CVII. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

III. A METHOD FOR THE MEASUREMENT OF RESPIRATORY QUOTIENT IN SERUM.

By FRANK DICKENS[1] and FRANTIŠEK ŠIMER.

From the Courtauld Institute of Biochemistry, Middlesex Hospital, London, W. 1.

(*Received May 10th, 1931.*)

In Part I of this series [Dickens and Šimer, 1930] the principles applicable to the measurement of the respiratory quotient of surviving tissues *in vitro* were summarised. It was concluded that the only existing principle by which the true R.Q. could be measured correctly, regardless of changes in acid-base equilibrium, was that in which the tissue is suspended in a CO_2-free medium, such as a Ringer solution buffered with phosphate, the gas-space containing pure CO_2-free oxygen. A reliable method involving such a principle was there described.

The method gives accurate results and is convenient and simple to use. Nevertheless, there are two obvious objections which may be brought against the conditions of the tissue in such a method; firstly, the low CO_2 tension, which approaches zero in the medium, and secondly, the fact that the whole of the buffering is due to phosphate, whilst in the body phosphate buffers are, at least quantitatively, much less important. Above all, the phosphate method cannot be applied if serum or other similar body-fluids are used as medium. There can be no doubt that at any rate in the case of certain delicate material, such as germinal and embryonic tissues, and probably for other tissues also, serum is a more suitable medium than a simple salt solution.

When an attempt is made to find a method which might be applied to the measurement of respiration and respiratory quotient in such a medium, very great practical difficulties arise. These are due to the circumstance remarked on by Warburg [1925] who wrote: "Up to now, no method exists for the estimation of the respiration CO_2, when fluids are employed whose bicarbonate content corresponds to that of serum, *i.e.* under conditions which are physiological for cells of the higher animals."

In the present paper, a method is described whereby the CO_2 formed in respiration by a piece of tissue suspended in such a medium may be directly measured, independently of any changes whatever in the bound CO_2, whether these changes arise from production or consumption of acids by the tissue, or

[1] A member of the scientific staff of the Medical Research Council.

whether they are due to retention of CO_2 by the buffer substances contained in the medium. The oxygen consumption of the same piece of tissue is given simultaneously by manometric readings, and hence the R.Q. is accurately determined. The method may therefore be used for determining the R.Q. and respiration, either with simple Ringer solution containing bicarbonate, or with serum or other similar fluid in equilibrium with the physiological tension of CO_2. A great advantage of the method is that exactly the same technique and calculation may be used with equal accuracy either for Ringer solution or serum, and no corrections, empirical or otherwise, are needed for the "retention" in the latter medium.

Principle. The method is a manometric one. A thin slice of the tissue is suspended in serum or Ringer solution in a vessel of special design, filled with a mixture of oxygen with the required percentage of CO_2. The vessel and contents, connected with a simple Barcroft-Warburg manometer, are shaken in a thermostat as in Warburg's method [1925]. If we denote the total pressure-change read on the scale of the manometer during the experimental period by h, this pressure, which may be either positive or negative, is made up of the following:

(1) h_{O_2} = pressure-change caused by disappearance of oxygen consumed in respiration;

and (2) h_{CO_2} = pressure-change due to respiratory CO_2;

and (3) h_b = pressure-change due to alteration in the amount of combined CO_2 during the experiment.

These magnitudes are related to the observed pressure-change h, by the following equation:

$$h = h_{O_2} + h_{CO_2} + h_b \qquad \text{.....(1).}$$

In this equation all pressures must be algebraically reckoned, h_{O_2} being always negative, h_{CO_2} positive, and h_b either positive or negative according to whether the total amount of chemically combined CO_2 is diminished or increased.

The magnitude of h_b is determined by liberation of the whole of the bound CO_2 present at the beginning and end of the experiment by acidification with excess of strong acid.

In order to determine h_{CO_2} a new principle has been introduced. A direct determination is made of the change during the experimental period in the total amount of CO_2, bound and free, present in the vessel. This quantity is the amount of CO_2 respired during the experiment and is denoted by x_{CO_2}. It is important to emphasise that by the determination of this magnitude, three of the four quantities in equation (1) are known, namely, h, h_{CO_2} and h_b. Hence the value of h_{O_2} is determined, and therefore the amount of respired oxygen (x_{O_2}) is also known.

Both x_{O_2} and x_{CO_2} being known, the R.Q. $= -x_{CO_2}/x_{O_2}$.

It will be noted that the above principle is quite general for any medium.

Method.

Apparatus and solutions. The special vessels[1] (Fig. 1) used to contain the tissue and solutions are attached by means of the ground-joint to Barcroft manometers. The vessels are conical in shape, with as nearly as possible equal volumes of about 20 cc. They contain an inner separate conical part (*A*) which receives the piece of tissue and the medium (1·5 cc. serum or Ringer solution).

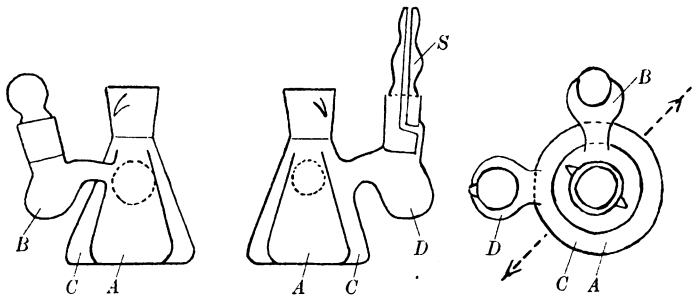

Fig. 1. The dotted line in the plan shows direction of shaking.

Communicating with compartment *A* by a fused-through join is the bulb *B*, containing excess of acid (0·1 cc. 3*N* HCl) which may at any desired moment be tipped into *A* by inclining the vessel. In this way, the respiration is stopped and the total bound CO_2 is liberated.

The outer annular portion *C*, and the bulb *D* communicating with it, serve for the absorption of the whole of the CO_2 contained in the vessel. For this purpose, use has been made of a principle of gas-analysis described by Krebs [1930]. The part *C* contains 2 cc. of a solution of $KMnO_4$ (*M*/5 with *M*/500 H_2SO_4), whilst bulb *D* has 0·2 cc. 30 % NaI, the latter solution being acidified to *N*/500 with H_2SO_4 immediately before use. When the contents of the bulb *D* are tipped into the part *C*, the iodide reacts with the $KMnO_4$ and a strongly alkaline solution results. The free CO_2 is thus rapidly and quantitatively absorbed.

The stopper *S* is of the type used by Warburg and Kubowitz [1929] for the determination of oxygen in gas-mixtures. It is closed by turning the stopper under the water of the bath, and permits the streaming of gas through the vessel whilst the latter is being shaken in the thermostat. Its use is essential for the accuracy of the method. An ordinary glass stopper is used for the other bulb (*B*).

Manometric fluid. The usual Brodie fluid (sp. gr. 1·033) is unsuitable for these experiments since its use would necessitate inconveniently long manometers in order to measure the large pressure changes involved. According to the carbon dioxide tension in the vessel, one or other of two different fluids has been found satisfactory.

[1] The vessels were made for us by Messrs C. Dixon and Co., 27, Devonshire Street, London, W.C. 1.

(1) For CO_2 tensions of the order of that occurring in serum, Clerici solution as used by mineralogists (a mixture of thallium malonate and thallium formate of sp. gr. approximately 4) is used. Sodium tauroglycocholate (0·1 %) is dissolved in the solution before use and, after filtration, the sp. gr. of the mixture at the prevailing temperature of the laboratory is accurately determined.

(2) In some experiments where a lower CO_2 tension than the physiological is employed, a solution of lead perchlorate, sp. gr. 2·07 [Krebs, 1930], has been used instead of the above solution.

These solutions must be protected from accidental dilution by droplets of water by means of loosely-fitting glass caps on the open ends of the manometers and by cotton-wool plugs inserted loosely in the tubes supplying the moist gas stream to the vessels. If new rubber tubing is used for the reservoirs of the manometers, it should be artificially aged by soaking in Clerici solution before use to remove traces of an impurity which otherwise slowly reacts with the solution, forming a dark-coloured deposit on the glass.

Supply of gas mixture. The gas mixture stored under pressure in the usual type of commercial gas-cylinder is first passed through a simple T-tube safety device, consisting of a vertical tube immersed to a suitable depth in water, which controls the maximum pressure of gas in the manometers. It then passes through a little water contained in a bubbler immersed in the bath, and the moist gas is distributed to the manometers through a six-way tube. Before entering the manometers the gas passes through the small tubes containing the cotton-wool plugs already mentioned; these tubes are attached by flexible rubber connections to the gas supply in order to allow vigorous shaking during the passage of the gas.

Procedure. For each determination, two vessels with tissue are necessary, or more if duplicate estimations are to be made. The solutions are first pipetted into the vessels as described above, serum having first been approximately saturated with the gas-mixture used in the experiment. Special care is necessary to measure exactly equal volumes of the serum or $NaHCO_3$-Ringer into all vessels. The fresh tissue is then cut with the razor in the usual way, with care to cause minimal damage to the tissue and to secure slices sufficiently thin to permit complete diffusion of metabolites [Warburg, 1925]. Before being transferred to the vessels, the slices are first suspended in serum or $NaHCO_3$-Ringer solution and a stream of 5 % carbon dioxide is passed briskly through for some 5 minutes, in order to arrive at a uniform preliminary condition in the various slices. They are then weighed in the moist state on a torsion balance, the weight for each vessel being equal, and the amount sufficient to give a respiration of some 100 $mm.^3$ per hour. After weighing each piece of tissue it is quickly rinsed in a $NaHCO_3$-free salt solution. This stage is not essential, but tends to lead to more consistent figures for the $NaHCO_3$-introduced with the tissue into the vessel. The tissue is then placed in the central compartment of the vessels, into the serum or $NaHCO_3$-Ringer. The vessels are attached to

their respective manometers and are together put into the bath with their stoppers closed. A third vessel without tissue and containing only the $KMnO_4$ and NaI is used for the analysis of the gas mixtures, and as usual the bath also contains a thermobarometric control vessel. The gas stream (see above) is now connected to the upper tubes of all manometers and a positive pressure of gas is set up in the vessels. The stoppers S of the vessels are now opened under the water, without removing the vessels from the bath, until a vigorous gas-stream is seen to bubble through the water of the bath. At the same time it is necessary to make sure that the vessels are firmly fixed to their manometers by means of their ground-joints. The shaking apparatus is now set in motion and the time noted. A period of 10 minutes, during which a steady stream of gas passes and the shaking is maintained at the rate of 90 swings per minute, suffices for thermal and gaseous equilibrium to be established. This rate of shaking must be used throughout the experiment. During the preliminary period the levels of the manometric fluid are roughly adjusted to a suitable height. The gas-stream is now stopped by turning the stoppers S without removing the vessels from the bath, the shaking being momentarily interrupted for this purpose. The upper taps of the manometers are closed, the tubing used for the gas-supply disconnected, and the final adjustment of level in the manometers is made. Shaking is recommenced, and the first reading of all vessels is taken 15 minutes from the time of first putting into the bath. Immediately after taking this reading, the acid from bulb B is at once tipped into the control vessels, which are momentarily removed from the bath for this purpose. The positive pressure caused by this acidification is read off and is denoted by h_{B_0} in the Protocol at the end of this paper, in which vessels Nos. 7 and 11 are the controls on the preformed CO_2, and Nos. 9 and 10 are the duplicate experimental vessels used for a double determination of R.Q., whilst No. 12 is solely for analysis of the gas-mixture from the cylinder.

During the experimental period of about 3 hours, the shaking of manometers is interrupted at intervals of half hour in order to follow the change of pressure in Nos. 9 and 10, the difference between the initial pressure and final pressures at the end of the 3 hour period, corrected of course for any small changes shown by the thermobarometer vessel, gives the value of h. Immediately after reading this quantity, the acid is tipped into the experimental vessels, causing a positive pressure (h_{B_1}) which is read off. After adding the acid, about 10 minutes is sufficient for the complete evolution of CO_2.

The whole of the CO_2 in the vessels is now in the free state, and is determined in all vessels, including No. 12, used for analysis of the gas-mixture, by tipping the iodide into the $KMnO_4$.

The absorption of CO_2 is complete in some 20 minutes, and the resulting negative pressures, H_0, H_1, and H_{gas} are then read off on the respective manometers of the control, experimental, and gas-analysis vessels.

Calculation. The basis of the calculation is the relationship given in equation (1):

$$h = h_{O_2} + h_{CO_2} + h_b \qquad \text{.....(1).}$$

Since the volumes of gas corresponding to these heights are merely the products of these heights in mm. and the appropriate vessel-constants, equation (1) may be re-written in the form:

$$h = \frac{x_{O_2}}{k_{O_2}} + \frac{x_{CO_2} + x_b}{k_{CO_2}} \qquad \text{.....(2).}$$

Vessel constants. For a manometric fluid of specific gravity S, the calculation of the vessel-constants for oxygen and carbon dioxide is as follows:

$$k = \frac{V_G \times (273/T) + V_F . \alpha}{760 \times (13{\cdot}60/S)},$$

where $V_G + V_F$ = total volume in mm.^{3} of vessel and capillary of manometer to the zero mark, V_F = volume of liquid placed in manometer vessel and α = Bunsen solubility coefficient of oxygen or CO_2 respectively. S must be accurately determined for each batch of manometer fluid prepared.

In the following calculations, quantities determined in the experimental vessel I (at time t_1), and at time t_0 in the control (vessel O) are denoted by the suffixes 1 and 0 and the vessel-constants for carbon dioxide by k_1 and k_0 respectively, whilst k_{O_2} is the constant for oxygen relating to the experimental vessel.

Determination of x_{CO_2}. The total amount of CO_2 in the vessel is made up of the following: (1) CO_2 introduced as gas at the time of filling the vessel, (2) bound CO_2 introduced with the tissue and serum or Ringer bicarbonate, (3) CO_2 formed in respiration. Each of these will now be considered.

(1) Since all the vessels are filled with the same gas-mixture under the same pressure, all contain the same percentage of CO_2 at the time of closing the taps. By absorption of this amount of CO_2 the negative pressure H_{gas} is set up, which is related to the percentage of CO_2 in the gas-mixture used by the following equation [Krebs, 1930]:

$$\%\ CO_2 = \frac{H_{gas} \times 100}{P_o - p_w} \qquad \text{.....(3),}$$

where P_o = total pressure inside the vessel at the beginning of the experiment, and p_w = pressure of saturated vapour above the solutions in the vessel, all pressures being reckoned as usual in heights of manometer fluid.

This equation shows that H_{gas} is independent of the size of vessel or amount of fluid contained in it, and for a certain gas-mixture is constant if the total pressure in the vessels is the same. In any particular experiment this is easily realised by making the initial levels of manometer fluid in the two limbs approximately equal. The actual amount of CO_2 is of course dependent on the size of the vessel and amount of fluid, and is given by $k_{CO_2} . H_{gas}$.

(2) If the quantities of solution and tissue are the same in experimental and control vessels, the amount of bicarbonate introduced is the same in both vessels and is affected to the same extent in both vessels up to the time t_0.

(3) The same applies to respiration, which up to the time t_0 is the same in both vessels. In any case, this term is very small, since it concerns only the amount of respiratory CO_2 produced in the interval between stopping the gas-stream and t_0, which is in general only 5 minutes or less.

The volume of CO_2 produced between the times t_0 and t_1 in the experimental vessel, *i.e.* respiratory $CO_2 = x_{CO_2}$, is given by the difference in initial and final contents of total CO_2 in the vessel. If the vessels were of exactly the same size, x_{CO_2} would be simply the difference in the amounts of CO_2 present in the control vessel at t_0 and the experimental vessel at t_1. Since in practice we are dealing with vessels of slightly different volume, this must be considered in the calculation, which is carried out by either of the two principles described below.

First principle.

The quantities actually measured are H_1 and H_0, from which we may at once subtract H_{gas}, since this is the same in both vessels. Hence, apart from the amount of free CO_2 introduced in the gas-mixture, the amounts of CO_2 at time t_0 and t_1 are:

$$(H_0 - H_{gas})\, k_0 \text{ and } (H_1 - H_{gas})\, k_1$$

respectively. The difference between these two amounts is the respiratory CO_2 between time t_0 and t_1, hence:

$$x_{CO_2} = (H_1 - H_{gas})\, k_1 - (H_0 - H_{gas})\, k_0 \qquad \text{.....(4).}$$

Determination of x_{O_2}. From equation (2):

$$x_{O_2} = \left(h - \frac{x_{CO_2} + x_b}{k_1}\right) k_{O_2} \qquad \text{.....(5).}$$

In this equation, x_b is the change in bound CO_2 during the experimental time $t_1 - t_0$. If B_0 = initial amount of bound CO_2 at the time t_0, and B_1 = bound CO_2 at time t_1 in the experimental vessel, then:

$$x_b = B_0 - B_1 = h_{B_0} k_0 - h_{B_1} k_1 \qquad \text{.....(6).}$$

The values x_{CO_2} and x_b and h in equation (5) being known, x_{O_2} is thus determined, and R.Q. $= - x_{CO_2}/x_{O_2}$ can at once be calculated.

Second principle.

By substituting the observed pressure changes from equations (4) and (6) in equation (5), this may also be written in the form:

$$h_{O_2} = h - H_1 + h_{B_1} + H_{gas}\{1 - (k_0/k_1)\} + (H_0 - h_{B_0})(k_0/k_1) \qquad \text{...(7).}$$

Hence if $k_0 = k_1$, $h_{CO_2} = H_1 - H_0$ (from equation (4)) .....(4*a*),

and $h_{O_2} = h - H_1 + H_0 + h_{B_1} - h_{B_0}$ (from equation (7)) .....(8).

If the volumes of the two vessels are nearly the same, this condition can be achieved in practice, by varying the volume of liquid in these two vessels so that k_{CO_2} is the same for both. This modification makes the separate determination of H_{gas} unnecessary, provided as before that the quantities of tissue and serum taken are equal in the two vessels.

Notes.

(1) *Time of saturation with gas-mixture.* The time allowed is sufficient to attain complete filling of the vessel with the gas-mixture, and in control experiments the analysis was unchanged by prolonging the passage of gas for a greater time.

(2) *Control experiments with $NaHCO_3$ solutions.* In the absence of tissue in the vessel, x_{CO_2} is zero and $H - H_{gas} = h_B$, where h_B is the pressure change caused by the liberation of CO_2 from the bicarbonate taken. In numerous control experiments h_B and B $(= h_B.k_{CO_2})$ have been determined (a) by acidification and (b) from measurements of H and H_{gas} with an accuracy governed only by the accuracy of the manometer readings, *i.e.* $\pm$ 0·5 mm. manometer fluid.

(3) *Blank correction for tipping of NaI.* It should be mentioned that in determining the actual percentage of CO_2 in the vessel, it is necessary to apply the small correction of a few mm. mentioned by Krebs, due to the slight change of pressure which accompanies the mixing of the NaI and $KMnO_4$ and having no relation to the absorption of CO_2. In the method described, where the absolute percentage of CO_2 is not required, this correction is unnecessary, since it cancels out, as is clear from equations (4) and (8).

(4) *Magnitude of respiration and gaseous diffusion.* The fundamental principle underlying manometric measurements is the existence of a virtually complete equilibrium between the liquid and gaseous phases. The quantities of tissue and rate of shaking referred to above are suitably chosen to ensure this, and should be adhered to.

(5) *Correction for different weights of tissue.* With a little practice it is usually possible to weigh sufficiently nearly equal pieces of wet tissue into each vessel to render special correction for this point unnecessary; particularly as the magnitude of the correction for tissue taken from the control vessel is always small compared with the actual measurement in the experimental vessel. In all cases, however, the dry weight is accurately determined by drying to constant weight at 110°, since it is required for calculation of Q values [Warburg, 1930] and also as a check on the equality of the wet weights taken.

Should there be an appreciable difference between the dry weights of tissue in the control and experimental vessels, the correction may be applied in the following way. In the first place, the total bicarbonate content of the volume of original medium used is determined in the usual way. Denoting

this by B_M mm.^{3} and the weights of tissue by m_1 (experimental vessel) and m_0 (control vessel) then

$$x_b = B_M + (B_0 - B_M)\, m_1/m_0 - B_1,$$
$$x_{CO_2} = (H_1 - H_{gas})\, k_1 - B_M - (H_0 k_0 - H_{gas} k_0 - B_M)\, m_1/m_0.$$

The remainder of the calculation is as given above.

Notes on the determination of glycolysis or oxidative removal of lactic acid.

(1) *In bicarbonate-Ringer solution.* In solutions containing no other buffer besides bicarbonate, the change in bound CO_2, x_b, is exactly equal to the amount of acid formed or consumed by the tissue (x_G):

$$x_b = x_G.$$

x_G is positive if acid is formed, *i.e.* CO_2 liberated, and negative if acid is consumed (*i.e.* CO_2 bound). Both quantities are expressed in mm.^{3} according to Warburg [1930].

From x_G the value of aerobic glycolysis is calculated in the usual way:

$$Q_M^{O_2} = x_G/(m.t),$$

where m is the dry weight of the tissue in mg. and t the duration of the experiment in hours.

The method described may be applied with equal accuracy to the measurement of oxidative removal of lactic, pyruvic or other organic acids metabolised by tissues.

(2) *In serum.* In serum the decrease of the amount of bicarbonate, x_b, is smaller than the amount of fixed acid, x_G, formed by the tissue. The difference, $x_G - x_b = \Delta x$, is due to the presence of buffers other than bicarbonate, which (1) neutralise part of the acid formed in glycolysis, and (2) combine with a certain amount of CO_2 formed in respiration.

Δx must be determined experimentally. Two vessels, O and I, of the type described in this paper (see Fig. 1) are used for the determination. Vessel I is the same vessel as that used in the main experiment.

Exactly equal amounts of a solution of tartaric acid are pipetted into bulb B of both vessels and evaporated to dryness at 100° [Warburg, 1925]. The concentration of the acid and the amounts pipetted must be chosen so that the quantity in bulb B of both vessels is as nearly as possible equal to x_G ($> x_b$). Into bulb D of both vessels is now pipetted a solution of tartaric acid of such concentration and volume that the quantity of acid is exactly equal to the respiratory CO_2, x_{CO_2}, of the main experiment. This condition is easily fulfilled as x_{CO_2} is accurately known from the main experiment. The volumes of tartaric acid solution in B and D are checked by weighing the vessel before and after pipetting, and a correction is applied if necessary.

The central compartment (A, Fig. 1) contains 1·5 cc. of serum in vessel I and 1·5 cc. bicarbonate-Ringer in vessel O. Into the outer part C of both vessels bicarbonate-Ringer solution is pipetted, the volume measured being

such that the total volume of fluid V_F is in both equal to that in the main experiment (3·80 cc.).

Both vessels are now filled with the same gas mixture in the same way as in the main experiment. After equilibrium has been established a reading of pressure is taken and in both vessels the acid in D is tipped into C, and the dried acid in B is washed into A simultaneously. Denoting the total resulting pressures set up in vessels O and I by h_0 and Δp respectively, the amounts of CO_2 liberated are $h_0 k_0$ and $\Delta p . k_1$. The difference $h_0 k_0 - \Delta p . k_1 = \Delta x'$, is the amount of acid retained by the serum in vessel I under the conditions of this particular experiment.

This amount $\Delta x'$ is equal to Δx only if the quantity of acid in bulb B is exactly equal to x_G, *i.e.* if

$$\Delta p = \frac{x_{CO_2} + x_b}{k_1} \qquad \text{.....(9).}$$

In choosing the amounts of acid some preliminary experiments with varying amounts of acid in B may be necessary. The amount of acid in D is of course in these estimations kept constant and equal to x_{CO_2} in the main experiment.

If the amount of acid taken is not exactly the same as that in the main experiment, but the conditions approximate fairly closely to those prescribed by equation (9), the observed value of $\Delta x'$ may be corrected for the conditions of the main experiment as follows:

$$\Delta x = \frac{\Delta x'}{\Delta p} . \frac{x_{CO_2} + x_b}{k_1} \qquad \text{.....(10).}$$

The use of the above principle of calculation of aerobic glycolysis in serum is made possible by the direct determination of x_{CO_2} by the new method, and has the following advantages.

(1) It is not necessary to calculate the retention for CO_2 from an observation of the retention for lactic acid, or *vice versa*.

(2) When both glycolysis and respiration occur together, as for example in tumour tissue, the effect of the lactic acid retention is to cause a change in the retention for CO_2 and *vice versa*, during the experiment. This mutual influence of the two retentions is allowed for in the above direct method of determination.

Accuracy. The probable error to be expected in the value of R.Q. may be conveniently considered on the basis of equations (4 *a*) and (8). Under these simplified conditions (weight of tissue, and constants for carbon dioxide equal in the different vessels) the relationship between the pressures directly observed and the R.Q. is as follows:

$$\text{R.Q.} = \frac{(H_1 - H_0)}{(H_1 - H_0) + h_{B_0} - h_{B_1} - h} \cdot \frac{k_{CO_2}}{k_{O_2}} \qquad \text{.....(11).}$$

In this equation the largest term, $H_1 - H_0$, is present in both numerator and denominator. This is very favourable to the accuracy of the method since any slight error in $H_1 - H_0$, due either to the error in readings themselves

$\pm$ 0·5 mm.), or to slight inequalities in the initial amount of CO_2 in the experimental and control vessels has a negligible effect on the value of R.Q.

The limiting factor in the accuracy is therefore the measurement of h_{B_0} and $(h_{B_1} + h)$. The difference of these two terms which occurs in the denominator of equation (11), is always smaller than $(H_1 - H_0)$, and the accuracy of measuring this quantity is controlled by

(1) accuracy of reading ($= \pm$ 0·5 mm.);

(2) accuracy in pipetting equal amounts of $NaHCO_3$ in the control and experimental vessels;

(3) agreement in bicarbonate content of the two pieces of tissue in the two vessels.

On the basis of the above error in scale-readings, and the experimentally observed accuracy in determining (2) and (3), and assuming a respiration of the order mentioned in this paper, the probable accuracy may be calculated to be 2 to 3 %, or 0·02 in the determination of R.Q. This order of agreement between duplicate determinations of R.Q. on the same tissue has been obtained in a number of experiments, which will form the subject of Part IV of this series.

We wish to express our indebtedness to Prof. O. Warburg, with whom we had the privilege of discussing the problem in the early stages of the development of the method.

We also wish to make acknowledgment to the Halley Stewart Trust Fund for a Scholarship held by one of us (F. Š.).

REFERENCES.

Dickens and Šimer (1930). *Biochem. J.* **24**, 905.
Krebs (1930). *Biochem. Z.* **220**, 250.
Warburg (1925). *Biochem. Z.* **164**, 481.
—— (1930). Metabolism of tumours. (Constable, London.)
—— and Kubowitz (1929). *Biochem. Z.* **214**, 7.

Protocol.

Rat liver in inactivated horse-serum containing 0·2 % glucose. Rat fasted 24 hours. Duration of experiment, 3 hours. Gas-mixture, oxygen with 4·50 % CO_2. Bicarbonate content of serum =461 mm.³ per cc. Hence p_H=7·41. Temperature 38°.

Vessel No.	7	9	10	11	12
Total volume (cc.)	19·73	21·38	21·00	20·16	—
Solutions:					
A (Fig. 1)		1·5 cc. serum			—
B „		0·1 cc. HCl			—
C „		2·0 cc. $KMnO_4$			—
D „		0·2 cc. NaI			—
Weight of tissue:					
Wet (mg.)	80	90	90	90	—
Dry (mg.)	15	18·7	21·2	21·3	—

Gas passed through vessels in bath for 10 mins. Taps then closed and first reading taken after further 5 mins. (t_0).

Readings:

Pressure changes corrected for thermobarometer (mm. Clerici solution of sp. gr. 3·975).

	At t_0 tip B into A	—	—	Tip B into A	—
	$h_{B_1}=+113$	—	—	$h_{B_1}=+111$	—
	At t_1	$h=-30·5$	$h=-34$	—	—
		Tip B into A	—	—	—
		$h_{B_1}=+103·5$	$h_{B_1}=+105·5$	—	—
		Tip D into C (all vessels)			
	$H_0=235$	$H_1=280$	$H_1=286·5$	$H_0=234·5$	$H_{gas}=111$
Calculation:					
Vessel constants					
k_{CO_2}	6·17	6·72	6·60	6·31	—
k_{O_2}	—	5·92	5·84	—	—
k_{O_2}/k_{CO_2}	—	0·889	0·884	—	—
$h_B k_{CO_2}=B$ (mm.³)	697	696	696	700	—
$B_0-B_1=x_b$ (mm.³)	—	3	3	—	—
$(H-H_{gas})\,k_{CO_2}=C$ (mm.³)	764	1136	1157	779	—
$C_1-C_0=x_{CO_2}$ (mm.³)	—	**364**	**385**	—	—
$hk_{O_2}-k_{O_2}/k_{CO_2}(x_{CO_2}+x_b)$ $=x_{O_2}$ (mm.³)	—	**−508**	**−540**	—	—
Respiratory quotient:	—	**0·72**	**0·71**	—	—
Q_{O_2} (mm.³ oxygen respired per mg. tissue per hr.)	—	**−9·1**	**−8·5**	—	—