CC. THE METABOLISM OF NORMAL AND TUMOUR TISSUE.

XI. THE MEASUREMENT OF RESPIRATORY QUOTIENT, RESPIRATION AND GLYCOLYSIS WITH THE AID OF THE CONSTANT-VOLUME DIFFERENTIAL MANOMETER.

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The methods previously described in this series make possible the accurate measurement of the true respiratory quotient and respiration of tissues suspended in a phosphate medium [Dickens and Šimer, 1930], or in bicarbonate media (including serum) in equilibrium with CO_2 at physiological tension [Dickens and Šimer, 1931]. Additional points of technique were described recently [Dickens and Šimer, 1933]. These methods may be applied to all types of tissue; but when the bicarbonate method is applied to tissues in which the aerobic acid production is high compared with the respiration, the glycolysis may not persist throughout the experiment at its initial level. For with such tissues, although it is adequate with others, the amount of bicarbonate solution which can be used may be unable to prevent an undue fall of p_H and increase of lactate concentration in the medium. With the simple manometer used, and also with the Barcroft manometer as applied to the measurement of respiratory quotient by Dixon and Keilin [1933], the permissible bicarbonate reserve is limited by the length of the manometer limb and the density of the manometer fluid. Increase of the former is inconvenient; increase in the latter results in loss of sensitivity. The difficulty has been overcome by the application of the constant-volume differential manometer [Dickens and Greville, 1933] to the respiratory measurements. It is now possible to use a sensitive manometer fluid (Brodie fluid of sp. gr. approximately 1), and to have a volume of bicarbonate solution such that, even with highly glycolysing tissues, the glycolysis remains nearly constant throughout. The conditions are strictly comparable with those of the Warburg [1924] two-vessel method; but whereas Warburg's method gives only the O_2 consumed and the total CO_2 produced by respiration and glycolysis, the method now described gives the true respiratory quotient and the true aerobic glycolysis, respiratory CO_2 and O_2-uptake.

Method.

Principle. The constant-volume differential manometer has both vessels rigidly attached to manometer limbs which may be moved independently up and down. By this means the meniscus in each limb is kept at a fixed point in the

scale, and the contents of the vessel at constant volume; the difference in pressure between the vessels is given in terms of height of manometer fluid by the vertical distance between the menisci in the two limbs. Each limb has a tap which enables the vessels, the limbs and the outside air to be connected in pairs or all together. When this manometer is used for the measurement of R.Q., equal weights of tissue are put into equal volumes of medium in the two vessels, which are of equal size. Excess of acid is tipped into the medium in one vessel at the beginning of the experiment, in the other at the end. The pressure difference between the two vessels immediately before addition of acid to the medium in the first vessel is zero; after acid addition in the second vessel it is equal to the pressure increase due to the respiratory CO_2 less the pressure decrease due to the absorbed O_2. The total CO_2 in both vessels is now absorbed, when the pressure difference between them represents the O_2 absorbed during the experiment. This subtracted from the previous pressure difference gives the respiratory CO_2, so that the R.Q. can at once be calculated. Hence, the measurement of respiration and R.Q. necessitates only two readings; that of aerobic glycolysis, which is deduced from the pressure change in the second vessel during the experiment, demands a third reading in conjunction with a thermobarometer. The latter is unnecessary if only R.Q. and respiration are to be measured.

Apparatus. The constant-volume differential manometer, its use including filling with gas and its calibration have already been fully described [Dickens and Greville, 1933]. For the measurement of R.Q. the only modification lies in the use of a special vessel of new design[1] (Fig. 1). This is of conical shape and of

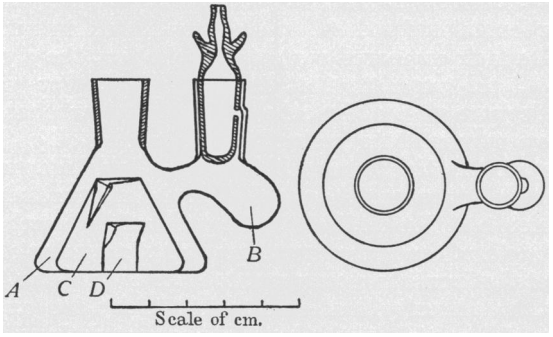

Fig. 1.

20–25 cc. volume. The diameter of the base should be about equal to the height: this gives stability to the vessel and large surface to the solutions. The vessel contains a separate inner conical part *C*, in the centre of the base of which there is a small cylindrical vessel *D* with a lip, like a small jug. *D* and *C* have each a groove at a suitable point in their rim to facilitate the insertion of pipettes and tissue. *B* is a bulb communicating with the outer part *A*; it has a bored stopper which can be closed by turning [Warburg and Kubowitz, 1929] and which is provided with hooks from which rubber bands may be stretched to hooks on the vessel. On inclining the vessel to the left (Fig. 1), liquid may be tipped from *B* into *A*; on inclining to the right, liquid first runs from *A* into *B*, and finally the contents of *D* drain almost completely into *C*.

[1] Made for us by Messrs C. Dixon and Co., 27, Devonshire Street, London, W.C. 1.

Having only one stopper, the vessel is more convenient and less likely to leak than that described for the original bicarbonate method [Dickens and Šimer, 1931]; it is also more stable; hence its use in that method is now recommended.

Procedure. The solutions are first measured into the various parts of the vessels as follows:

A. Bicarbonate-containing medium (3·0 cc. are usually taken).

B. 2·5 *N* hydrochloric acid, 0·25 cc.

C. 43 % NaI, $2H_2O$, 2 cc. (acidified immediately before experiment with 0·1 *N* sulphuric acid, methyl orange as indicator).

D. 30 % $NaMnO_4$, $3H_2O$ containing *N*/400 H_2SO_4, 0·5 cc.

The medium must be accurately measured with a grease-free pipette with two marks and a fine tube; such a pipette delivered 3 cc. with an extreme error of ± 4 mg. in twelve consecutive measurements. After the solutions have been measured, the stoppers of the vessels, greased with a good rubber lubricant (Stevens's rubber grease), are worked in by turning. The tissue is then prepared for the experiment. In order to equalise as nearly as possible the conditions of the two pieces of tissue, the portions to be used are suspended for 7 minutes in bicarbonate-containing medium through which oxygen containing 5 % carbon dioxide is passing and are then rinsed for a further 3 minutes in oxygenated bicarbonate-free salt solution. Portions are then weighed on a torsion balance after draining on filter-paper, the weights for each vessel being equal to the nearest mg., and are inserted in the medium in the spaces *A*. The vessels are attached to the manometer by the ground joints lubricated with rubber-grease, and placed in the bath. The gas stream (5 % CO_2 in O_2), passing first through a small water-containing gas-bubbler immersed in the bath and then through a cotton-wool plug, enters the apparatus through the upper tubes of the manometers and leaves through the stoppers of the vessels below the water-level in the bath. Extra gas-pressure should be applied by means of a screw-clip or tap on the safety device [Dickens and Greville, 1933, Fig. 3]. After the gas has passed for 10 minutes, during which the manometer has been continuously shaken, the extra pressure is released, the stoppers of the vessels turned and worked in until they will no longer move and the manometer shaken for a further 2 minutes with the vessels open to the air through the taps. The shaking is then interrupted, the vessels shut to the air and connected to the limbs, the menisci in the limbs being level and at the centre (150) mark. The right-hand tap is turned so that the limb is open to the air and the vessel closed: the acid is then tipped from the bulb into the medium in the right-hand vessel, the limb being temporarily taken out of the clips. After the right-hand vessel has been replaced, shaking is resumed. If glycolysis is to be measured, a simple manometer should have been placed in the bath at the same time as the differential manometer, and readings of both manometers are taken at intervals until the end of the experiment. When the differential manometer is used as a simple manometer, the two menisci are still kept to their appropriate marks, the position of the mark on the left-hand limb being read off as usual on the graduated scale on the right-hand limb.

At the end of the experimental period (usually 2 hours), during which 120–200 $mm.^3$ oxygen should have been consumed, a final reading for use in calculation of glycolysis is taken, the meniscus in the left-hand limb is brought to the mark, the left-hand tap is turned so that the vessel is closed, and the acid is

tipped from the bulb. After shaking has been continued for a few minutes, both menisci are levelled at their marks, the limbs being open to the air. Both limbs are put in communication with their vessels and closed to the air, and 10 minutes after adding the acid a reading is taken, the constancy of which is checked 5 minutes later. This reading less 150 = H_1. The permanganate is tipped into the iodide in both vessels and the manometer shaken until constancy of the reading shows that gas absorption is complete. This usually requires 35 minutes, but longer times, up to 1 hour, are occasionally necessary. The final reading less 150 = H_2.

Calculation. Let h_{O_2} = pressure-change caused by consumption of oxygen and h_{CO_2} = pressure change due to respiratory carbon dioxide.

All pressures are reckoned algebraically in terms of mm. of Brodie fluid. With the procedure described, h_{O_2} is always negative, h_{CO_2} is always positive, whilst gas evolution in the left-hand (experimental) vessel always causes an increase in the reading.

After complete liberation of the bound carbon dioxide in both vessels, equal amounts of bound carbon dioxide having been present at the beginning of the experiment, any difference of pressure between the vessels is due only to the respiratory exchange during the experimental period.

Hence $$H_1 = h_{O_2} + h_{CO_2} \qquad \text{.....(1).}$$

After complete absorption of the carbon dioxide in the two vessels, the pressure difference is due to the oxygen consumed in the experimental vessel during the experimental period.

$$H_2 = h_{O_2} \qquad \text{.....(2).}$$

Subtracting, $h_{CO_2} = H_1 - H_2$, and, as usual,

$$x_{O_2} = H_2 . k_{O_2}; \quad x_{CO_2} = (H_1 - H_2) . k_{CO_2},$$

where k_{O_2} and k_{CO_2} are the simple manometer vessel-constants [Warburg, 1926].

Hence, $$\text{R.Q.} = -\frac{x_{CO_2}}{x_{O_2}} = \left(1 - \frac{H_1}{H_2}\right)\frac{k_{CO_2}}{k_{O_2}} \qquad \text{.....(3).}$$

Now let h = pressure change read off on the instrument during the period in which it is used as a simple manometer, corrected for thermobarometer changes.

Then $h = h_{O_2} + h_{CO_2} + h_b$ where h_b = pressure-change due to alteration in the amount of bound CO_2 during the experimental period. By (1), $h_b = h - H_1$, so that

$$x_b = (h - H_1)\, k_{CO_2} \qquad \text{.....(4).}$$

Table I.

Both vessels contained:

In *A*, 3 cc. bicarbonate solution, containing *ca.* 1700 mm.³ bound CO_2.
In *B*, 0·3 cc. 2·5 *N* HCl.
In *C*, 2·0 cc. 43 % sodium iodide solution.
In *D*, 0·5 cc. 30 % sodium permanganate solution.

Filled with gas mixture containing 5 % carbon dioxide.

Pressure change on acidification of both vessels (corresponding to error in H_1):

+1, 0, +1, 0, +0·5, 0, −0·5 mm. Brodie fluid.

Pressure change on acidification followed by CO_2 absorption in both vessels (corresponding to error in H_2):

+1, +2, 0, 0·5, 0, 0 mm. Brodie fluid.

Table II.

Tissue	Moist wt. in each vessel (mg.)	Bicarbonate concentration (mM/litre)	Pressure difference (mm. Brodie fluid) After acidification	Pressure difference (mm. Brodie fluid) After absorption
Rat testis	100	26	0	−1·5
Rat kidney	50	26	0	−1
Jensen rat sarcoma	95	26	−1	−3
,,	100	26	−1	−1
,,	100	34	−1·5	−1
,,	100	34	−2	0

Protocols.

The protocols show results with examples of two types of tissue, (1) a normal tissue of known R.Q. = 1, with little aerobic glycolysis, and (2) a tumour tissue with high aerobic glycolysis and low R.Q.

(1) *Rat brain.*

Thin slices of cerebral cortex; rinsed 5 mins. in bicarbonate-Ringer in stream of $O_2 + 5\ \% CO_2$, 5 mins. in salt solution in stream of O_2, before experiment.

Gas mixture, O_2 with 5·46 % CO_2. Bicarbonate content of Ringer 530 mm.^{3} CO_2/cc.

Glucose 0·2 %. 37·2°. p_H 7·4.

Differential manometer filled with Brodie fluid: vessel vols. 23·23 cc. (both).

Contents of vessels:

A, 3·0 cc. bicarbonate-Ringer-glucose solution + tissue.
B, 0·25 cc. 2·5 *N* HCl.
C, 2·0 cc. 43 % sodium iodide solution.
D, 0·5 cc. 30 % sodium permanganate solution.

Vol. fluid 5·75 cc. Vessel consts. $k_{O_2} = 1{\cdot}55$, $k_{CO_2} = 1{\cdot}84$.

Experiment:

Differential manometer. Pressure readings (mm. Brodie fluid).

Time (mins.)		
0	150	
	———	Acidify closed R.H. vessel.
2	150	
		$h = +3$ (corrected for thermobarometer).
120	156	
	———	Close L.H. vessel and acidify. Level at 150. Open both vessels to manometer.
130	136·5	
135	137	
	———	$H_1 = -13$. Add $NaMnO_4$ to NaI, both vessels.
170	70	
175	69·5	$H_2 = -80$.
185	70	

Weight of tissue: wet (L.H.) 60 mg.; (R.H.) 60 mg. dry (L.H.) 5·5 mg.; (R.H.) 6·3 mg.

Calculation:

Time of experiment: 2 hrs. 0 min.

$H_2 = h_{O_2} = -80$ mm. $x_{O_2} = -124$ mm.^{3}

$h_{CO_2} = H_1 - H_2 = +67$ mm. $x_{CO_2} = +123$ mm.^{3}

$h_b = h - H_1 = +16$ mm. $x_b = +29$ mm.^{3}

Whence $Q_{O_2} = -11{\cdot}3$ (mm.^{3} O_2 per mg. dry tissue per hour).

$Q_M^{O_2} = +2{\cdot}6$ (mm.^{3} acid produced per mg. dry tissue per hour).

R.Q. = 0·99.

(2) *Jensen rat sarcoma.*

11-day tumour; thin slices; pre-treatment, vessels, vessel-constants, gas mixture and solutions, as for brain (1), except that bicarbonate content of Ringer solution = 666 mm.³/cc.; hence p_H (initial) = 7·5; (final) = 7·3. 37·2°.

Experiment:

Differential manometer.

Time (mins.)		
0	Level at 150 mm. and acidify closed R.H. vessel.	
30	+ 20·5	Pressure changes in 30-min. periods corrected for thermobarometer.
60	+ 22·5	
90	+ 25	
120	+ 27	
150	+ 27	
	$h = +122$	Close L.H. vessel and acidify. Level menisci at 150 mm. Open both vessels to manometer.
	Pressure readings	
160	109	
165	110	$H_1 = -40$. Add $NaMnO_4$ to NaI, both vessels.
210	36	
220	32·5	
235	32·5	$H_2 = -117·5$.

Weights of tissue { wet (L.H.) 55 mg.; (R.H.) 55 mg. / dry (L.H.) 5·3 mg.; (R.H.) 6·2 mg.

Calculation:

Time of experiment 2 hrs. 30 mins.

$$H_2 = h_{O_2} = -117·5 \text{ mm.} \quad x_{O_2} = -182 \text{ mm.}^3$$
$$h_{CO_2} = H_1 - H_2 = +77·5 \text{ mm.} \quad x_{CO_2} = +142·5 \text{ mm.}^3$$
$$h_b = h - H = +162 \text{ mm.} \quad x_b = +298 \text{ mm.}^3$$

Whence

$$Q_{O_2} = -13·7.$$
$$Q_M^{O_2} = +22·4.$$
$$\text{R.Q.} = 0·78.$$

Simultaneously made experiments on slices of the same tumour suspended in 1·5 cc. bicarbonate-Ringer by Dickens and Šimer [1931] method gave R.Q. = **0·81**, $Q_{O_2} = -12·7$; whilst a determination of aerobic glycolysis by Warburg's 2-vessel method gave $Q_M^{O_2} = +22·3$, if the value of R.Q. found by the constant-volume differential manometer experiment given above be assumed for the calculation.

Notes.

(1) *Absorption of carbon dioxide.* The production of an absorbent for carbon dioxide by the mixing of two solutions, such as NaI and $KMnO_4$, avoids the necessity of having taps for the introduction of alkali into the vessel during the experiment. The quantities of the reagents used for carbon dioxide absorption by Dickens and Šimer [1931] are insufficient for the present method; more concentrated solutions are used, and it is necessary to use the more soluble sodium permanganate. The pressure change accompanying the mixing of iodide and permanganate which has no relation to CO_2 absorption may become large with concentrated solutions. It is probably due to alterations either of vapour pressure or of volume. The pressure changes observed on adding 30 % permanganate to sodium iodide solutions of various concentrations are shown in Fig. 2, from which it is seen that by choosing suitable concentrations the pressure change on mixing strong solutions may be reduced below that found with the original dilute solutions. We have therefore used in all experiments 30 % $NaMnO_4$, $3H_2O$ and 43 % NaI, $2H_2O$, 0·5 cc. of the former being added to 2 cc. of the latter. Carbon dioxide absorption by these quantities is rapid and complete. Since they were found to absorb completely 6·4 cc. carbon dioxide, *i.e.* 25 % of the vessel volume and twice as much carbon dioxide as is likely to be in the vessel during a R.Q. determination, it is clear that an ample excess of alkali is produced.

(2) *Accuracy.* In the absence of tissue, errors may be introduced by inaccurate measurement of the bicarbonate solutions and unequal filling of the two vessels with gas. The magnitude of the errors occurring in absence of tissue with careful measurement and technique as described is shown by Table I. When equal wet weights of tissue are inserted into the two vessels, the accuracy is limited by the agreement in their initial bicarbonate contents and in their glycolyses during the preliminary period. In the control experiments in Table II amounts of tissue suitable for R.Q. determinations were prepared and the vessels filled by the technique described, but 2 minutes

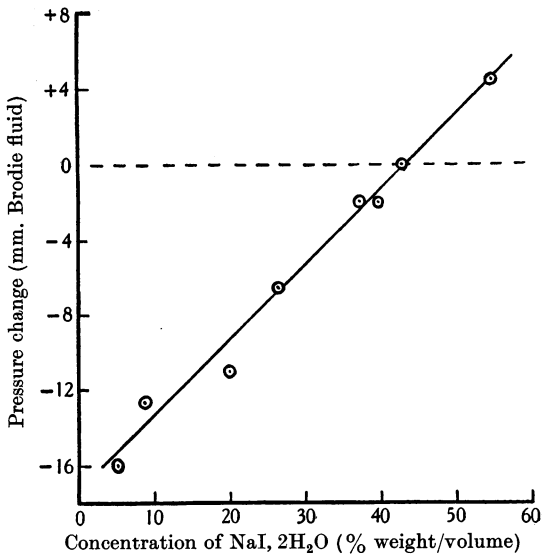

Fig. 2. Pressure changes on adding 0·5 cc. 30% $NaMnO_4$, $3H_2O$ to 2 cc. of sodium iodide solutions of various concentrations; 37·3°; air in gas space. 0·2 cc. *N* NaOH in vessel as CO_2 absorbent. Permanganate solution contained *N*/400 H_2SO_4, iodide contained *N*/100 H_2SO_4.

after stopping the gas stream the acid was tipped from the bulbs into both vessels. After a steady reading had been obtained, the carbon dioxide was absorbed in both vessels. The figures in the fourth column correspond to errors in H_1, those in the fifth to errors in H_2. In the last two experiments the strong bicarbonate-Ringer solution now used for tumour experiments (see Protocol) was taken. H_2 is always much larger than H_1, and errors of a few mm. in H_2 have a negligible effect on the R.Q. The possible error in the latter, governed by the error in H_1, with a respiration corresponding to 100 mm. Brodie fluid, is $\pm 2{\cdot}5$ %. This is equal to the error of the Dickens and Šimer method [1931]; but it is to be noted that in the present method the necessary respiration is only one-half and the bicarbonate reserve over twice that obtaining in the earlier one.

(3) Any oxygen uptake or carbon dioxide production during the experimental period by the tissue in the vessel in which the acid is added from the bulb at the beginning of the experiment will lead to faulty values of x_{O_2} and x_{CO_2}. The significance of oxygen uptake by acidified tissue in the measurement of R.Q. is discussed by Needham [1932, 1, 2]. We have found, however, that various types of tissue, when acidified under conditions comparable with those of the acidified tissue in the present method, show an oxygen uptake always less than 1 % of that of the non-acidified tissue. Further, in absence of carbon dioxide absorbent, such acidified tissue causes no pressure change. It is therefore concluded that the presence of acidified tissue in this method has a negligible effect on the observed value of the R.Q.

If at the beginning of the experiment, the metabolism of the tissue in one vessel could be immediately and completely stopped without altering the acid-base equilibrium, the presence of acidified tissue during the experimental period would be avoided, and also there would be no need

for a thermobarometer to assist in the measurement of h. The method would thereby be improved, but no suitable agent for killing the tissue has yet been found.

(4) This method has been developed during the last two years independently of that described recently by Dixon and Keilin [1933], which is essentially an application of the principle of the Dickens and Šimer [1931] method to the Barcroft manometer. Not only has the application of the constant-volume differential manometer, in preference to the Barcroft instrument, made possible a method which combines an increased sensitivity with a larger bicarbonate reserve, but also the flexibility of the rubber connection between the two limbs allows the use of rigidly fixed vessels without the necessity for ground-in bulbs and for the introduction of extraneous fluids by taps attached to the vessel. It is our experience that only stoppers which have been worked in until they will no longer turn are consistently capable of withstanding without danger of leak the large pressure differences involved in the determination of a respiratory quotient.

SUMMARY.

The constant-volume differential manometer previously described by the authors has been applied to the measurement of respiratory quotient, respiration and glycolysis in bicarbonate media, with the help of the principle used by Dickens and Šimer [1931] in the earlier method for this purpose. Advantages of the new method are:

1. It is not necessary to use dense manometric fluids.
2. The sensitivity is increased above that of the previous method, so that for the same accuracy less tissue and a shorter experimental period may be used.
3. At the same time the bicarbonate reserve in the vessels may be greatly increased. This enables accurate measurement of the aerobic acid production of highly glycolysing tissue, for which the previous method gave low values.
4. The conditions for measurement of glycolysis are comparable with those in the Warburg two-vessel method; but the new method, like the other two described in this series, has the great advantage that the metabolism is measured on a single piece of tissue.

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REFERENCES.

Dickens and Greville (1933). *Biochem. J.* **27**, 213.
—— and Šimer (1930). *Biochem. J.* **24**, 905.
—— —— (1931). *Biochem. J.* **25**, 973.
—— —— (1933). Abderhalden's *Handbuch der biologischen Arbeitsmethoden*, IV, **13**, 435.
Dixon and Keilin (1933). *Biochem. J.* **27**, 86.
Needham (1932, 1). *Proc. Soc. Exp. Biol. Med.* **30**, 182.
—— (1932, 2). *Proc. Roy. Soc. Lond.* B **112**, 98.
Warburg (1924). *Biochem. Z.* **152**, 51.
—— (1926). Über den Stoffwechsel der Tumoren. (Springer, Berlin.)
—— and Kubowitz (1929). *Biochem. Z.* **214**, 7.