A RESPIROMETER FOR METABOLIC STUDIES AT HIGH GASEOUS PRESSURES[1]

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The use of carbon monoxide as a specific inhibitor of cytochrome oxidase is accompanied by one serious complication—the inhibition is reversed by oxygen (Warburg, 1949). The degree to which one can inhibit the enzyme is therefore dependent, not alone on the carbon monoxide pressure, but also on the pressure of oxygen that is simultaneously present. In short, the inhibition of cytochrome oxidase is a function of the carbon monoxide/oxygen ratio. To achieve an effective inhibition of the enzyme, this ratio must be high. Fifty per cent inhibition requires approximately a 10 to 1 ratio; 75 per cent inhibition, a 20 to 1 ratio. For greater degrees of inhibition, still higher ratios are necessary (Warburg, 1949; Ball *et al.*, 1951).

To achieve this goal it has been customary to use an atmosphere containing 95 per cent carbon monoxide and 5 per cent oxygen; indeed, an atmosphere of 98 per cent carbon monoxide and 2 per cent oxygen has occasionally been employed. However, oxygen at these low pressures fails to satisfy the normal respiratory requirements of most plants and animals *in vivo* or *in vitro* (Tang, 1933). Experimental results are thereby complicated by anoxia, and there is uncertainty as to whether an observed effect is due to the presence of carbon monoxide or a deficiency in oxygen. The earth's atmospheric pressure (760 mm. Hg) is too low to permit one to inhibit cytochrome oxidase effectively by substituting carbon monoxide for the nitrogen in air. If the oxygen tension is to be maintained at its normal value ($\frac{1}{5}$th atmosphere), then several atmospheres of carbon monoxide must be superimposed.

For this reason there has long been a need for a simple and practical method for measuring oxygen consumption and carbon dioxide production at high gaseous pressures. This objective seems doubly attractive in view of the insensitivity of most biological preparations to pressure *per se*, as long as the latter is not extremely high.

Two methods have been described for this purpose; namely, that of Libbrecht and Massart (1937) and that of Stadie and Riggs (1944). Both of these methods utilized a pressure chamber containing a manometric apparatus of the Warburg type. The chamber designed by Stadie and Riggs had a capacity of 60 liters and enclosed 6 Warburg manometers and vessels. The apparatus was con-

[1] This study was aided by grants to Professor C. M. Williams from the Lalor Foundation and the U. S. Public Health Service, and by an Institutional Grant to Harvard University from the American Cancer Society.

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structed so that the necessary manipulations and readings were made from the outside, while high pressure and constant temperature were maintained inside. As might be anticipated, such an apparatus was extremely costly, presented an explosive hazard, and required careful checks to police a dozen separate gaskets and fittings.

The present paper describes a simple and practical technique for measuring gas exchange at positive pressures up to seven atmospheres. The respirometer itself is inexpensive, safe, leak-proof, and yields results of the same degree of accuracy as conventional manometric and volumetric techniques. In its present form it is suitable for studies of intact animals, plants, and tissues where agitation is not required. However, the principle of the technique is readily adaptable to studies of solutions, slices, and homogenates, and also to studies at pressures below atmospheric.

Principle of Method

A small glass respiration chamber, containing the experimental animal plus a carbon dioxide absorbant, is joined to a graduated capillary tube. Provisions are made so that the capillary can subsequently be sealed with a fluid index drop. After assembly, the respiration chamber is enclosed in a large polymethyl methacrylate (Lucite) chamber capable of withstanding high internal gas pressure. At the start of the experiment the capillary tube is patent; the lumen of the capillary therefore affords a direct connection between the gas space of the respiration chamber and the Lucite compensation chamber. Consequently, when the latter is filled with gas to a desired pressure, gas passes through the capillary and fills the respiration chamber at the same pressure. When a desired pressure is attained, an index drop is tipped into the graduated capillary, thus sealing the respiration chamber. The measurements are then performed in the same manner as in an ordinary volumeter of the Fenn type (Fenn, 1935). Since the plastic compensation chamber is closed off from the outside air, excursions of the index drop are independent of changes in atmospheric pressure (*cf.* Gerard and Hartline, 1934).

Apparatus (see Fig. 1)

a. Compensation chamber. The plastic compensation chamber is a transparent Lucite cylinder fitted with brass endplates, gaskets, and needle valves. Figure 2 shows the chamber and its component parts. The Lucite cylinder is 4″ I. D. × 4.50″ O. D. × 18″ long. The endplates are 5″ × 5″ × 0.5″ brass plates with a 0.25″ deep circular channel milled on the inner surface to receive the Lucite cylinder. A rubber gasket is inserted into this channel. Half-inch Hoke needle valves are threaded and silver brazed in the center of each endplate. The two endplates are held together by four brass rods, 0.5″ in diameter. Endplate A is bolted to the rods, while endplate B is removable. On endplate A the rods extend 0.5″ beyond the reducing valve so that the tank may be placed on end. The internal volume of the assembled compensation chamber was 3460 cc.

b. Capillary volumeter. The size of the respiration chambers and capillaries is dictated by the dimensions of the experimental animal, the rate of oxygen consumption, and the desired sensitivity. The size most frequently used in this laboratory

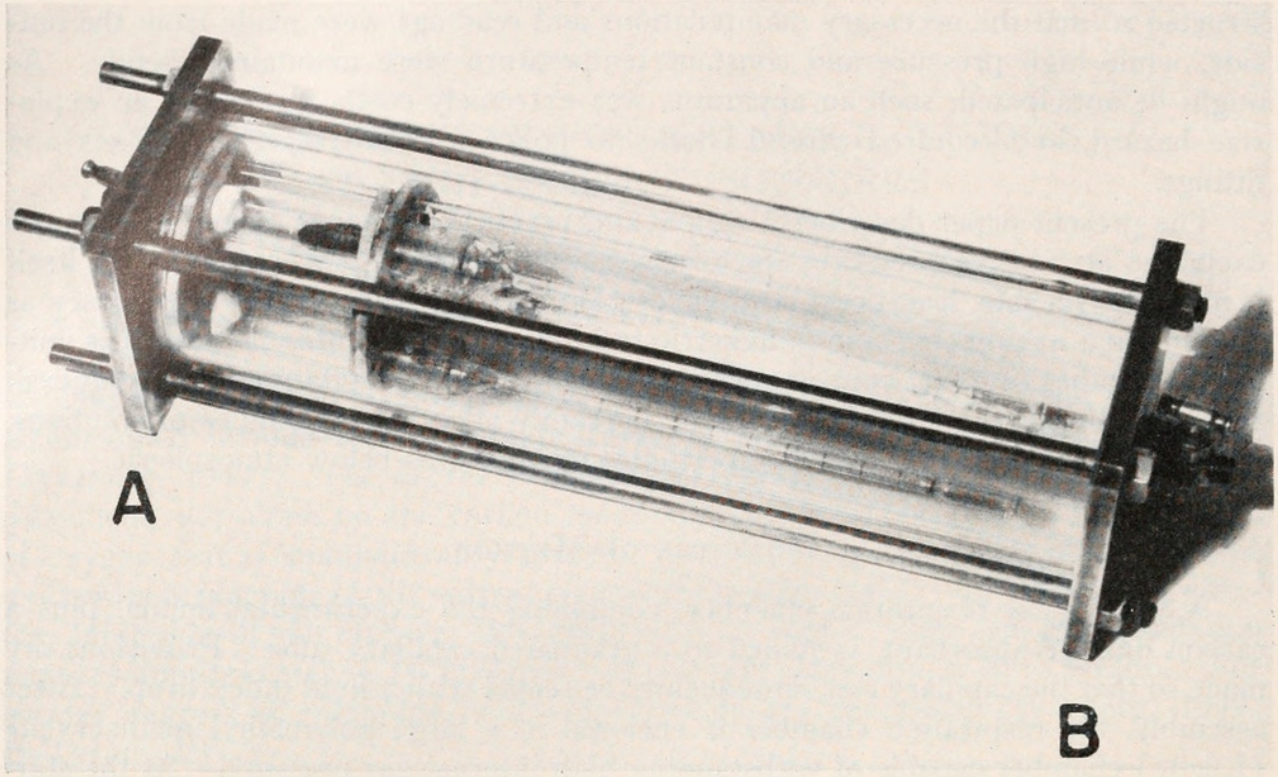

FIGURE 1. High pressure respirometer: an animal-containing *capillary volumeter,* a *reference volumeter,* and a *capillary barometer* are mounted on the *capillary volumeter frame.*

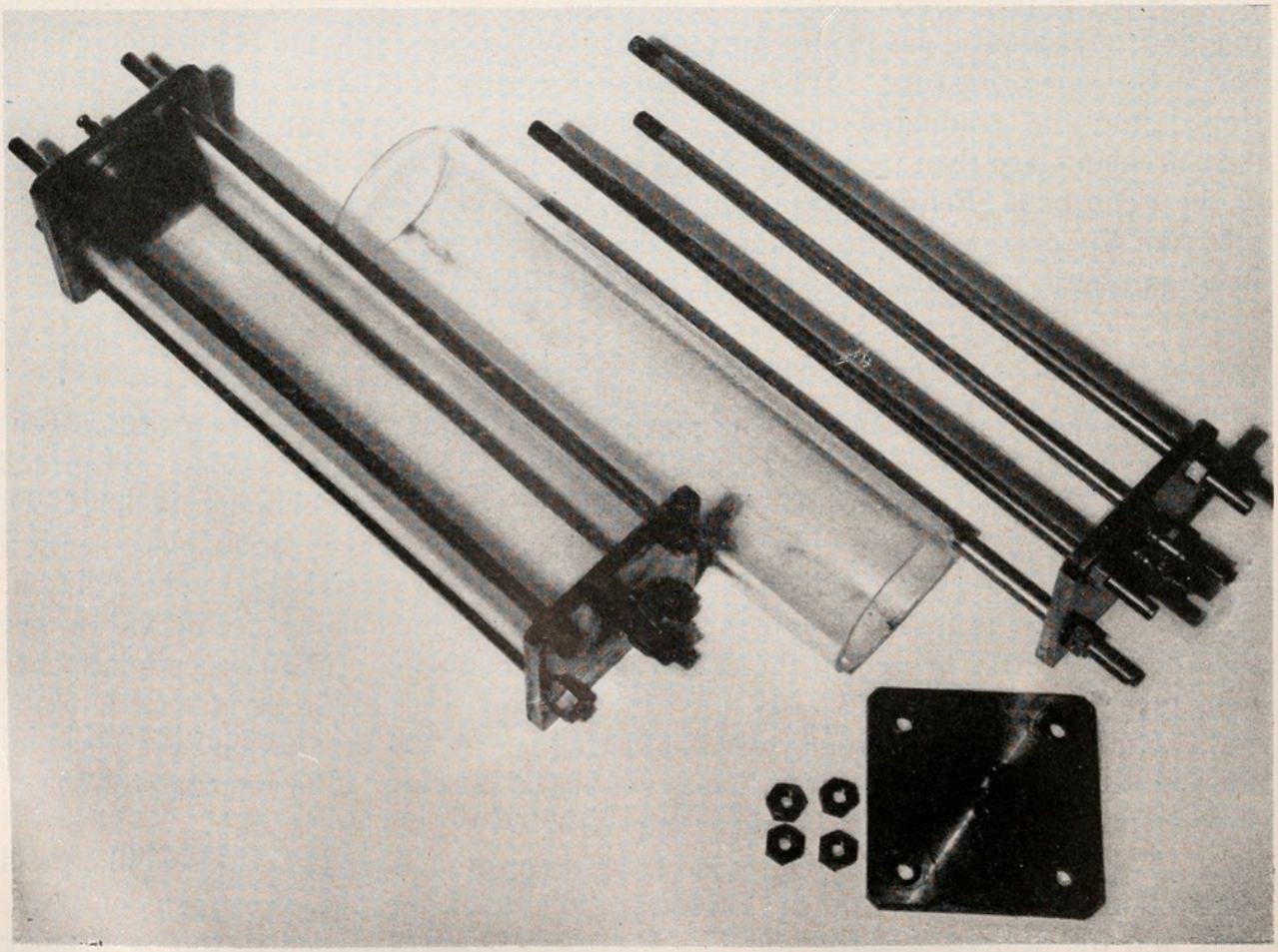

FIGURE 2. A 3460-cc. Lucite compensation chamber and its component parts.

is shown in Figure 3.[4] It consists of a 20-cm. length of 2 mm. bore capillary tubing, calibrated in 0.005 cc. units, and fitted by a 20/40 standard taper joint to a 30-cc. Pyrex shell vial, 85 mm. long. To the tip of the capillary is fused a 3.5 cm. length of 7-mm. bore Pyrex tubing. The latter is slightly constricted at its distal open end and serves as a reservoir for the index drop solution. The calibrated volume of each capillary is 550 mm.[3] The total volume of each volumeter to the tip of the capillary is 45 cc.

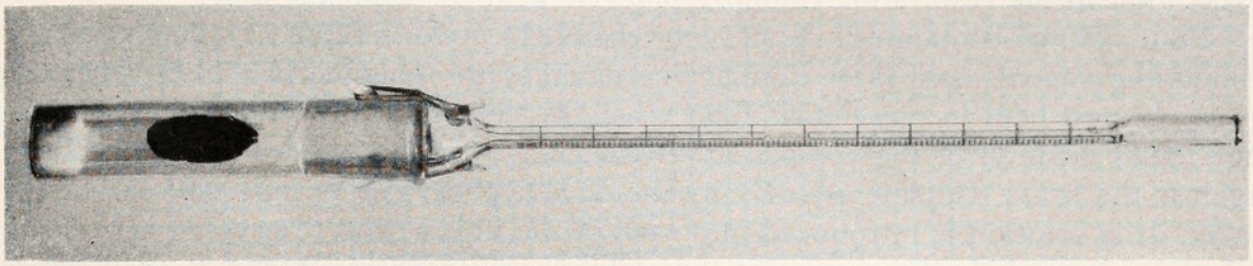

FIGURE 3. A 45-cc. capillary volumeter.

The respiration chamber can be attached to a standard Warburg manometer by means of an adapter previously described (Schneiderman and Williams, 1953). Thus the respiration of the animal in air before and after exposure to high pressure may be conveniently measured by conventional methods without removal of the animal from the respiration chamber.

c. Capillary barometer. Measurements of absolute pressure, accurate to within one per cent, are required for the proper determination of gas exchange in the present apparatus (see discussion of calculations below). Since the standard Bourdon type pressure gauges are subject to errors in excess of 5 per cent, a capillary barometer is utilized. Each such barometer consists of a one-cc. pipette of approximately 2 mm. internal diameter, graduated in 0.01 cc., and sealed at one end. By means of a long hypodermic needle a drop of colored detergent solution is placed in the closed end of the capillary and another drop at the beginning of the graduations. In each experiment three capillary barometers of this type are enclosed in the Lucite compensation chamber. The pressure in the closed system is calculated by application of the gas law from readings of the capillary barometer taken before and after compression and from a reading of the local barometric pressure.

d. Water bath. Glass aquaria make the most satisfactory water baths since the glass sides facilitate reading the capillaries.

e. Reagents. (1) The *index drop solution* has the following formula: 1 part "Aquet" (detergent of Emil Greiner Co.), 500 parts distilled water, a few drops of dilute H_2SO_4 to prevent carbon dioxide absorption by the index drop, and a few crystals of acid fuchsin to give the solution a red color. This fluid flows easily, keeps almost indefinitely at room temperature, and forms an index drop which responds regularly to slight pressure changes. (2) The *carbon dioxide absorbant* is carbonate-free 1 N KOH. (3) The grease used on the ground-glass joints connecting the respiration chambers to the capillaries and on the gaskets of the brass endplates is Dow-Corning silicone stopcock grease. Conventional organic greases

[4] The assistance of Dr. Conrad Yocum in the design of the final capillary volumeter is gratefully acknowledged.

have a tendency to oxidize or react in other ways with oxygen and carbon monoxide under pressure.

Procedure

A roll of filter paper is deposited in the bottom of each respiration chamber and moistened with 0.5 cc. of 1 N KOH. A small paraffin-coated tube is placed in the chamber to support the experimental animal. The latter is inserted and the ground-glass joint in the respiration chamber plugged with the capillary tube.

Four animal-containing respiration chambers, two reference volumeters not containing animals, and three capillary barometers are mounted in a plywood frame (Fig. 1) and held in place with rubber bands. The assembly is then placed in a horizontal position in the compensation chamber with the base of the frame flush against the brass endplate A. By means of a hypodermic syringe 0.05 cc. of the index drop solution is introduced into each of the index drop reservoirs, care being taken not to occlude the capillaries themselves. With valves A and B open, brass endplate B is now bolted on. One then records the temperature of the room, the position of the index drop in the capillary barometers, and the atmospheric barometric pressure.

The experimental gas is supplied from standard cylinders through a manually controlled reduction valve connected by a flexible 0.25″ bore copper tubing to valve A. About 10 liters of experimental gas are flushed slowly through the compensation chamber.[5] Valve B is closed and the experimental gas introduced under pressure through valve A to approximately the desired pressure, as indicated by the capillary barometers. Valve A is then closed.

The compensation chamber is now tilted to a vertical position so that the index drops flow into the lumina of the calibrated capillaries. Valve A is opened slightly and sufficient gas introduced under pressure to force a drop of index fluid a few centimeters into each of the six capillary tubes. Valve A is then closed and the compensation chamber returned to a horizontal position. Valve A is reopened and gas under pressure is slowly admitted until the drops have traversed the lengths of the capillary tubes to the proximal end of the calibrations. Valve A is then closed, the flexible coupling disconnected, and the compensation chamber immersed in a water bath controlled to $\pm 0.01^\circ$ C.

Valve A is now opened carefully until a barely detectable outward movement of the index drops is observed. The rate of gas escape is adjusted so that 10 to 20 minutes are required for the drops to migrate to the distal end of the capillaries. Valve A is finally closed when the distal ends of the index drops are about 2 centimeters from the distal ends of the calibrations. By running the drops up and down in this manner, one wets the walls of the measuring capillaries and thereby assures both a sensitive response and a constant size in the index drops.

Temperature equilibration requires 80 to 100 minutes. After equilibration, the

[5] If this flushing procedure is not carried out, then, upon compression of the compensation chamber with the experimental gas mixture, some of the air in the compensation chamber will be forced into the respiration chambers along with the experimental gas. This introduces considerable error; for example, if the air-filled compensation chamber is compressed with 5 atmospheres of carbon monoxide without prior flushing, the carbon monoxide/oxygen ratio in the compensation chamber will be approximately 25 : 1 while the carbon monoxide/oxygen ratio in the volumeters will be less than 6 : 1.

bath's temperature and the position of the drops in the capillary barometers are recorded to determine the absolute pressure in the compensation chamber. The two barometric pressures that agree most closely are averaged. Readings of the position of the index drop in each calibrated capillary also begin at this time. The positions of the drops are recorded at intervals ranging from 30 minutes to 6 hours, as dictated by the rate of oxygen consumption and the duration of the experiment. Thermobarometric corrections are applied to each reading, taking into account the fact that the actual volume of gas in the reference volumeters is slightly larger than that in the animal-containing chambers. Calculation of thermobarometric corrections may be simplified by enclosing in each thermobarometer a glass rod of approximately the same volume as the animal in the experimental chamber.

To calculate the oxygen consumption in mm.^{3} at S. T. P. from the excursion of the index drop, the following calculations are employed:

Let: v = *volume* in mm.^{3} of capillary that the index drop traversed.
P = absolute pressure in atmospheres after compression.
V_r = gas volume of respiration chamber in cc. (*i.e.*, 45 cc. minus volume of organism and reagents).
V_c = gas volume of compensation chamber in cc. (*i.e.*, 3460 cc. minus volume of 6 respiration chambers and frame = ca. 3000 cc.).
T = Temperature of bath.

A calibration factor F is calculated at each pressure and temperature to convert the measurements of v to mm.^{3} oxygen. Thus:

$$Fv = \text{mm.}^3 \text{ oxygen consumed at S. T. P.}$$

Fenn (1935) has shown that the value of F is provided by the formula

$$F = \left[\frac{V_c + V_r}{V_c}\right] \times P \times \frac{273}{T}.$$

Under ordinary experimental conditions the quantity in brackets is very nearly 1.01.

Thus

$$F = P \times \frac{273}{T} \times 1.01$$

$$\text{mm.}^3 \text{ oxygen consumed} = Fv = P \times \frac{276}{T} \times v.$$

Corrections for the vapor pressure of water and for the solubility of oxygen in the insect and the reagents were not applied since the combined errors thereby introduced were less than one per cent.

At the conclusion of the experiment, the compensation chamber is slowly decompressed and unbolted and the animals removed from the respiration chambers. By the addition of acid the total carbon dioxide produced in each respiration chamber during the experiment is displaced from the alkali and measured volumetrically in the gas analyzer described by Bliss (1953) or manometrically by coupling the respiration chamber to a standard Warburg manometer. The average carbon dioxide output may then be calculated.

Accuracy of Method and Range of Application

The calibrated capillaries can be read to ± 0.1 division. This and the total capillary excursion (110 divisions) establish the theoretical limits of accuracy. The actual limits are, of course, determined in large measure by the degree of agreement between the thermobarometers. Table I records the results of a series of readings on two sets of thermobarometers in two typical sets of experimental conditions. The maximum standard deviation of ± 0.16 corresponds to an error of about 0.1 division in reading the positions of the index drops, in close agreement with the theoretical limits. Therefore any individual reading corrected for thermobarometric change is accurate to within ± 0.2 division. A capillary excursion of 30 divisions would thus be accurate to ± one per cent.

Table I

Typical series of thermobarometric readings

Time reading taken (hours)	Difference between initial and subsequent thermobarometric readings					
	T_1	T_2	T_3	T_4	T_5	Standard deviation
Series 1						
Five thermobarometers each containing 1 ml. 1 N KOH compressed with 5 atmospheres of nitrogen						
2.00	.8	.8	.8	.9	.7	.07
4.00	1.4	1.4	1.4	1.6	1.4	.08
4.70	1.7	1.8	1.6	1.7	1.6	.07
16.90	2.6	2.8	2.6	2.7	2.4	.16
18.65	2.9	3.0	2.9	3.0	2.8	.07
Series 2						
Two thermobarometers each containing 1 ml. 1 N KOH compressed with 5 atmospheres of carbon monoxide						
1.02	.7	.7				0
2.12	1.1	1.3				.15
7.50	2.2	2.3				.1
20.75	1.7	1.5				.15

The experimental method has been applied without difficulty to studies of organisms and tissues having oxygen uptakes between 20 and 1000 mm.^{3}/ hour, and by the use of high pressures of carbon monoxide the role of cytochrome oxidase has been studied in both animals (Schneiderman and Williams, 1954), and plants (Hackett, Yocum and Thimann, personal communication).

We wish to express our sincere appreciation to Professor Carroll M. Williams in whose laboratory these experiments were performed.

Summary

A simple and practical apparatus is described for the measurement of oxygen consumption and carbon dioxide production at positive pressures up to seven atmospheres. It consists, essentially, of a series of capillary respirometers enclosed in a large Lucite compensation chamber capable of withstanding a positive pressure. The details of the construction and operation of the apparatus and the accuracy and range of application are considered.

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