1282	480
30	600		204	1235	451
39	540		182	1100	420
49	530		180	1090	445
59	500		168	1019	394

(Data plotted in Fig. VIII B)

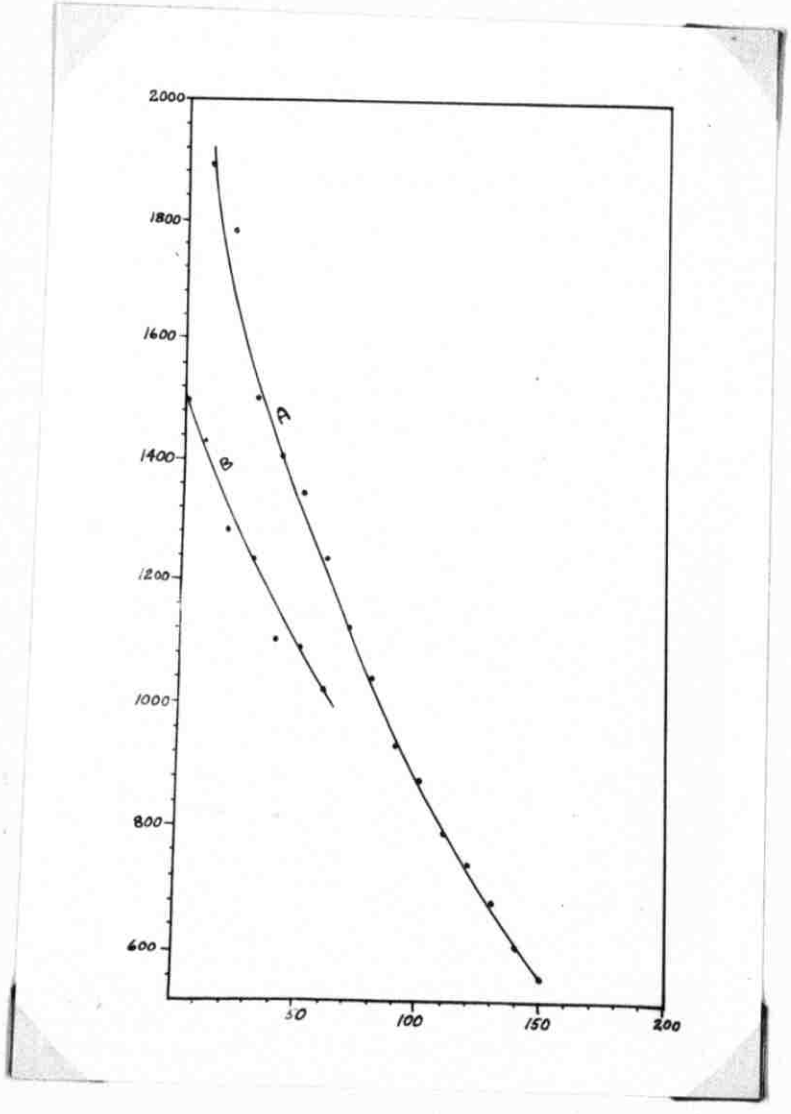


Fig. VIII

Variation of dissolved oxygen content of the cyst fluid with time.

(Data from Table XVIII & XIX)

Table XX*

oxygen $mm^3/gm./hr.$	oxygen $mm^3/gm./25$ ml. fluid.
1260	1740
750	1530
685	1400
540	1310
550	1228
528	1120
528	1020
480	940
480	860
400	788
400	728
360	660
300	608

* The values in this table were calculated from Fig. VIII.A.

(Data plotted in Fig. IX)

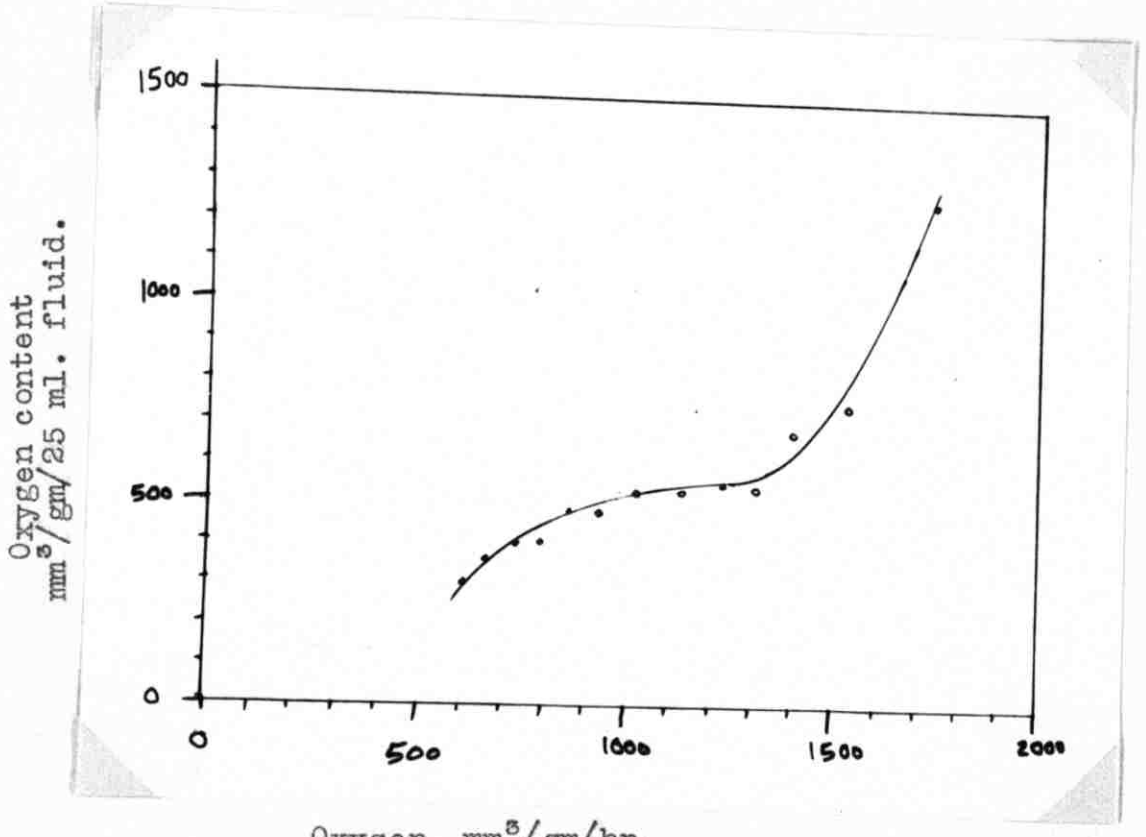


Fig. IX

Variation in Rate with content of dissolved oxygen.

(Data from Table XX)

Experiment No. 4

Dry weight of sample: 450.5 mgs.

Conditions A: Sample studied as received.

Table XXI

t (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml. fluid	oxygen mm^3/25 ml fluid/gm.
0	98		24	53
10	66	± 2	14	31
22	62		12	27
30	70		14	31
35	66	± 1	14	31
40	68		14	31
45	70		14	31

(Data plotted in Fig. XA)

Conditions B: Oxygen bubbled through the previous sample to a level of 302 mm^3/25 ml. fluid.

Table XXII

t (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml. fluid	oxygen mm^3/25 ml. fluid/gm.	oxygen mm^3/gm./hr.
0	870		302	670	-
11	700	± 10	240	532	1190
20	480		162	359	620
30	340		112	248	569
41	230	± 5	72	160	480
50	160		48	106	354
61	88	± 2	22	49	164
70	79		18	40	0
80	79	± 1	18	40	0
90	80		18	40	0
100	82		18	40	0

(Data plotted in Fig. X B as □)

Conditions C: Oxygen rebubbled through sample to a level of 308 mm^3/25 ml. fluid.

Table XXIII

t (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml. fluid.	oxygen mm^3/25 ml. fluid/gm.
0	890		308	682
12	690	± 10	236	522
20	490		174	386
40	205		62	137
51	142	± 5	40	89
61	84		20	43
72	84	± 1	20	43
80	93		22	49
88	87		20	43

(Data plotted in Fig. X C as •)

Since both these points and those of Table XXII fall on the same curve this is taken as evidence that mercury does not poison the parasites under these conditions the rate remains essentially the same and not affected by mercury.

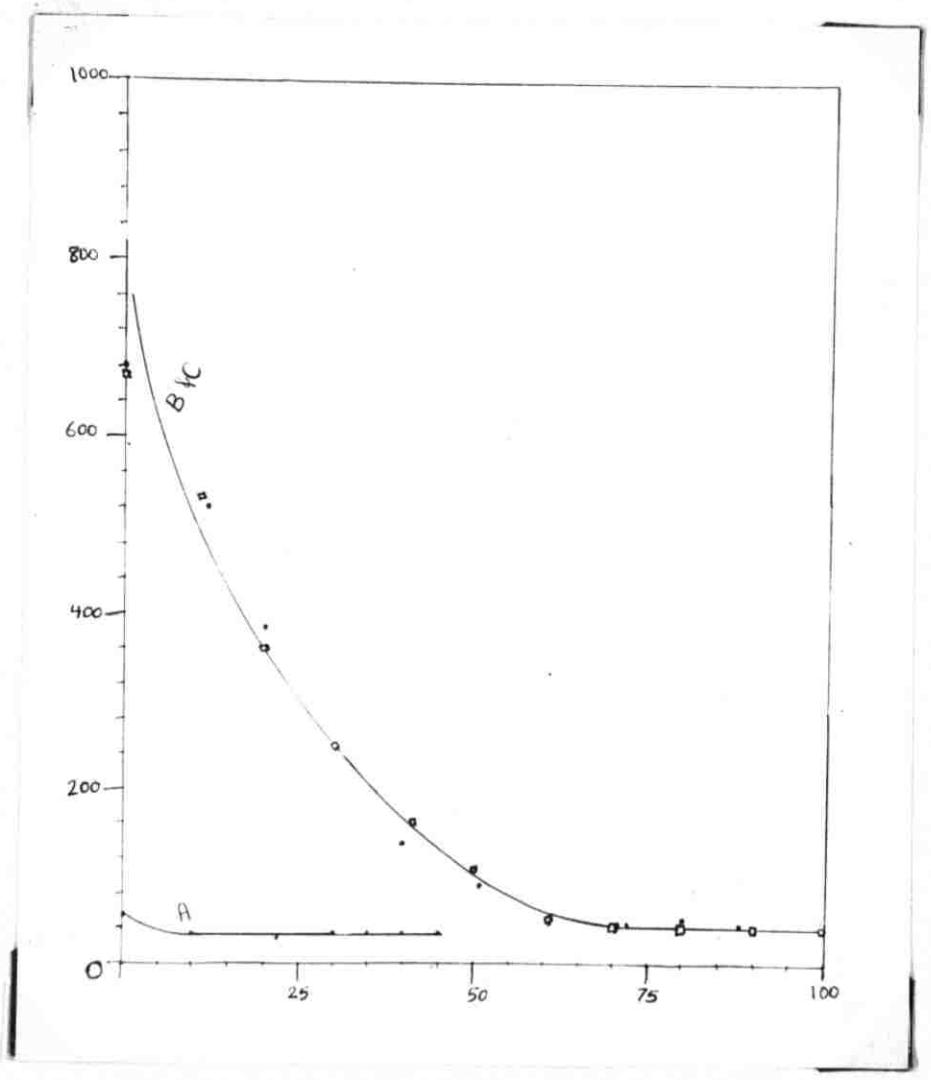


Fig. X

Variation in dissolved oxygen content of the cyst fluid with time.

(Data from Tables XXI, XXII & XXIII)

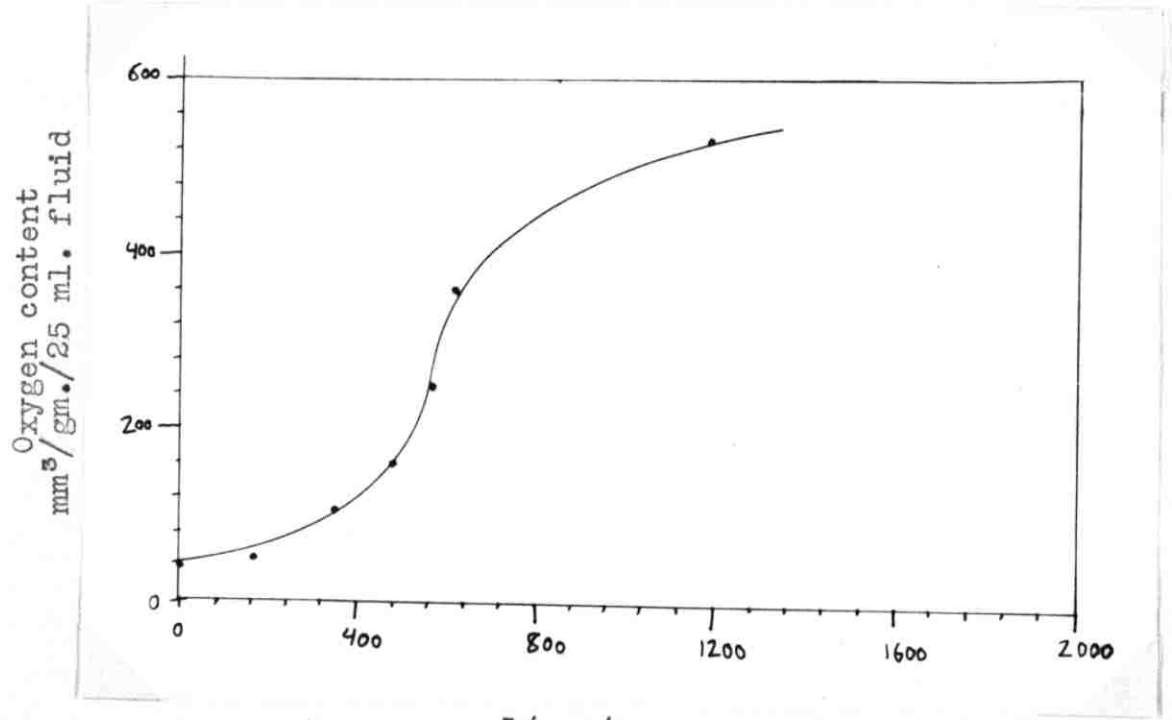


Fig. XI

Variation in rate with content of dissolved oxygen.

(Data from Table XXII)

Experiment No. 5

Dry weight of sample: 241.3 mgms.

Conditions A: Sample studied as received.

Table XXIV

t (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml. fluid.	oxygen mm^3/25 ml. fluid/gm.	oxygen mm^3/gm./ hr.
0	335		110	456	-
6	260		82	341	830
15	194	→ ± 5	58	241	400
20	186		56	233	262
26	160		48	200	170
30	146		42	175	92
50	140		40	166	0
60	140	→ ± 2	40	166	0
70	144		42	175	0
80	146		42	175	0
90	146		42	175	0

(Data plotted in Fig. XII A)

Conditions B: Oxygen bubbled through sample to a level of 254 mm^3/25 ml. fluid.

Table XXV

t (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml. fluid.	oxygen mm^3/25 ml. fluid/gm.	oxygen mm^3/gm./hr.
0	740		254	1070	-
11	700	± 10	240	1000	-
20	600		204	850	655
30	530		180	748	545
40	470		158	661	480
50	415		138	574	427
60	375		124	515	415
72	320	± 5	104	425	416
80	270		86	358	366
90	240		76	316	352
100	224		70	291	260
110	194		58	221	168
120	182	± 2	54	224	80
133	160		48	200	0
140	164		48	200	0
150	160		48	200	0
160	156		46	191	0
168	162		48	200	0
195	166		50	208	0

(Data plotted in Fig. XII B & XIII)

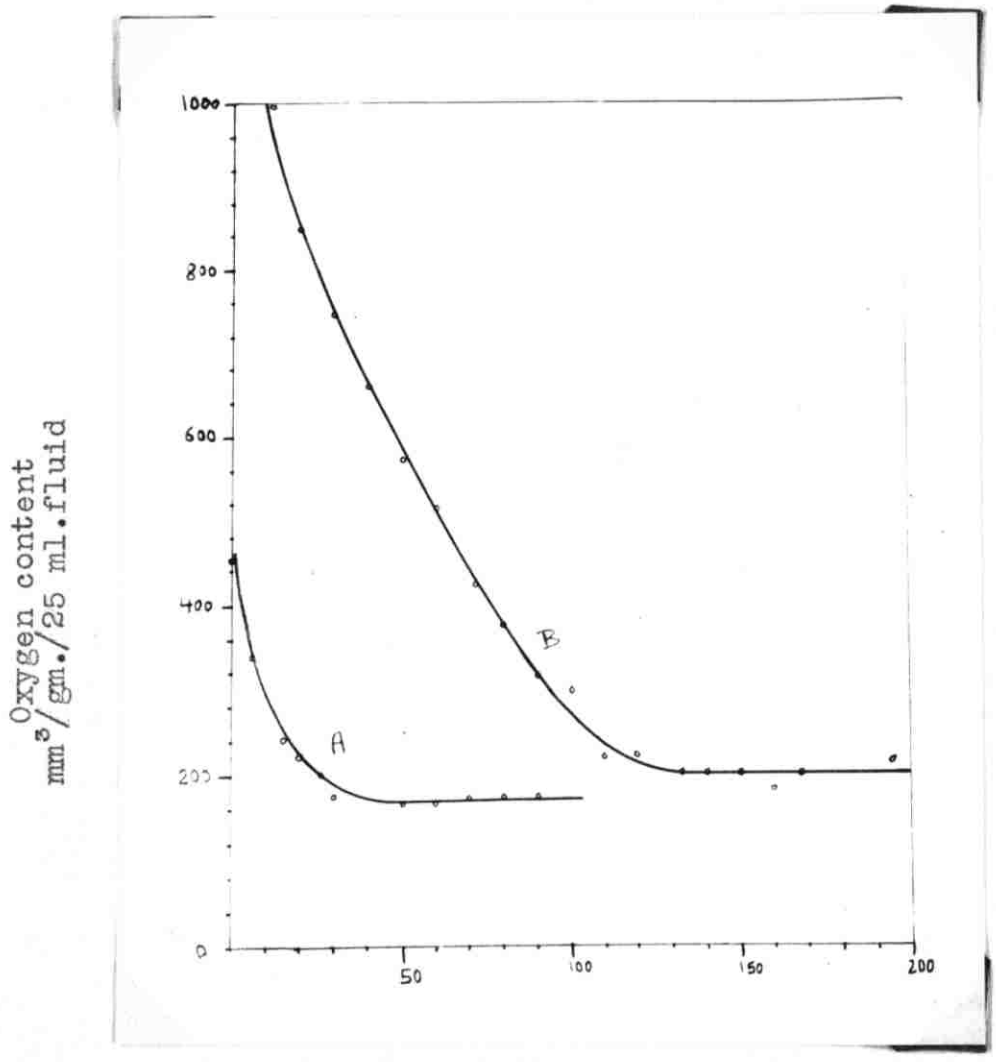
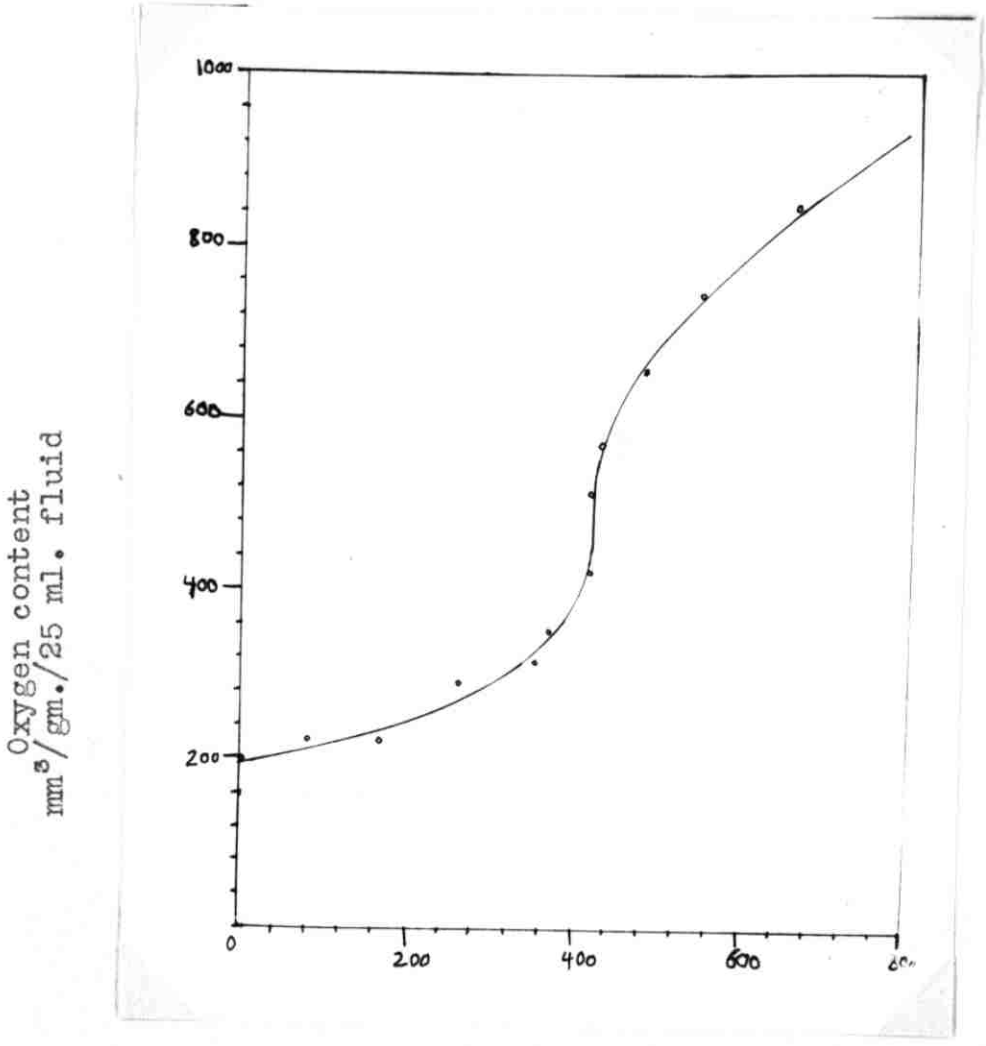


Fig. XII

Variation in dissolved oxygen content of the cyst fluid with time.

(Data from Tables XXIV & XXV)



Oxygen, mm^3/gm./hr.

Fig. XIII

Variation in rate with content of dissolved oxygen.

(Data from Table XXV)

Experiment No. 6

Dry weight of sample: 306 mgms.

Conditions A: Sample studied as received

Table XXVI

t (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml. fluid.	oxygen mm^3/25 ml. fluid/gm.	oxygen mm^3/gm./ hr.
0	295		96	314	-
10	165	± 5	50	163	737
15	118	± 2	32	104	501
21	95		24	79	374
25	66		14	46	240
30	64	± 1	12	39	0
40	58		10	33	0
50	63		12	39	0
60	59		10	33	0
70	59		10	33	0

(Data plotted in Fig. XIV & XV)

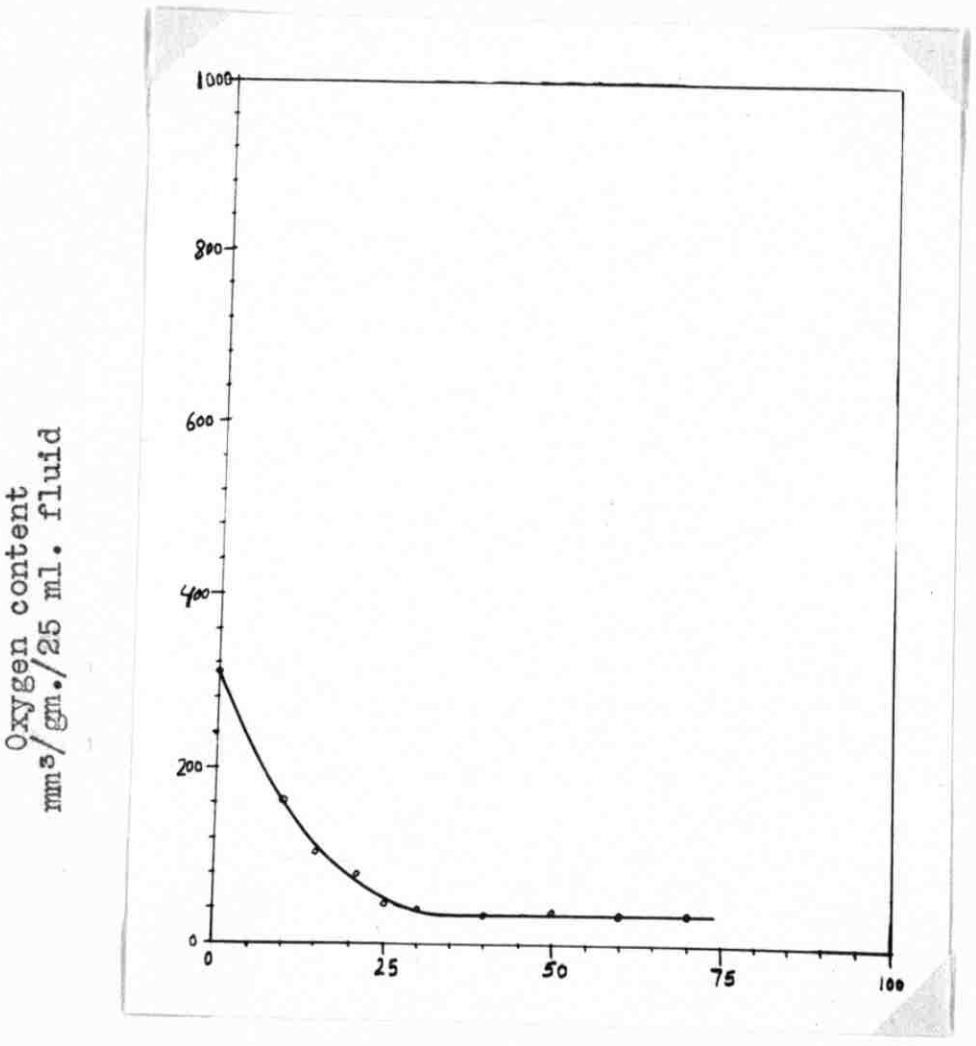
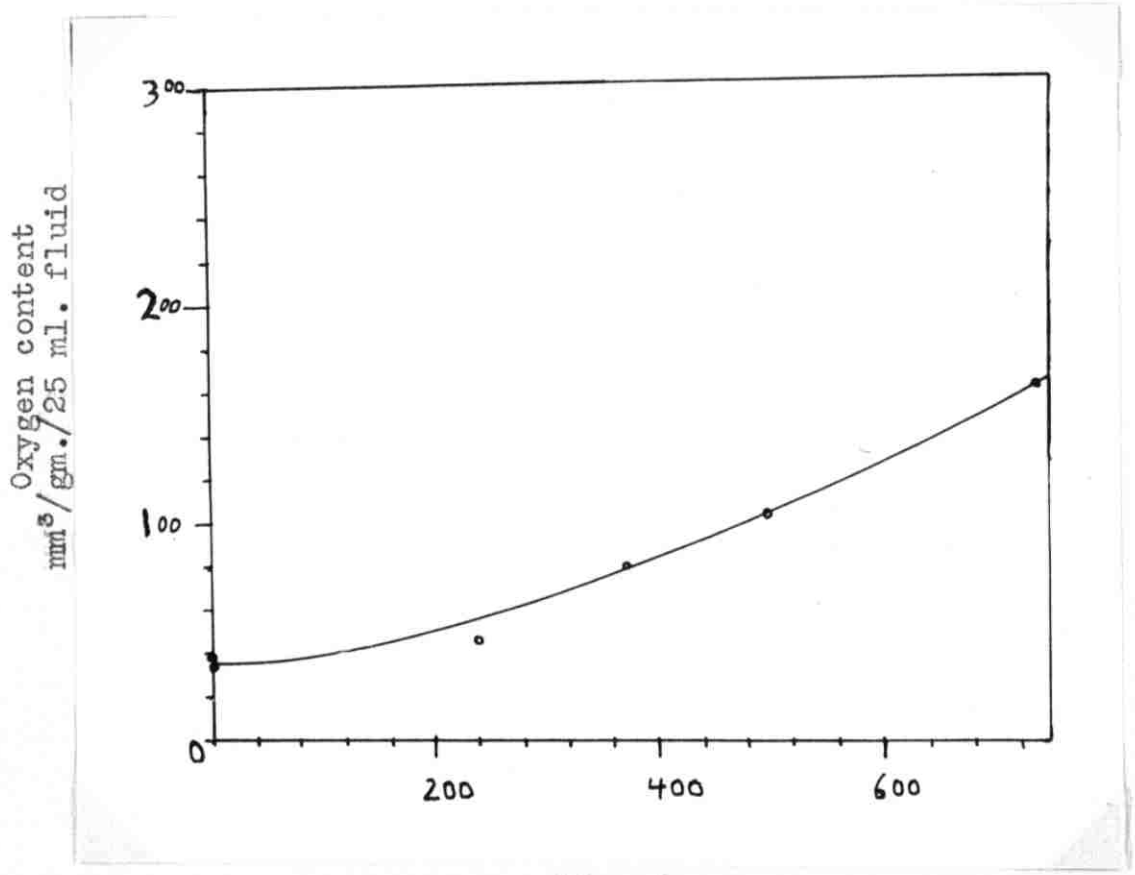


Fig. XIV

Variation in dissolved oxygen content of the cyst fluid with time.

(Data from Table XXVI)



Oxygen, $mm^3/gm./hr.$

Fig. XV

Variation in rate with content of dissolved oxygen.

(Data from Table XXVI)

Conditions B: Sample bubbled with oxygen to the level of 234 mm^3/25 ml. fluid.

Table XXVII

t (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml. fluid	oxygen mm^3/25 ml. fluid/gm.	oxygen mm^3/gm./hr.
0	680		234	765	-
5	520	± 10	176	575	1960
10	420		140	457	1600
15	310		100	327	1530
21	190	± 5	58	189	1330
25	100		26	85	686
30	62		12	39	267
35	64		12	39	0
40	62	± 1	12	39	0
50	64		12	39	0
60	67		14	46	0
70	68		14	46	0

(Data plotted in Fig. XVI & XVII)

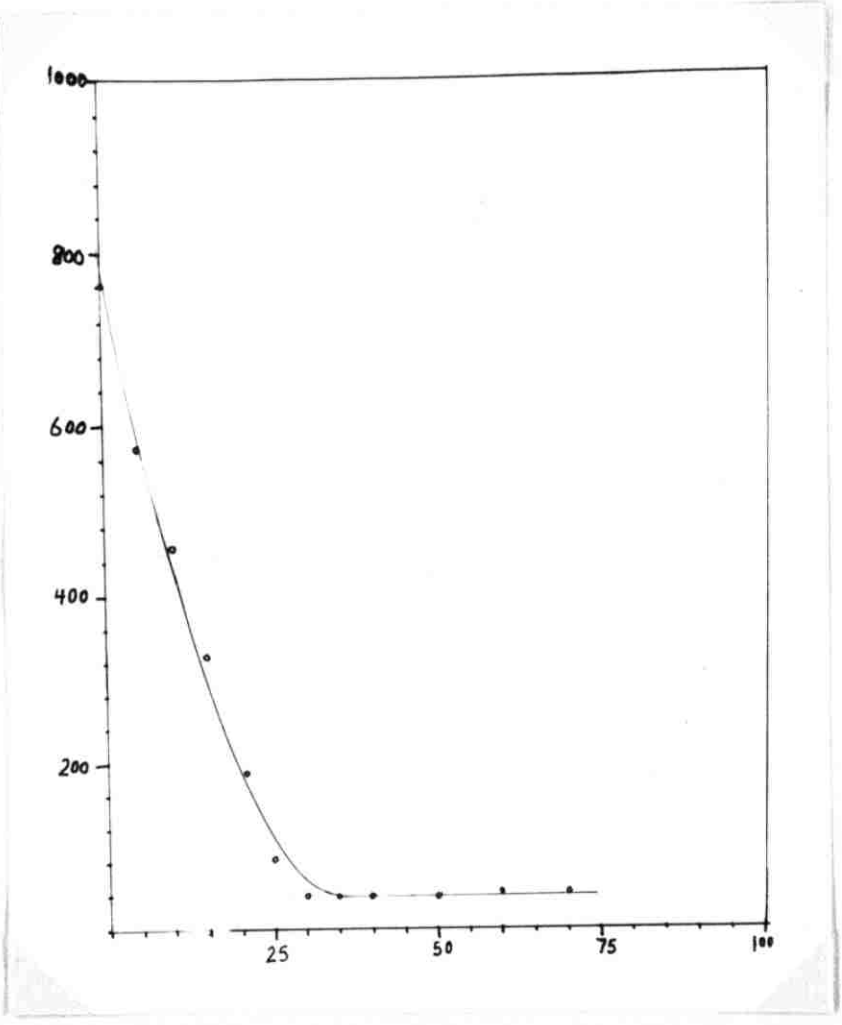
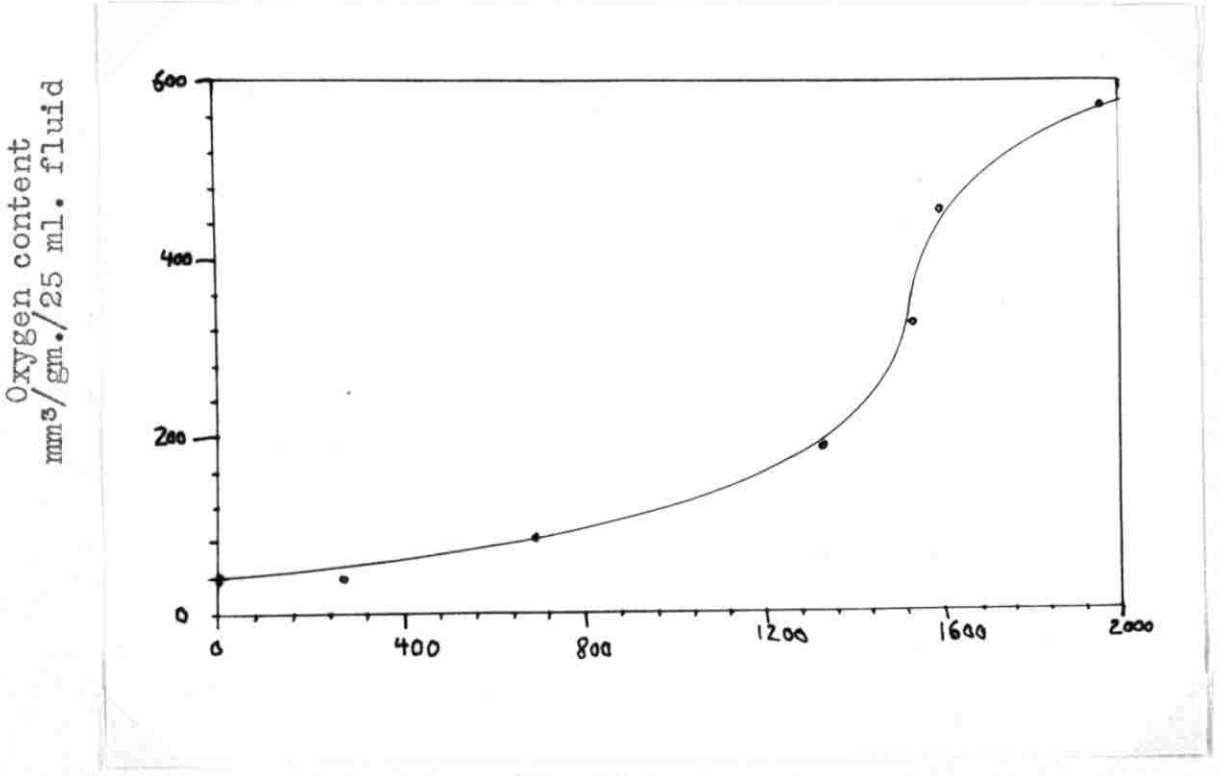


Fig. XVI

Variation in dissolved oxygen of the cyst fluid with time.
(Data from Table XXVII)



Oxygen, $mm^3/gm./hr.$

Fig. XVII

Variation in rate with content of dissolved oxygen.

(Data from Table XXVII)

Conditions C: Sample rebubbled with oxygen to the level of 258 mm^3/25 ml. fluid.

Table XXVIII

t (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml. fluid.	oxygen mm^3/25 ml. fluid/gm.	oxygen mm^3/gm./ hr.
0	750		258	844	-
6	610	± 10	208	680	1880
10	490		164	535	1320
15	390		130	425	1005
20	335	± 5	110	360	960
25	275		88	287	800
31	204		62	202	740
35	166	± 2	50	163	540
45	92		22	72	240
55	68		14	46	0
65	69	± 1	14	46	0
75	67		14	46	0
85	69		14	46	0

(Data plotted in Fig. XVIII & XIX)

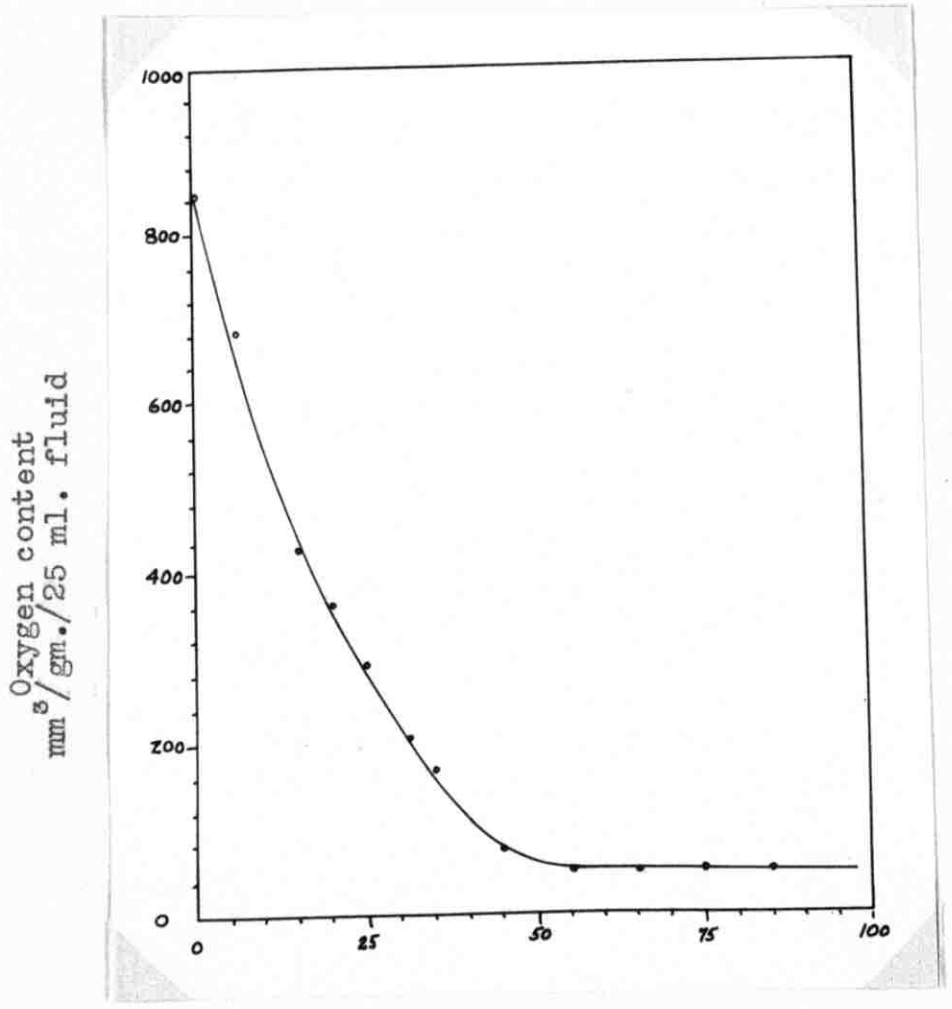
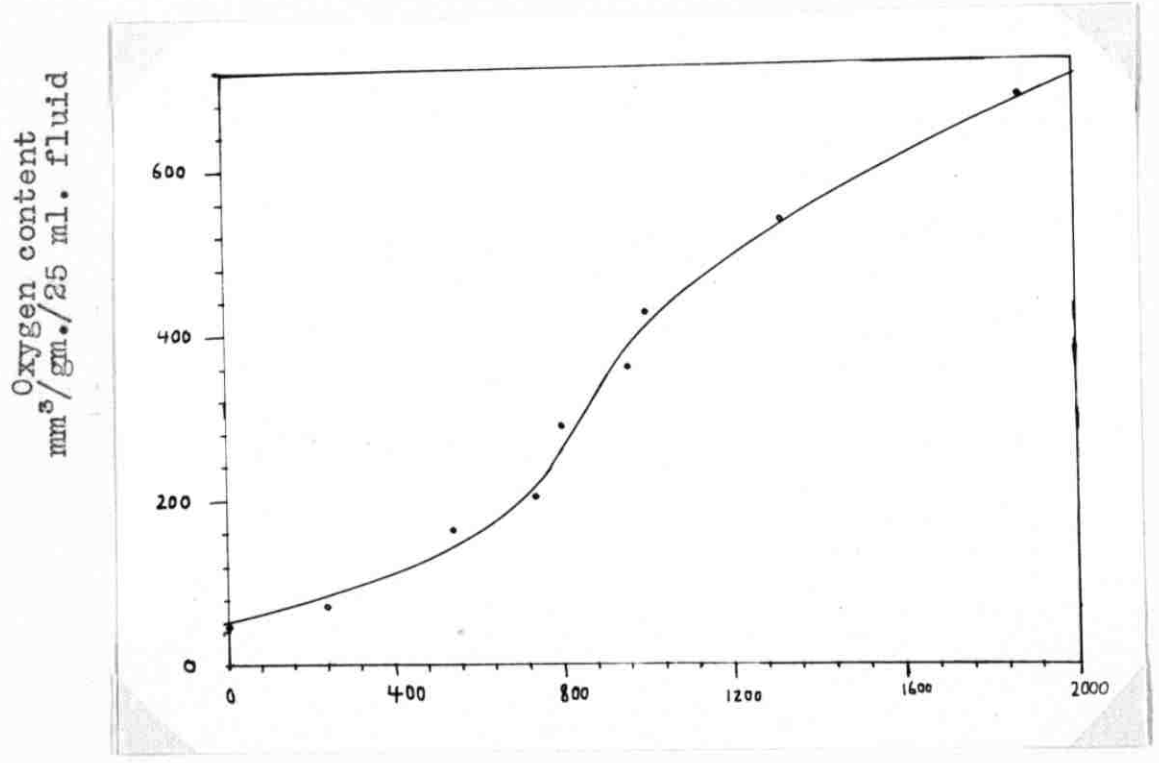


Fig. XVIII

Variation in dissolved oxygen content of the cyst fluid with time.

(Data from Table XXVIII)



Oxygen, $mm^3/gm./hr$

Fig. XIX

Variation in rate with content of dissolved oxygen.

(Data from Table XXVIII)

Conditions D: Sample deoxygenated by bubbling nitrogen through the system until the oxygen level was 12 mm^3/25 ml. fluid, then the sample was bubbled with oxygen to a level of 306 mm^3/25 ml. fluid.

Table XXIX

t (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml. fluid	oxygen mm^3/25 ml. fluid/gm.	oxygen mm^3/gm./ hr.
0	880		306	1000	-
10	690	± 10	236	770	1820
20	550		186	606	1080
31	420		140	457	1050
41	285	± 5	92	300	985
61	80	± 2	18	59	203
71	62		12	39	0
80	55	± 1	10	33	0
90	64		12	39	0

(Data plotted in Fig. XX & XXI)

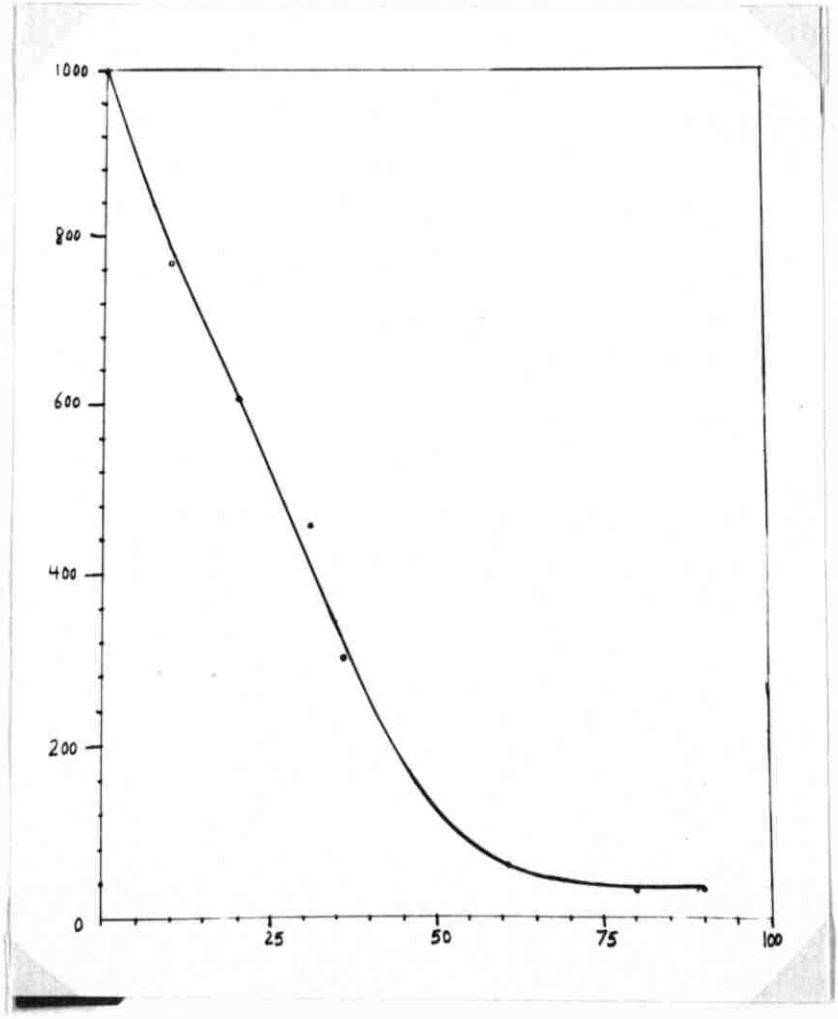
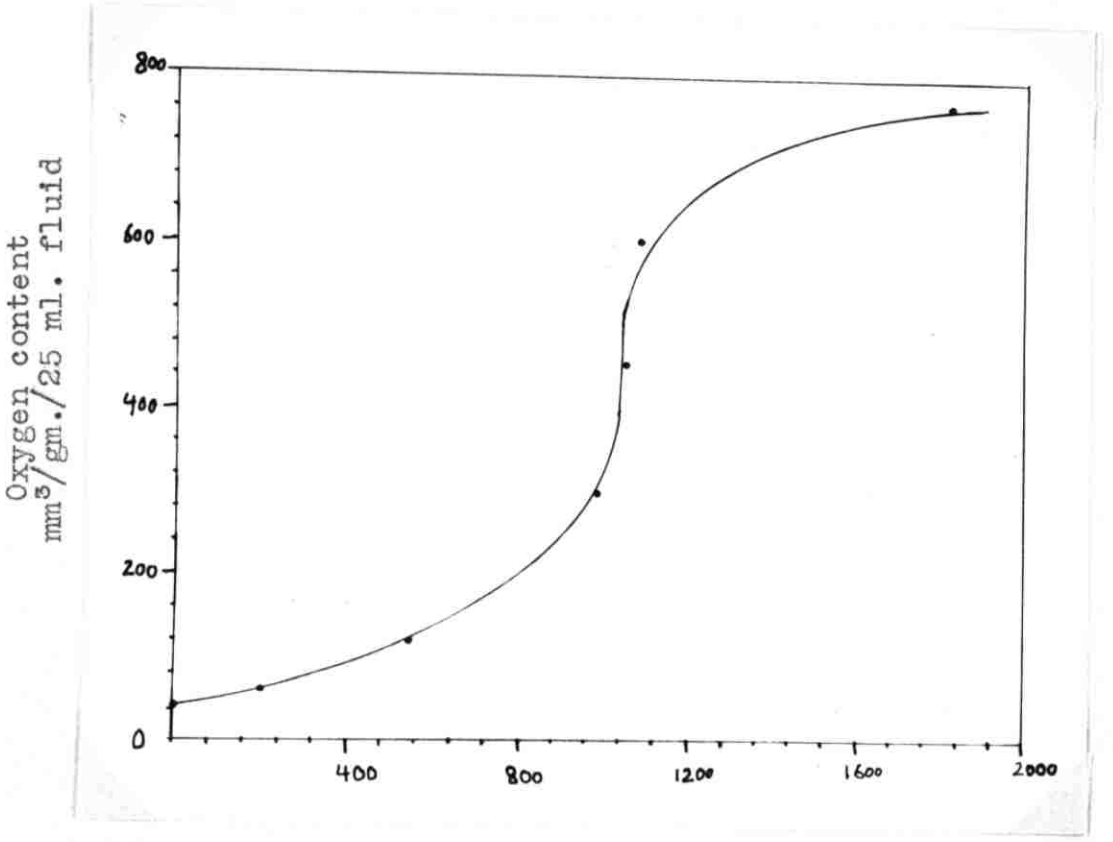


Fig. XX

Variation in dissolved oxygen content of the cyst fluid with time.

(Data from Table XXIX)



Oxygen, $mm^3/gm./hr.$

Fig. XXI

Variation in rate with content of dissolved oxygen.

(Data from Table XXIX)

Conditions E: Sample D with an oxygen level of 12 mm^3/ 25 ml. fluid, was placed in an air tight container for 8 hours (and stored in a refrigerator) after which time its oxygen level was found to be constant at 12 mm^3/25 ml. level. The sample was then rebubbled with oxygen to a level of 296 mm^3/25 ml. fluid. Microscopic examination of the scolices showed them to be alive after the 8 hours isolation period.

Table XXX

t (min.)	i_d (mm)	error (mm)	oxygen mm^3/25 ml. fluid.	oxygen mm^3/25 ml. fluid/gm.	oxygen mm^3/gm./ hr.
0	850	± 10	296	966	2600 *
12	390	± 5	130	425	2060
21	175	± 2	52	170	1390 1000 **
30	64	± 1	12	39	0
40	66		14	46	0
50	69		14	46	0
60	69		14	46	0

* Rate 2600 mm^3/gm./hr. was calculated from Fig. XXII at oxygen content 748 mm^3/gm./25 ml. fluid.

** Rate 1000 mm^3/gm./hr. was calculated from Fig. XXII at oxygen content 80 mm^3/gm./25 ml. fluid.

(Data plotted in Fig. XXII & XXIII)

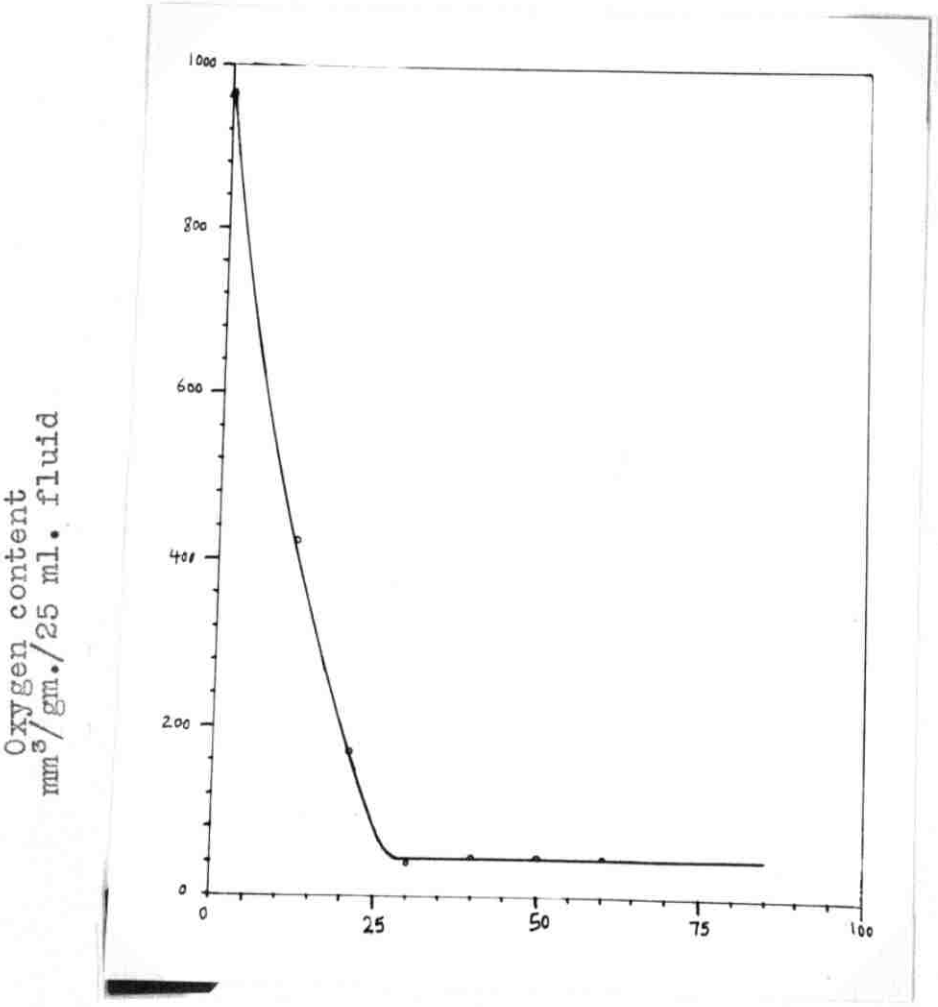


Fig. XXII

Variation in rate with content of dissolved oxygen.

(Data from Table XXX)

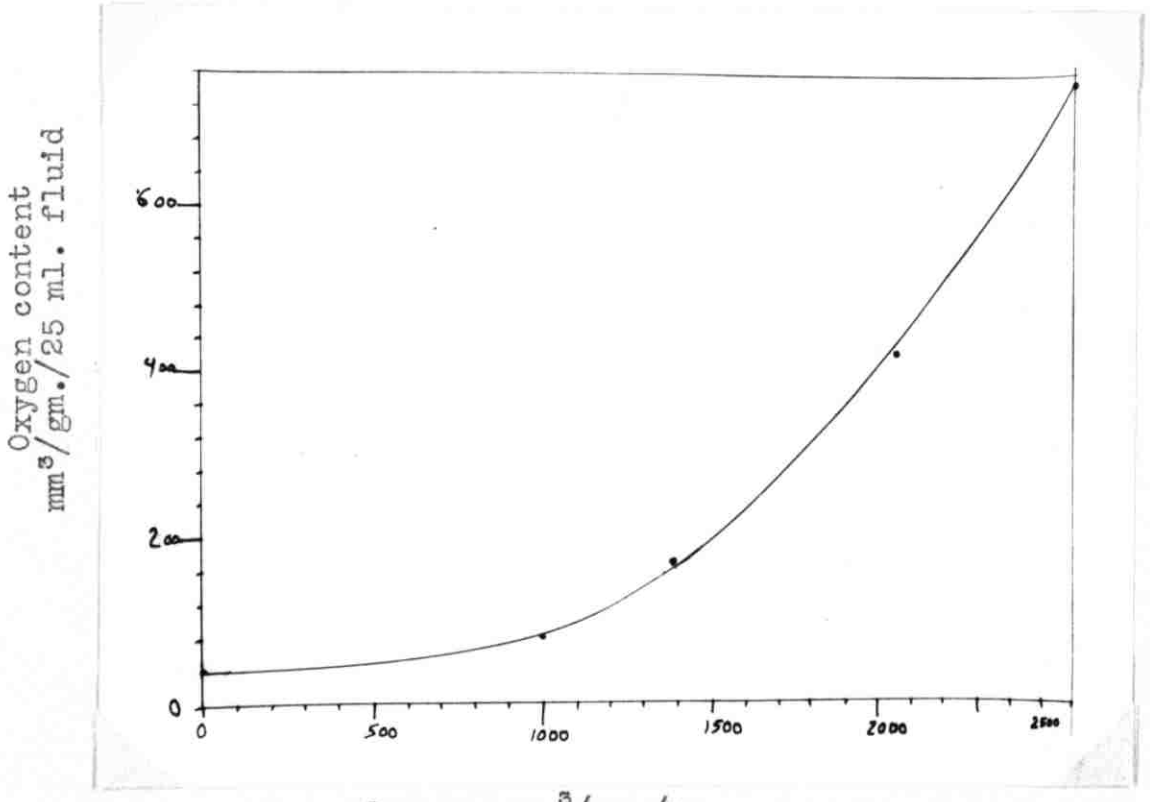


Fig. XXII

Variation in rate with content of dissolved oxygen.

(Data from Table XXX)

DISCUSSION

As was pointed out in the introduction, the ultimate purpose of the present investigations was to learn something about the respiratory mechansim of hydatid cyst parasites.

The experimental controls that were run indicate that the polarographic method is admirably fitted for respiratory studies of the type carried out here. The results may be considered reliable and reproducible.

Consideration of the graphs showing the variation of the rate of oxygen consumption with decreasing content of dissolved oxygen in the cyst fluid, reveals that below a well defined level of oxygen concentration (ca. 40 $mm^3/gm/25$ ml. fluid) the rate rapidly approaches and does indeed become zero, (there is one exception to this behavior that at present remains unexplained, see Fig. XII). This content of dissolved oxygen is well above the minimum limit detectable by the polarographic method. This suggests that below this limiting oxygen concentration the respiration of the parasites is not aerobic. Parasites maintained in the cyst fluid (containing the minimal oxygen concentration) for a period of eight hours were shown to still be living (by microscopic examination) and that no change in this limiting oxygen concentration could be detected. Furthermore, these same parasites were observed to respire at an increased rate when the oxygen concentration of the fluid was again brought to a high level. This series of experiments indicates that

for moderate length periods, at least, the parasites are capable of anaerobic respiration. On the other hand, the very fact that the dissolved oxygen content of the cyst fluid containing parasites decreases in a regular manner indicates that the organisms also respire aerobically. The point at issue is to determine, if possible, which mechanism is predominant in a given case and in particular which mechanism, if either, predominates in vivo.

This phenomenon, that an aerobically respiring organism may respire at an increased rate after prolonged oxygen defficiency, is termed the oxygen debt. Its exact cause has not been ascertained but it may be due to the accumulation of partially oxidized waste products during the anaerobic respiratory phase. When the supply of oxygen is again increased these by products must perhaps be further oxidized before, or concurrently during, the usual respiratory processes resulting in an apparent increase in the rate of aerobic respiration. In the particular case observed it was noted that after the parasites had existed for eight hours in fluid in which the dissolved oxygen content was at its minimal level, restoring the oxygen concentration to a high level resulted in a rate of respiration that was 80% greater than its previous value (see experiments 6D & E, pages 82, 85). In another case in which the oxygen deficiency was maintained for only one hour the final rate was only 15% greater than the original rate (see experiments 6C & D, pages 79, 82). This implies that

the increase in rate after oxygen deficiency may be proportional to the period of deficiency. The data obtained thus far are too meager to draw any firm conclusions and must await further substantiation.

With one exception (Experiment 6E, Fig. XXIII) the experiments yield data in which the variation in rate with decrease in dissolved oxygen concentration is of the same general form. This general relation might be briefly described in the following way: below a certain concentration of dissolved oxygen the rate of decrease is zero; over a small range of oxygen concentrations greater than this minimal level the rate of decrease in the rate of consumption is extremely rapid and essentially linear with decrease in oxygen concentration (here after this will be referred to as zone A-B); over a moderate range of oxygen concentrations greater than those of zone AB the rate of decrease in oxygen consumption tends to remain constant or at least does not change extremely rapidly (hereafter referred to as zone BC); and finally over a small range of oxygen concentrations greater than those of zone BC the rate of decrease in the rate of oxygen consumption with decrease in oxygen concentration again becomes very rapid and essentially linear (here after referred to as zone DC; for an explanation of these designations see Figure XXIV. In none of the experiments did the rate of oxygen consumption appear to approach a limiting value with increasing oxygen concentration. On the contrary

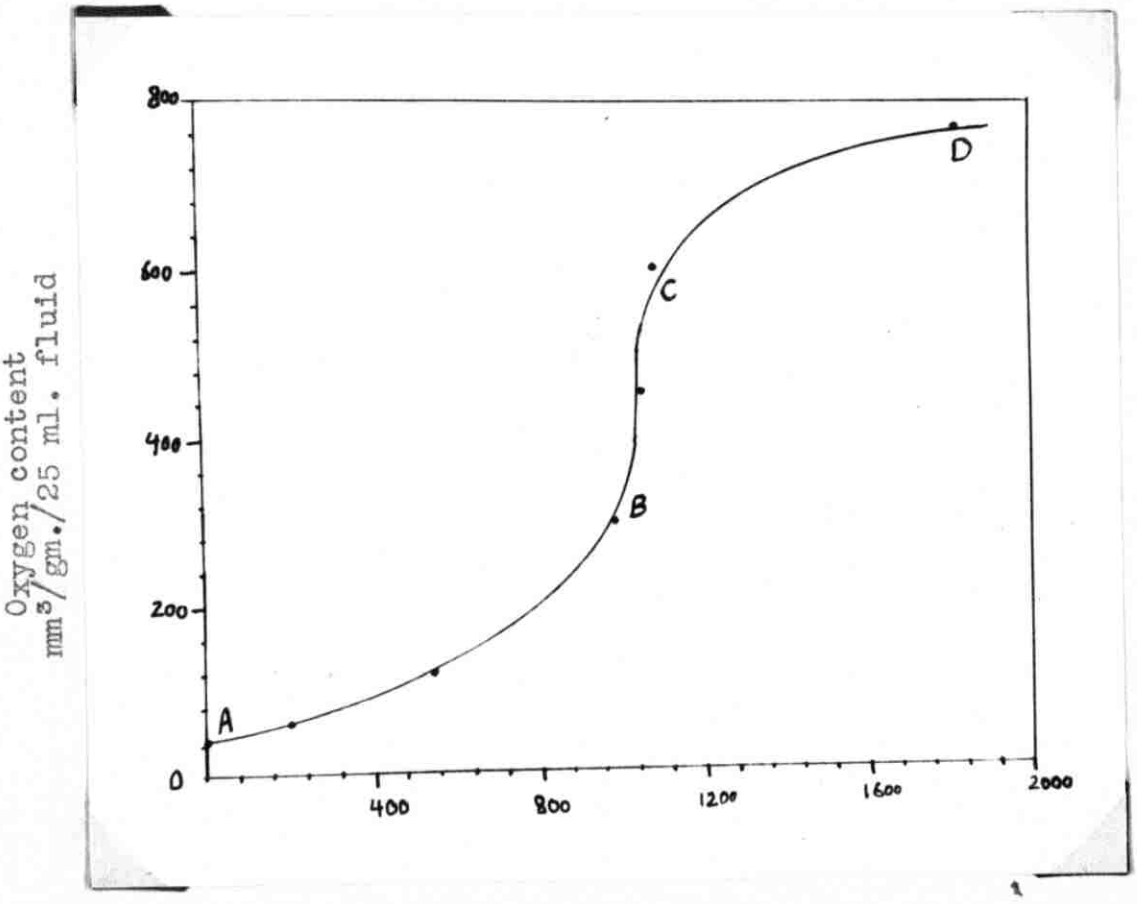


Fig. XXIV

Fig. XXIV showing the different zones of rate of consumption of oxygen (as referred to in the text).

the data (with the exception of experiment 6E, Fig. XXIII) indicate that above a certain range of oxygen concentration (the region beyond point C) the rate increases without limit. Whether or not this would be true for much higher oxygen concentrations is not known and must be subsequently determined.

A comparison of the various figures shows that there seems to be no relationship between the rate of consumption of oxygen at a given concentration of oxygen in one experiment and the rate of consumption for the same oxygen concentration in another experiment.

Although no comparison between the rate of oxygen consumption and dissolved oxygen content can be made between various experiments on an absolute basis, some very interesting relative comparisons can be made.

From two experiments on the concentration of dissolved oxygen in the cyst fluid, in which the oxygen content was established by equilibrium with the air, the concentrations 104 mm^3 oxygen /25 ml. fluid and 84 mm^3 oxygen /25 ml. fluid were found. As was pointed out before (Table IV, page 20) these different values probably arise due to differences in the salt concentrations of the samples (also to be discussed below). If these values are converted to a weight basis for a number of different samples the following results are obtained:

Table XXXI

Exp. No.	oxygen content corresponding to 104 mm^3/25 ml.	oxygen content corresponding to 84 mm^3/25 ml.
4B	233	186
5B	436	349
6B	349	274
6C	349	274
6D	349	274
6E	349	274

Oxygen level in the cyst fluid in equilibrium with air in mm^3/gm/25 ml. fluid.

By drawing horizontal lines on the figures at these two levels for the different experiments it will be seen that the rate of consumption curves are intersected at approximately point B and above. In other words zone BC, which is the region in which the variation in rate of oxygen consumption with change in oxygen concentration is small, corresponds approximately to the region where the oxygen concentration of the fluid is that which it would have were it in equilibrium with the air.

Secondly, if for the purpose at hand, the rate of

consumption of oxygen at the midpoint of zone BC is designated the optinum rate, the following comparison may be made. Experiments to determine the oxygen content in vivo (page 51) showed that the oxygen concentration of the cyst fluid varied depending on the source of the fluid. Selecting the two values 73 mm^3 oxygen/25 ml. fluid and 33 mm^3 oxygen/25 ml. fluid for the samples of fluid from the lungs and liver respectively and converting these values to a weight basis for the different samples the following results are obtained:

Table XXXII

Exp. No.	Oxygen content in liver	Oxygen content in lungs
4B	78	173
5B	145	324
6B	114	255
6C	114	255
6D	114	255
6E	114	255

Oxygen level in the cyst fluid in vivo (in mm^3 oxygen/gm./25 ml. fluid).

By comparing the rates corresponding to these concen-

trations (from experiments 4B, 6C and 6D) to the optimum rates (as defined above) it is found that for the oxygen concentration existing in vivo in the liver the average rate is ca. 48% of the optimum value. And for the lung the average rate is ca. 90% of the optimum value (see Table below):

Table XXXIII

Exp. No.	rate at O_2 level in liver	rate at O_2 level in lungs	optimum rate
4B	260	496	560
5B	0	344	420
6B	944	1456	1520
6C	415	800	860
6D	505	920	1040
6E	1170	1660	?

Rate of oxygen consumption corresponding to the oxygen level in the cyst fluid in vivo (in mm^3/oxygen/gm./hr.).

It would seem to be significant that the deviation in these values is not large.

Before deciding on the ultimate significance of these values the importance to be attatched to the optimum rate must be decided. As an aid to doing this recourse will be made to some results obtained by Rogers (26) on studies of

some nematode parasites of the alimantary tract. By plotting the rate of consumption of oxygen vs the oxygen concentration of saline solutions containing the parasites, Rogers found that definite limiting rates were obtained with increasing oxygen concentrations. In this respect his results differed from those obtained here since no limiting rates were found. Rogers' data was obtained from Warburg manometric experiments. Careful inspection of Rogers' results shows that the maximum partial pressures of oxygen that he used were 150 mm of Hg and that this is approximately the partial pressure of oxygen in air at standard atmospheric pressure. Since his rates had attained their maximum values under these conditions, it means that the rate maxima correspond to the rate when the concentration of dissolved oxygen was determined by equilibrium between the saline solution and air. By comparing this maximum rate with the rate corresponding to the in vivo oxygen tension Rogers estimates the percentage respiratory activity in vivo.

In the experiments carried out here the optimum rate usually was about the same as the rate of consumption at an oxygen concentration of 104 mm^3 oxygen/25 ml. fluid. This is the maximum oxygen concentration that was obtained in fluid in equilibrium with air. This suggests, in our case as in Rogers, that the respiratory activity in vivo in the liver is ca. 48% and in the lung ca. 90% of the maximum rate attainable - when that maximum rate is limited by the

dissolved oxygen concentration in equilibrium with air. It is unfortunate that greater oxygen pressures were not used by Rogers so that it could be ascertained if true maxima were reached.

As was pointed out in the introduction, the results obtained here can not be directly compared with those obtained by Von Brand et al. (4). Von Brand's experiments were carried out manometrically at atmospheric pressure. He found that the rate remained essentially constant for a period of one hour at any given temperature. Although most of his experiments were carried out in Rogers' solution, one was carried out in the cyst fluid. In this case the rate was 2125 mm^3 oxygen/gm./hr. By comparing the rates of consumption corresponding to the oxygen concentration in the cyst fluid (in equilibrium with air) in a series of different samples, it was found that the rates obtained here vary from ca. 20 to 90% of his value. It would seem that little significance can be attatched to this comparison at present.

Von Brand also found that the rate of oxygen consumption varied with the ionic strength of the solution in which the studies were made. As has been shown (Table IV, page 20) the concentration of dissolved oxygen varies significantly with the ionic strength of the solution (estimated on the basis of Cl^-). Consequently, since as the graphs of rate of consumption vs dissolved oxygen concentration show, the rate of consumption varies over

wide limits with the dissolved oxygen concentration, Von Brands results are qualitatively explicable. Further experiments should be carried out to obtain more quantitative data for a satisfactory comparison.

By determining the rate of anaerobic CO_2 evolution manometrically and comparing it with the rate of oxygen consumption it is possible to estimate the relative importance of the aerobic and anaerobic mechanisms. Sucha a comaprison is not possible when using the polarographic method since CO_2 is not detectable. This results in an inability to estimate the relative importance of anaerobic respiration. The results obtained in these investigations do conclusively show the existence of an anaerobic and an aerobic mechanism. Utilizing the general classification of Moulder (20) the cyst parasites would then be designated as faculative aerobes - meaning they can "survive more or less indefinitely either in the presence or absence of oxygen". Furthermore, since the rate of respiration in vivo attains ca. 48 - 90% the optimum value attainable under atmospheric conditions it may tentatively be concluded that the aerobic mechanism plays a significant role in the cysts respiration in vivo.

As is apparent from the foregoing, these preliminary investigations have established the value and applicability of the polarographic method in respiratory studies of this type. Further investigation to more completely elucidate the

metabolic process of the cyst parasites is encouraged by the results already obtained.

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