MANOMETRIC TECHNIQUES
and
TISSUE METABOLISM

by

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Chapters on Specialized Techniques

by

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龍門聯合書局影印
1951

INTRODUCTION TO THE SECOND EDITION

The experience gained during the past few years and the rather gratifying acceptance of the first edition have prompted us to revise and rewrite portions of this volume. In doing this we have attempted to correct errors appearing in the original edition, to add new and valuable techniques which have been developed or with which we have become familiar recently, and to omit certain specialized sections which were rarely used. These changes have not altered the size of the book appreciably, but the content is significantly different from that of the first edition; we hope the alterations will improve its usefulness.

The objectives and the principles are still the same--we regard this as an attempt to place in the hands of the beginner sound basic methods which will aid his work. This is a practical manual to be used in the laboratory rather than a reference volume to be kept on the shelf. Therefore, the book has been retained in its original form with only minor modifications designed to save space; the substitution of a heavier cover should improve its durability. It is anticipated that the volume will be revised whenever warranted.

We are indebted to all of the contributors for their cooperation, for only through a cooperative effort has the publication of this volume been possible. Permission to reproduce certain of the figures has been granted by Arthur H. Thomas (Figs. 45, 46), Central Scientific Company (Fig. 47), G. M. E. (Fig. 18), and by E. Machlett and Son (Figs. 13, 16, 28, 30, 34).

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Chapter I

THE WARBURG CONSTANT VOLUME RESPIROMETER

INTRODUCTION

Manometric methods for estimating exchange of gases have been in use in the study of both chemical and biological reactions for generations. A wide variety of techniques have been employed and many types of apparatus have been developed. The type of respirometer which has met with widest use is almost universally known as the "Warburg" instrument, although, as pointed out by Warburg (1926), it has a long history. In essence the present instrument is a modification of a "blood-gas manometer" described by Barcroft and Haldane (1902) or of that described by Brodie (1910). The essential principle involved is that at constant temperature and constant gas volume any changes in the amount of a gas can be measured by changes in its pressure. This method is most commonly applied to measurements of oxygen uptake. We shall therefore first describe its principles in terms of oxygen uptake and later consider other uses to which the instrument may be applied.

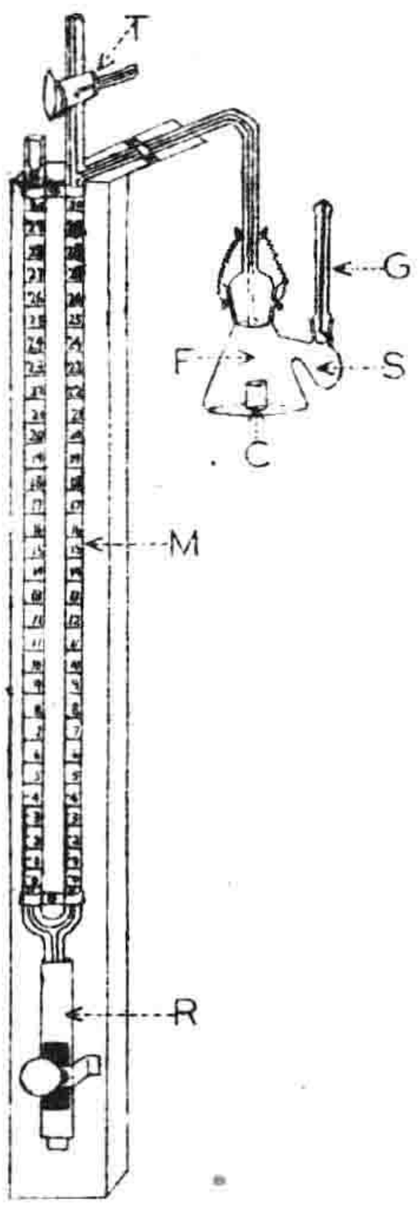

FIG. 1

The Warburg constant volume respirometer.

APPARATUS

F = flask
S = sidearm
G = sidearm stopper with gas vent
C = center well (for alkali)
M = manometer proper
R = fluid reservoir which, by adjustment of the screw clamp, serves to alter the level of the fluid in the manometer
T = three-way stopcock

The scale of the manometer is graduated in centimeters (numbered) and in millimeters. Normally one records readings in terms of millimeters.

The apparatus (Fig. 1) consists of a flask (F) (detachable) sometimes equipped with one or more sidearms (S), attached to a manometer (M) cortaining a liquid of known density. The flask is immersei in a water bath at a constant temperature, and between readings the system is shaken or whirled to promote a rapid gas exchange between the fluid and the gas phase. It is assumed that the temperature of the manometer, which is not immersed, does not differ greatly from that of the flask. Details of the apparatus have been described by Burk and Milner (1932), Dixon (1943), Perkins (1943), Warburg (1923, 1924, 1926) and others. Further details of shaking apparatus will be found in Chapter 5.

The manometer has (as shown in Fig. 1) an open and a closed end. A given point on the closed side of the manometer (usually 150 or 250 mm.) is chosen, and the liquid in the closed arm of the manometer is always adjusted to this point before recording pressure changes.

GENERAL PRINCIPLES

Suppose that one has an oxygen consuming reaction going on in the flask. One adjusts the closed side of the manometer (with stopcock open) to 250 mm. (by adjusting the screw clamp on the fluid reservoir of the manometer), closes the stopcock, and reads the open

side of the manometer. Assume that the level of the liquid in this arm is 249 mm. (Fig. 2). This reading of 249 is recorded. After 10 minutes time the liquid has gone up in the closed arm and down in the open arm. One again adjusts the closed arm to 250 mm. and thus holds the volume of gas in the flask constant. The reading on the open arm is now 220 mm. For both the initial and 10 minute readings the fluid in the closed arm of the manometer was adjusted to 250 mm., but during the interval the reading on the open arm dropped from 249 to 220 mm. (29 mm.) as a result of oxygen consumption in the flask. If one knows the gas volume of the flask (V_g), the volume of fluid in the flask (V_f), the temperature of operation, the gas being exchanged and the density of the fluid in the manometer, it is possible to calculate the amount of gas used up (or given off), providing only one gas is being changed. There are methods for handling alterations in the amount of more than one gas; these will be described later. The essence of the method is to hold the gas and fluid volumes constant and to measure the decrease or increase in pressure when one gas alters in amount.

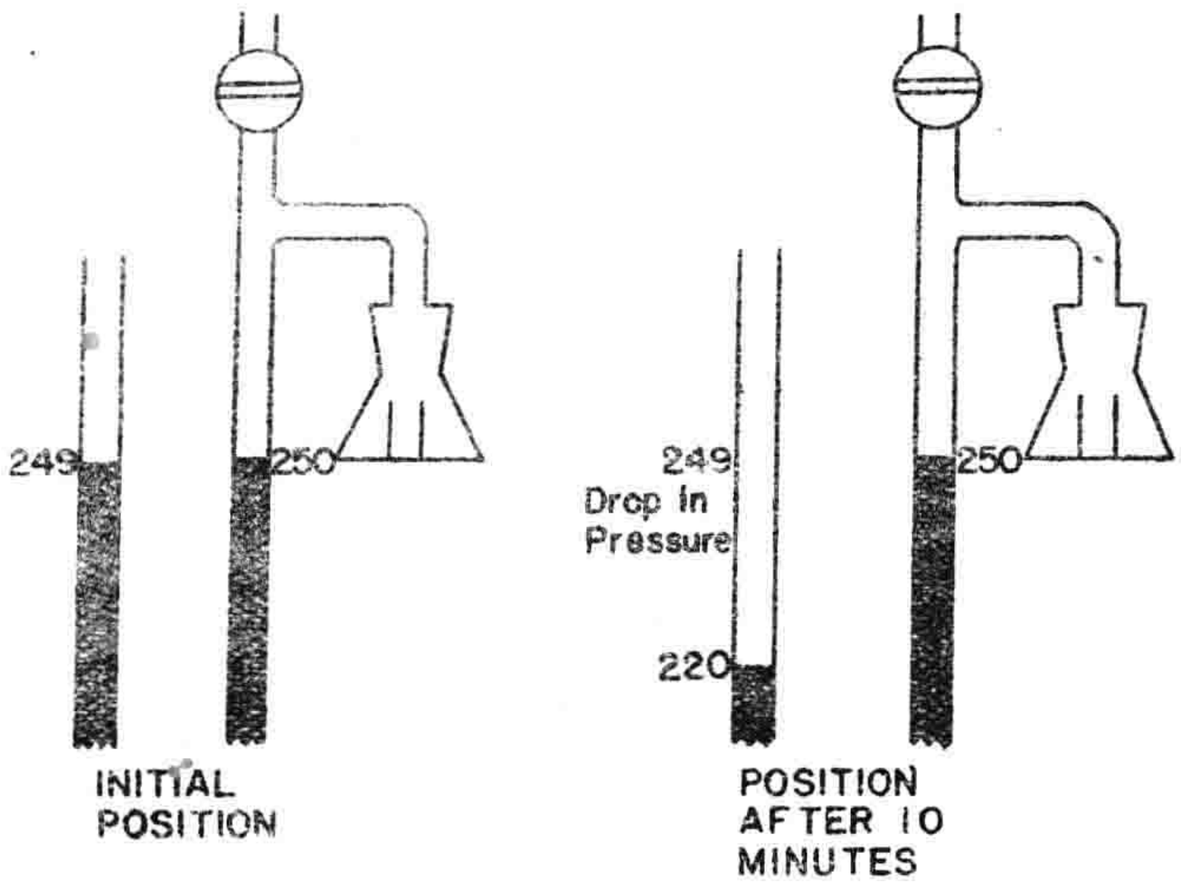

FIG. 2

Diagram illustrating the determination of pressure change.

DERIVATION AND MEANING OF FLASK CONSTANT

Fundamentally this consists of so calibrating the system that from the observed pressure changes one can calculate the amount (in $mm.^3$ or micro liters (µl) at 0°C. and 760 mm. pressure) of gas utilized or given off.

The following symbols are employed:

Let h = the observed change in the manometer (open side) reading in mm.
x = µl. gas (0°C., 760 mm. pressure)
V_g = Volume of gas phase in flask including connecting tubes down to the "zero" point (150 or 250 mm. on closed end of manometer).
V_f = Volume of fluid in vessel.
P = Initial pressure in vessel of the gas involved in the determination. This is actually the partial pressure of the particular gas in a gas mixture. If this gas mixture contains water vapor, the partial pressure of the gas involved in the determination will be less than its partial pressure in the dry condition. Hence if P is defined as dry gas, P - R should be used in equations involving moist gases.
P_o = 760 mm. Hg (standard pressure) expressed in terms of the manometer fluid:

$$P_o = 760 \times \frac{13.60 \text{ (where 13.60 is the specific gravity of mercury)}}{\text{Specific gravity of manometer fluid.}}$$

T = Temperature of bath in absolute degrees (= 273 + temp. in °C.).
α = Solubility in liquid in vessel of gas involved (expressed as ml. gas/ml. liquid when gas is at a pressure of one atmosphere (760 mm. Hg) at the temperature T).
R = Vapor pressure of water (or other fluid) at temperature T. Inside the flask one has both fluid and gas. This fluid will exert a vapor pressure (R) in the gas phase and some gas will dissolve in the fluid.

In the gas phase one has gas (V_g) at a temperature (T) and at a pressure P - R (P - R = partial pressure of gas involved less the vapor pressure or the fluid). One can change this gas volume to standard conditions using this formula:

$$PV/T = P'V'/T'$$

(let prime symbols be standard conditions, i.e. V' = gas volume standard conditions, P' = P_o = 760 mm. Hg, T' = 273 = 0°C.). Hence in the flask:

$$(P - R)\ V_g/T = P_o\ V'/273$$

$$\text{and gas at standard conditions} = V' = \frac{V_g \frac{273}{T} (P-R)}{P_o}$$

Some gas is dissolved in the fluid initially. The amount of gas in the fluid is:

$$V_f \alpha\ (P - R)/(P_o)$$

Where α is the solubility of the gas (in ml. gas/ml. fluid) at a partial pressure of one atmosphere. The $(P - R)/P_o$ converts the solubility at one atmosphere to that actually existing in the flask.

This relationship holds, as Henry's law states, "The concentration of dissolved gas is directly proportional to the concentration (pressure) above the fluid." Hence if α is the solubility at P_o (one atmosphere) the solubility at the actual pressure existing in the flask, P - R (atmospheric pressure less than that due to water vapor), will be $\frac{P - R}{P_o}$. Virtually nothing is known of the relationship between chemical structure and solubility of gases so that one has to determine the solubility empirically. There is thus a different solubility for each gas in each solution. It is known, however, that the solubility of individual gases in a mixture is almost independent of the pressure of other gases, i.e., the solubility of oxygen at a given pressure and at a given temperature will be the same whether N_2, CO_2 or other gases are present or not.

From the considerations above, the gas present at the start was that in the gas phase plus that in the fluid phase or:

$$\text{Gas at start} = \underbrace{V_g \frac{273}{T} \frac{(P - R)}{P_o}}_{\text{Gas phase}} + \underbrace{V_f\ \alpha \frac{(P - R)}{P_o}}_{\text{Fluid phase}}$$

At the end of the observation period this gas has been changed by the amount x which has resulted in a pressure change of h mm. If gas is taken up, h is negative; if gas is given off, h is positive. We will here assume that it is taken up. The pressure is now (P - R - h) rather than the initial value (P - R).

$$\underline{\text{Gas phase is thus}}: \quad V_g \frac{273}{T} \frac{(P - R - h)}{P_o}$$

$$\underline{\text{Liquid phase}}: \quad V_f\ \alpha \quad \frac{(P - R - h)}{P_o}$$

$$\text{Gas at end} = V_g \frac{273}{T} \frac{(P - R - h)}{P_o} + V_f\ \alpha \frac{(P - R - h)}{P_o}$$

Gas taken up (x) is that which was present initially less that which appears at the end.

x = initial gas - final gas

$$x = \left[V_g \frac{273}{T} \frac{(P - R)}{P_o} + V_f \alpha \frac{(P - R)}{P_o}\right] - \left[V_g \frac{273}{T} \frac{(P - R - h)}{P_o} + V_f \alpha \frac{P - R - h}{P_o}\right]$$

$$= V_g \frac{273}{T} \frac{(P - R)}{P_o} + V_f \alpha \frac{(P - R)}{P_o} - V_g \frac{273}{T} \frac{(P - R - h)}{P_o} - V_f \alpha \frac{P - R - h}{P_o}$$

$$= V_g \frac{273}{T} \frac{h}{P_o} + V_f \alpha \frac{h}{P_o}$$

$$x = h \left[\frac{V_g \frac{273}{T} + V_f \alpha}{P_o}\right] = h\ k$$

Note that V_g, T, α, V_f and P_o are known and, for a given experiment, are constant; these values determine the flask constant k with which one can convert mm. pressure change into μl. O_2 taken up.

Summary:

x = amount of gas exchanged = h (alteration in reading on open arm of manometer) k (flask constant)

$$k = \text{flask constant} = \frac{V_g \frac{273}{T} + V_f \alpha}{P_o}$$

Example: A Warburg flask has a total volume of 12.616 ml. up to the 250 mm. mark on the manometer. To measure oxygen uptake in this flask by yeast at 28°C., we add 1 ml. of yeast suspension, 1 ml. of 0.1 M glucose, 1 ml. of M/50 phosphate buffer. In the center cup of the flask we place 0.2 ml. 10% KOH to absorb the carbon dioxide the yeast may produce. What flask constant should be employed?

V_f = 3.2 ml. = 3,200 μl.

V_g = total volume - fluid volume = 12.616 ml. - 3.2 ml. = 9.416 ml. = 9,416 μl.

$T = 273 + 28 = 301$ $\qquad \alpha = 0.027$ $\qquad P_o = 10{,}000$

$$k_{O_2} = \frac{V_g \frac{273}{T} + V_f \alpha}{P_o} = \frac{9{,}416 \times \frac{273}{301} + 3{,}200 \times 0.027}{10{,}000} = \frac{8540 + 86.0}{10{,}000} = 0.863$$

Difference in pressure in mm. times 0.863 = μl. gas.

(Chapter Six describes methods of determining the flask constants.)

Two points may be confusing. One, the choice of 0.027 for α, will be explained in the next section. The other is the use of P_o = 10,000. The manometer in this case was filled with "Brodie's Solution" composed as follows:

23 gram NaCl
5 gram Sodium Choleate (Merck)
in 500 ml. water
Usually colored with dye, e.g. Evan blue or acid fuchsin.

This has a density of 1.033 so that

$$P_o = 760 \times \frac{13.60}{1.033} = 10,000$$

THE SOLUBILITY OF OXYGEN

The solubility of oxygen is expressed in Table I as ml. O_2/ml. fluid when the gas is at one atmosphere pressure. This term, α, is sometimes referred to as the "Bunsen Coefficient".

The α value of a particular gas is influenced by two factors. First, as is apparent from the values in Table I, the solubility of gases decreases as the temperature rises. Second, the solubility of gases is appreciably diminished by the presence of dissolved solids (or liquids, but not gases) in the fluid. This is thought to be due to the hydration ("solvation") of the solute, which leaves less free solvent available for dissolving the gas. These effects are shown in Table II constructed from data in the International Critical Tables, Volume III, p. 271 (1928). Tables on the solubility of gases other than oxygen will be found in Chapter 6.

TABLE I

The Solubility of Oxygen

Data as ml. gas dissolved per ml. fluid
when gas is at 1 atmosphere pressure (α value)

Temperature °C.	Ringer's Solution	Water
0		0.04872
10	0.0480	0.03793
15	0.0340	0.03441
20	0.0310	0.03091
25	0.0285	0.02822
30	0.0260	0.02612
35	0.0245	
40	0.0230	
	Dixon (1943)	International Critical Tables (1928)

TABLE II

The Influence of Salts Upon the Solubility of Oxygen

Data in terms of ml. O_2 dissolved per ml. solution (α value)

	HCl, 15°C	HCl, 25°C	1/2 H_2SO_4, 25°C	NaCl, 25°C
Conc.				
0.0 M	0.034	0.028	0.028	0.028
0.5 M	0.033	0.027	0.027	0.024
1.0 M	0.031	0.026	0.025	0.020
2.0 M	0.028	0.025	0.023	0.016

Although the effect of salts on oxygen solubility appears large, it actually has little effect on flask constants for oxygen uptake. For example, a change from pure water to 2 M NaCl changes the α value from 0.028 to 0.016. This lowers the flask constant by $\frac{0.012 \times Vf}{10,000}$. In the case described above, the k instead of being 0.863 would be, with 2 M NaCl, 0.859.

THE THERMOBAROMETER

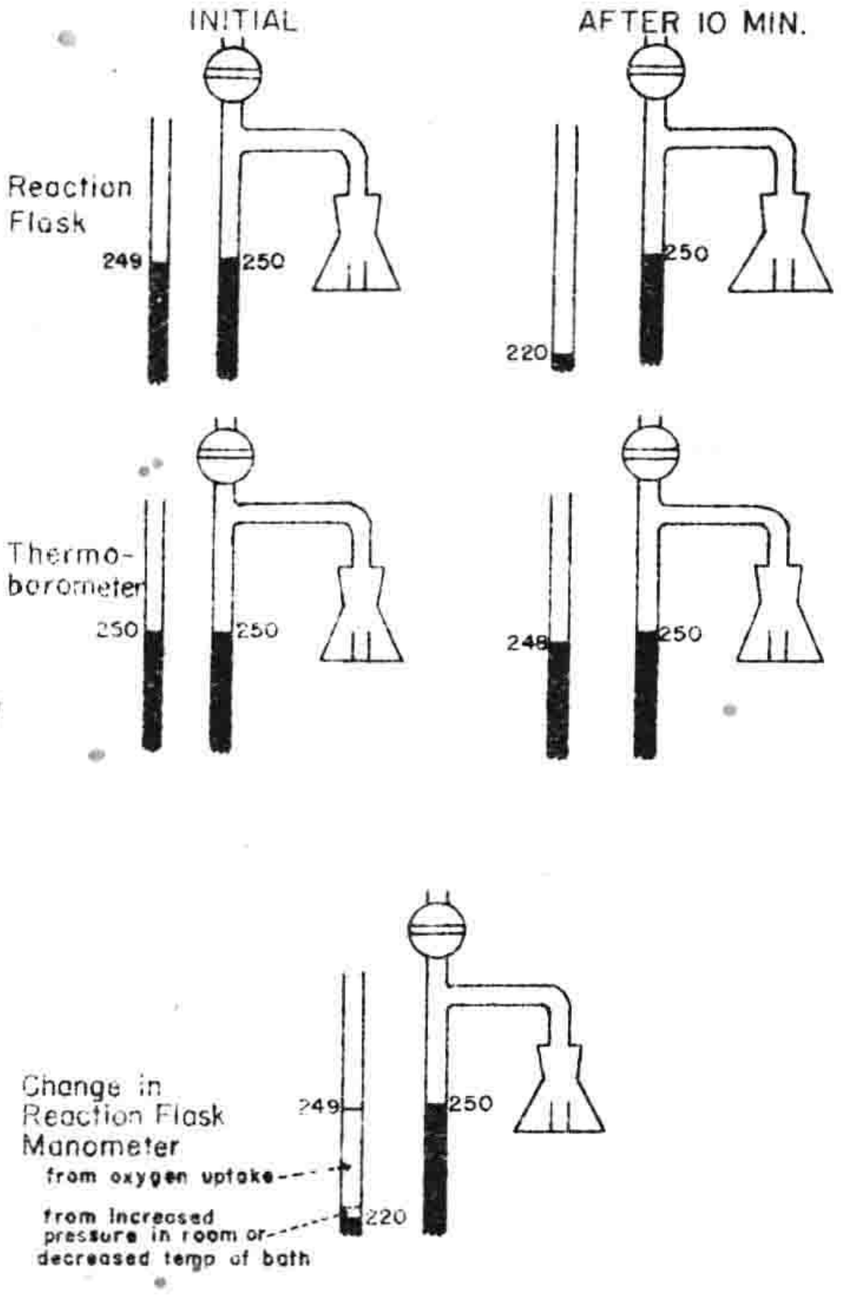

FIG. 3

Diagrams illustrating the use of the thermobarometer.

In the development of the flask constant, k, a value P was employed which was assumed to remain constant from the beginning to the end of a given period. This "P" represented the initial atmospheric pressure. The pressure in the room and the temperature of the water bath are likely to change, however, and these changes are corrected for by a thermobarometer. The thermobarometer consists merely of a Warburg manometer with a flask containing water attached; the volume of water is not critical.

Refer first to Fig. 3, initial, which represents the first reading; the reaction flask reads 249, the thermobarometer 250. At the end of a given period the reading on the reaction flask has dropped to 220, i.e., 29 mm. During the same time changes in the temperature of the bath or increased pressure in the room have caused the reading on the thermobarometer to drop to 248, i.e., 2 mm. The decrease in pressure observed in the reaction flask was due to two things: the use of some of the oxygen in the gas space inside the flask (27 mm.) and the external changes of temperature and pressure (2 mm.). Correction of the readings obtained for changes registered by the thermobarometer are obvious after a brief study of Table III which illustrates actual experimental data.

If the level of liquid in the open arm of the thermobarometer has risen there has been a decrease in pressure in the room or an increase in temperature of the bath. For reaction flasks which have registered a drop in pressure the observed decrease is smaller than the actual decrease by the amount the thermobarometer fluid has risen; hence the rise in the thermobarometer reading is added to the observed pressure drop. If the reaction flasks have registered a rise in pressure, the increase in the thermobarometer reading is subtracted from this observed rise. Examples to illustrate these points are given in Tables III and IV.

TABLE III

Corrections for Thermobarometer Changes

Time	Thermobarometer		Respiring Flask #1 (Method 1)			Respiring Flask #1 (Method 2)			
		Change (total)	Reading	Change	True Change	Reading	Chg. Interval	True Chg. Interval	Sum
0	250 mm.		249			249			
60	257 mm. (+7)	+7	248	-1	-8	248	-1	-8	-8
120 min.	259 mm. (+2)	+9	243	-6	-15	243	-5	-7	-15
0	250		249						
60	236	-14	111	-138	-124				

TEMPERATURE CONTROL

How accurately must the temperature of the bath be controlled? Two situations must be considered:

I. The whole bath is at a constant temperature but has dropped 1° below that accepted for calculation. The thermobarometer has responded to this drop in temperature so the main error involved arises from using the wrong flask constant. Suppose that in the case described in Table IV the temperature throughout the bath dropped to 27° C. and the data were calculated for 28° C. At 28° the k_{O_2} is 0.942; at 27° it is 0.945. If the measurements are actually made at 27° C., but the factor for 28° C. is used, an error of about 0.3% is introduced.

II. If the bath is not uniform in temperature a flask at a point 1° C. higher in temperature than another flask would indicate a pressure corresponding to about 33 µl. of gas more per 10 ml. of gas volume (difference of 0.05° C. = 1.7 µl.).

Hence two factors are important; first, that the temperature be held at the point desired, and second and more important, that the temperature of the entire bath be held uniform to within 0.05° C. This latter factor necessitates vigorous stirring of the water in the bath.

SAMPLE CALCULATIONS

The data of Table IV illustrate the method of calculating the µl. oxygen uptake from the observed changes in the level of the manometer fluid of the thermobarometer and of the reaction flask manometer. Two methods of calculation follow:

Total uptake method:

The application of this method is illustrated in columns 4, 5, 6 and 7 of Table IV. The uptake in mm. is calculated by subtracting the initial reading (246) from all subsequent readings (column 4). The thermobarometer correction is obtained by subtracting the initial reading (265) from all subsequent readings (column 5). Since in the interval from 10^{55} to 11^{00} the total uptake of 19 mm. in the reaction flask was due in part (1 mm.) to thermal or barometric changes, the real uptake was 19 - 1 = 18 mm. (column 6). This value times the flask constant for the conditions employed gives the ul. oxygen taken up (column 7).

TABLE IV

Calculating Oxygen Uptake from Manometer Readings

Time	Reading, Thermobarometer	Reading, flask	Total method				Interval method				
			Change, in mm.	Thermobarometer correction	Actual change, in mm.	O_2 uptake	Change, in mm.	Thermobarometer correction	Actual change, in mm.	µl. O_2 uptake	Sum
	mm.	mm.									
10^{55}	265	246	-	-	-	-	-	-	-	-	-
11^{00}	264	227	-19	-1	-18	17.0	-19	-1	-18	17.0	17.0
11^{05}	264	194	-52	-1	-51	48.1	-33	0	-33	31.1	48.1
11^{10}	264	159	-87	-1	-86	81.0	-35	0	-35	32.9	81.0
11^{15}	264	122	-124	-1	-123	115.9	-37	0	-37	34.9	115.9
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)

Flask: 1 ml. yeast suspension, 1 ml. M/200 KH_2PO_4, pH 4.5, 0.5 ml. water, 0.5 ml. 0.032 M glucose: glucose in sidearm, tipped in at 10^{55}.

Volume flask = 13.5 ml.; k_{O_2} = 0.942 Temp. 28° C.; 0.2 ml. KOH in center cup.

Interval uptake method:

This method is applied in the calculations shown in columns 8 to 12 of Table IV. Each reading is subtracted from the one following it (i.e., 246 from 227; 227 from 194, etc.) giving the change (column 8) over the interval. A similar calculation is made for the thermobarometer (column 9) from which the actual change (column 10) is readily apparent. These interval values are multiplied by the flask constant (column 11) to yield the uptake per interval and are added to yield the total uptake (column 12).

Although this method appears more laborious, it offers some advantages, especially when the rate of oxygen uptake is changing. In this case, for instance, the uptake during the first five minutes (17.0 µl.) is not the same as in the succeeding 5 minute intervals, for there is a tendency for the rate to increase throughout the determinations. This increase is not readily apparent when calculated by the "total method" and may even be overlooked in graphing.

Many laboratories have found it convenient to employ mimeographed or printed tabular forms for recording manometric data. The data may be recorded there permanently or temporarily before transfer of pertinent information to a permanent notebook. Two examples of such data sheets are shown in Fig. 4. The upper section is a reproduction of a form which is printed on the back of $8\frac{1}{2}$ x 11 inch graph paper and spirally bound into a notebook. Data are recorded and calculated on the printed sheet, and the results are plotted on the opposite sheet of graph paper. The readings for any one flask are recorded horizontally across the page. In the lower portion of Fig. 4 is shown a mimeographed sheet on which data for each flask are recorded in vertical columns.

It is convenient to record flask constants on a card and to secure the card inside the front or back cover of the notebook by means of a Scotch tape hinge. The card can then be flipped over, so that it projects beyond the cover of the book, where it can be referred to readily during the calculation of results.

THE USE OF THE WARBURG INSTRUMENT FOR THE MEASUREMENT OF RESPIRATION OF LIVING CELLS

Physiologically there are two meanings for the word "respiration". The older meaning confines the term to the actual uptake of gaseous oxygen. It was later realized that oxidations could occur (by the removal of hydrogen or electrons) without employing gaseous oxygen and so the term respiration was broadened to include any reaction by which the cell obtained energy, whether or not it involved gaseous oxygen as such. This has resulted in some confusion since the meaning of the term thus differs with various groups of investigators. For the purposes of this outline the following definitions are employed:

Respiration: The uptake of gaseous oxygen.
Fermentation: The transformations which occur in living cells (or enzymes therefrom) which do not employ gaseous oxygen.

In the case of most cells, as contrasted to many enzyme preparations, the utilization of oxygen results in a release of carbon dioxide. If these two gases (CO_2, O_2) are the only ones involved, one can measure the respiration (O_2 uptake) by absorbing the liberated carbon dioxide in alkali. In the presence of alkali the carbon dioxide pressure in the air is zero within the limits of measurement. The gas exchange caused by the respiration is oxygen absorption plus carbon dioxide liberation. But the alkali keeps the carbon dioxide pressure zero, hence the change noted on the barometer is due solely to the oxygen utilization. The excess of carbon dioxide in solution, of course, continually distills over into the alkali, but it does not affect the observed pressure changes.

THE ABSORPTION OF OXYGEN

The absorption of oxygen by the respiring tissue takes place almost entirely from the oxygen in solution. This is the principle reason for shaking the fluids in the respiro-

Title: Run No.:

Date: Temp.: Contents of inner well: Gas phase:

No.	Flask Contents	Time; Reading in mm.; Change in mm.; Gas change in c. mm.																		
1																				
2																				
3																				
4																				
5																				
6																				
7																				
8																				
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Signed ....

Prep: pH: Temp: Date: Noteb: Page:

Flask		T.B.																													
In cup																															
Side I																															
"2/well				/				/				/				/				/				/				/			
K______																															
Time	Int.	A	△	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
1047 1M																															

FIG. 4

Forms for recording manometric data.

meter, i.e. to obtain a fluid phase saturated with the gas phase. But one must, under practical circumstances, take care that the rate of oxygen uptake by the tissue is not greater than can be replaced by the diffusion of oxygen from the atmosphere into the fluid. If the rate of oxygen uptake is so high that the oxygen is used up faster than it can diffuse into the liquid, then the rate of respiration observed is dependent upon the rate at which oxygen diffuses into the fluid and has little to do with the potential rate of the reaction itself.

The rate at which gas diffuses into a liquid is dependent upon the surface layer of the liquid. The gas may be thought of as moving across a film of surface, and the theory of such diffusion has been well worked out. Roughton (1941) has described methods for correcting for diffusion errors when they exist. However, for virtually all respiratory measurements it is sufficient to note that by shaking the flasks a continual new surface is exposed to the gas by virtue of the turbulence of the fluid in the flask. Hence, the greater the rate of shaking, the greater the rate of diffusion of the gas into the liquid, and the greater the rate of respiration one may measure without diffusion errors.

Dixon and Tunnicliffe (1923) and Dixon and Elliott (1930) have studied these effects in the Barcroft differential manometer (see Chapter 7) and have concluded that 600-700 µl. O_2 per hour can be safely measured without diffusion errors when a shaking rate of 100 oscillations per minute is employed (over 1500 µl.O_2/hr. at a rate of 138 oscillations per minute).

In the Warburg respirometer the flasks used are usually smaller so the surface exposed to the gas is less than in the Barcroft type; hence, limiting rates of oxygen uptake are reached sooner. The actual rates measureable without errors arising from gas diffusion in flasks of approximately 15 ml. volume containing 3 ml. of fluid have been determined in the experiment described below. This illustrates one method for determining whether the rate of oxygen diffusion is the limiting factor in any results one might obtain (Fig. 5)

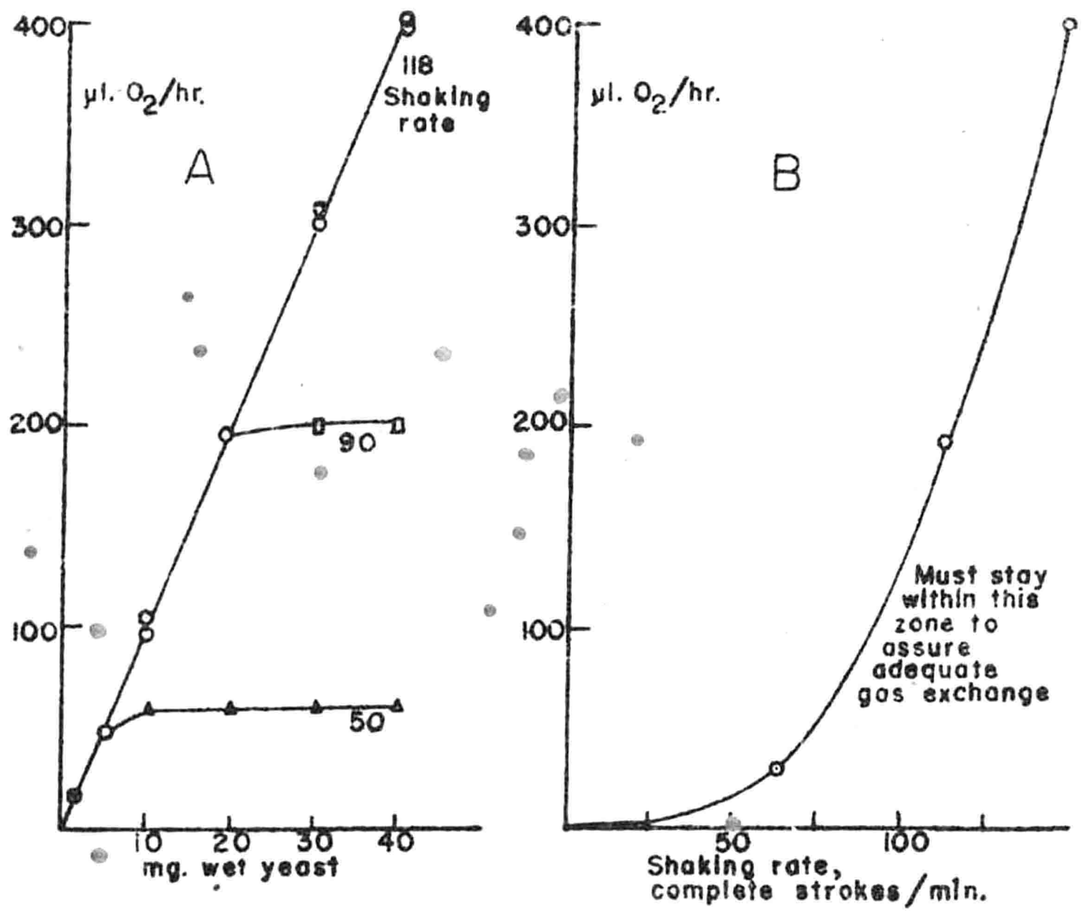

FIG. 5

Influence of shaking on rate of oxygen uptake in flasks of about 15 ml. capacity. Section A - Each flask has amount of yeast indicated on abscissa made to 2 ml. with 0.02 M KH_2PO_4 (pH 4.8), 1 ml. of 3% glucose, and 0.2 ml. 20% KOH in center well. Shaken at 50, 90 and 118 complete two-centimeter strokes per minute at 28°C. Section B - Data from Section A plotted to show adequate shaking rate for conditions described.

The basis of the experiment described is as follows: the rate of oxygen diffusion from the gas phase into the fluid phase is dependent upon the surface boundary. This boundary is altered more rapidly with faster shaking allowing more rapid oxygen exchange. If more rapid shaking (and thus more rapid oxygen exchange) does not increase the rate of oxygen uptake, then the rate of oxygen diffusion is not the limiting factor in the system being studied.

Another principle which may be used (but only in certain circumstances) depends upon the fact that the higher the concentration of the gas the greater will be its rate of diffusion into a liquid. Hence, one can vary the percentage oxygen in the atmosphere above the liquid, use various quantities of tissue, and determine the maximum rate of oxygen uptake that can be achieved before diffusion factors become significant. This method is, however, not only more laborious but also more complex, for there are reports that some

types of respiration are affected by the pressure of oxygen, per se. Therefore changing the shaking rate is the preferred method. Increasing the oxygen pressure is useful, however, when one finds it necessary to supply adequate concentrations of oxygen throughout a solid tissue. In this case the diffusion into the liquid is not the limiting factor, but diffusion into the respiring solid controls the oxygen level at its center. Obviously increased shaking will not alter these surfaces, so that the only practical solution is to increase the oxygen pressure. This is discussed under "tissue slices" in Chapter 10.

It sometimes happens that a reaction is dependent upon a contact between particles and that shaking disturbs this contact. One such example reported is the sulfur oxidation by bacteria (Vogler, LePage and Umbreit, 1942) in which a contact between the bacteria and the solid sulfur particles is necessary before oxidation can occur (Vogler and Umbreit, 1941, Umbreit, Vogel and Vogler, 1942). Shaking at rapid rates actually disturbs such contact and results in lowered oxidation. However, it is notable that if one employs rates of oxygen uptake lower than those at which limited diffusion becomes significant (i.e., at 100 strokes per minute, 300 μl.O_2/hr.) any variation in the rate of oxygen uptake with increase in shaking rate is not dependent upon the diffusion of oxygen since the fluid is already saturated. Therefore it is always desirable to determine the effect of alterations in the shaking rate to be certain that the results are independent of the rate of shaking. If they are not, one can readily determine whether the shaking rate is affecting the diffusion of oxygen or other factors (such as contact) by comparison with the rate of oxygen uptake which can be measured without diffusion effects under the conditions employed. Frequently important clues as to the nature of the reactions involved are obtained in this way.

THE ABSORPTION OF CARBON DIOXIDE

In the "direct method" of Warburg the oxygen uptake by living tissues, which also liberate CO_2, is measured by absorbing the CO_2 continuously in alkali during the determination. If the alkali employed fails to absorb the CO_2 completely and instantaneously, the CO_2 pressure in the gas phase will not be zero, and the readings on the manometer will not represent the true oxygen uptake. An example of circumstances of this type is given in the report of Brock, Druckrey, and Richter (1939); they observed that because of the large amounts of CO_2 liberated, readings on the manometer dropped only slightly or in some instances actually rose, yet oxygen was being consumed at a rapid rate. These workers found that the absorption of CO_2 was virtually instantaneous and that its pressure was held at approximately zero if the rate of CO_2 liberation was not more than 600 μl. per hour. Dixon and Elliott (1930) found that in the Barcroft apparatus (in the presence of adequate surface, see below) 1000 μl. of CO_2 per hour was almost instantaneously absorbed.

In absorbing CO_2 from the gas phase the same difficulties are encountered as in the absorption of oxygen. Here, however, because alkali is usually confined to the small center cup, an increased rate of shaking has little effect on increasing the surface. Hence some other method must be employed to increase the surface of the alkali. Usually small rolls or accordion folded pieces of filter paper are placed in the alkali cup. These should project beyond the side walls of the center cup into the open gas space above. A desirable projection is about 5 mm. Such "KOH papers" are usually prepared in quantity by cutting filter paper into squares with 2 cm. sides (the exact dimensions will vary with the depth of the cup employed; this varies from instrument to instrument, but the size need be only approximate). These papers are then folded three or four times, accordion fashion, and inserted into the center cup with tweezers. When wet by the alkali, previously added to the cup, they provide a large surface for the absorption of CO_2.

Sufficient alkali should be added to moisten the entire paper and still leave a well of free liquid in the bottom of the cup. For the papers described 0.15 to 0.20 ml. is adequate. Sometimes difficulty is experienced with the alkali "creeping over" the cup into the outer compartment of the flask. This can be prevented by greasing the top of the cup before inserting the papers. A convenient way of doing this is to wind a small amount of cotton about the end of a glass rod so that when placed over the center cup it will completely cover its top. After the cotton is saturated with grease, it is rotated in contact with the top surface of the center cup to give it a light coat of grease. A tapered 15 ml. centrifuge tube also makes a convenient tool for greasing alkali cups; the

bottom of the tube is greased and then rotated in the top of the alkali well. The relation of flask design to "creeping" of alkali is discussed in Chapter 6.

The concentration of alkali employed by various investigators differs widely, but KOH (because of the solubility of the potassium carbonate) is almost universally employed. Two things must be kept in mind in choosing the concentration. One is the capacity of the alkali employed to absorb CO_2; the other is the ease with which the alkali can be handled. Whereas the pressure of CO_2 above any solution of KOH is zero, very dilute solutions of KOH may be completely neutralized rather soon. Under most circumstances 1% KOH is undoubtedly sufficient. Most workers use 5, 10 or 20% KOH to be sure that an adequate supply is present to last throughout the experiment. 20% KOH offers no difficulty in handling. It is claimed by some that rather concentrated solutions of KOH (10-20%) react with the filter papers employed and that an oxygen uptake results from this reaction. While we have never experienced this, the recommendation that analytical grade filter papers be used for KOH papers should be followed whenever possible.

It is obvious that the conditions for obtaining adequate oxygen diffusion and CO_2 absorption are easily met. Usually the shaking rates employed are 100 to 120 two or three centimeter strokes per minute. Under these conditions (employing flasks of about 15 ml. capacity) one should use amounts of tissues that take up less than 300 µl. of O_2 per hour and give off less than 500 µl. CO_2. This usually means the use of about 100 mg. (wet weight) of animal tissues or somewhat less wet weight of yeast and bacteria. For the beginner it is well to choose tissue concentrations which take up about 200 µl. O_2 per hour.

PROCEDURE EMPLOYED

The actual procedure in setting up systems for the measurement of respiration of living cells varies widely. A common procedure is listed as follows:

1. To clean, dry, Warburg flasks equipped with a center well, add materials (except cells) to the main compartment of flask.
2. Add materials (if any) to the sidearm.
3. Add 0.2 ml. alkali (usually 5, 10 or 20% KOH) to the center well.
4. Grease attachment joint on manometer and grease and insert plug for sidearm. Grease top of alkali cup.
5. Add cells.
6. Add filter paper strip to alkali in center cup (see absorption of carbon dioxide).
7. Attach flask to manometer.
8. Place in constant temperature bath.
9. Adjust and tighten flask after about 5 min. shaking in bath. (This is done since sometimes the grease becomes softer and the flask tends to creep slightly.)
10. Allow to equilibrate, with shaking, for 10-15 minutes.
11. Adjust manometer fluid to zero point on closed side of manometer with stopcock open.
12. Close stopcock.
13. Begin readings.

LIMITATIONS OF METHOD

The method described in the previous sections, in which any carbon dioxide formed is absorbed by alkali, is known as Warburg's "Direct Method". It is the method most widely used for determining respiration. As with any other method it has certain limitations; these are:

1. The gases exchanged must be only O_2 and CO_2. In most cases this condition is not difficult to meet since in the majority of tissues these are the only gases involved. Warburg (1926), however, points out that "the metabolism of bacteria is rarely so simple that it can be measured by this method". This is a somewhat pessimistic viewpoint, and many bacteria can be studied adequately by this method. However, one should always take care to check that the only gases involved are O_2 and CO_2 before relying upon data derived by this method.

2. One must work in an atmosphere free from carbon dioxide. For some tissues, this is of no consequence, i.e. they respire at the same rate, to the same extent, and follow the same pathways whether CO_2 be present or not. But for others, this is by no means true. Carbon dioxide may inhibit, may stimulate, or may alter the path of metabolism of a given cell, hence measurements in the absence of CO_2 may not give a reasonable estimate of the reactions occurring in its presence. For this purpose the Warburg "Indirect Method" may be used (see Chapter 4).

3. The rate of oxygen uptake, and the rate of carbon dioxide liberation and absorption must be within a particular range so that the assumptions of the method hold, i.e., that the fluid is always saturated with oxygen gas (or air) and that the pressure of carbon dioxide in the gas phase is always zero.

Thus, in spite of the limitations of the "Direct Method" the conditions necessary for its adequate functioning can be met with ease in most cases.

RESULTS OF DETERMINATIONS

The Warburg "Direct Method" is suitable for two general types of use:

1. The determination of the rate of oxygen uptake.
2. The determination of the amount of oxygen uptake.

Both are usually measurable in the same determination. In expressing the rate of oxygen uptake, a quotient ("Q") is commonly employed. Several of these are in general use. These are defined as follows:

Q_{O_2} = µl. O_2 taken up per mg. dry weight of tissue per hour.

$Q_{O_2}(N)$ = µl.O_2 taken up per mg. tissue nitrogen per hour.

$Q_{O_2}(P)$ = µl.O_2 taken up per mg. tissue phosphorus per hour, or per mg. nucleic acid phosphorus per hour.

$Q_{O_2}(C)$ = µl.O_2 taken up per mg. tissue carbon per hour.

$Q_{O_2}(\text{cell})$ = µl. O_2 taken up per cell per hour.

In short, one specifies in the Q term the conditions under which the rate was measured and the basis used to estimate the amount of tissue. In a general way:

$$Q \begin{matrix} (\text{gas atmosphere}) \\ (\text{gas measured}) \end{matrix} \quad (\text{tissue basis})$$

For example:

$Q_{O_2}^{O_2}(N)$ means µl.O_2 taken up per mg. nitrogen of tissue per hour in an atmosphere of pure oxygen.

$Q_{CO_2}^{N_2}(P)$ means µl.CO_2 given off in an atmosphere of nitrogen (or under anaerobic conditions) per mg. of tissue phosphorus per hour.

Uptake of a gas is indicated by a minus (-) sign, release by a plus (+). Two conventions are employed:

1. When the gas atmosphere is air, the condition indicator is omitted.
2. When the tissue basis is dry weight, this indicator is omitted.

Thus, the term Q_{O_2} is used rather than $Q_{O_2}^{air}(\text{dry weight})$, and $Q_{O_2}(N)$ means oxygen uptake in air per unit nitrogen per hour, while $Q_{O_2}^{O_2}(N)$ means oxygen uptake in pure oxygen per unit nitrogen per hour.