# C. CARBOHYDRATE METABOLISM OF NORMAL AND TUMOUR TISSUE.

## PART I. A METHOD FOR THE MEASUREMENT OF THE RESPIRATORY QUOTIENT.

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Metabolic measurements upon isolated tissues *in vitro* have attained a high degree of accuracy in so far as the consumption of oxygen, or the measurement of glycolysis, alone is concerned. However, when the measurement of $CO_2$ actually formed in the tissues is considered, the existing methods are found to be generally unsatisfactory. Since the whole significance of the respiratory quotient is dependent upon very slight variations in its numerical value, it is of the utmost importance that the accuracy of the determination of $CO_2$ should be as nearly as possible equal to that of the oxygen measurement. The methods in common use are as follows.

(1) The oxygen consumption of a piece of tissue is measured manometrically, the $CO_2$ being absorbed by KOH. Comparison of the readings with those obtained with another piece of tissue in a second manometer vessel without KOH allows the $CO_2$ to be calculated [Büchner and Grafe, 1924]. A differential arrangement may also be used, as in Thunberg's respirometer [Fenn, 1927].

(2) In order to overcome the difficulties due to insufficient buffering and at the same time to use a physiological concentration of bicarbonate in the medium, Warburg introduced his "improved method" [Warburg, 1926]. This method is based on the different solubilities of oxygen and $CO_2$, and the respiration is calculated from readings made with two vessels with similar pieces of tissue but with different volumes of fluid in each. It is of great value for the measurement of aerobic glycolysis but cannot be used for the true respiratory quotient, since the figure for $CO_2$ obtained includes the "extra $CO_2$" liberated from the bicarbonate by the production of acid, in addition to the respiratory $CO_2$. Referring to the estimation of true respiratory quotient in bicarbonate-containing solutions, Warburg [1926] states: "On account of the high physiological bicarbonate concentration it is difficult to estimate the increase of bound $CO_2$ with sufficient accuracy, and it is questionable whether this difficulty can be overcome by a differential procedure."

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(3) Recently Crabtree [1929] has described a method in which the "improved method" of Warburg is combined with estimation of chemically bound $CO_2$ by acidification before and after the experiment. The objection quoted above greatly limits the accuracy of this method, since the change of bound $CO_2$ necessary for calculating the R.Q. appears as a small difference between two large readings.

(4) Analysis of the gas-phase in equilibrium with the solution containing the tissue is the basis of a further method. The application of the catharometer to the problem has been described by Slater [1926]. The apparatus is necessarily complicated.

(5) A method has been described by Fenn [1928] in which the $CO_2$ is absorbed by barium hydroxide solution in a special vessel arranged for the measurement of electrical conductivity. The total $CO_2$ liberated may be calculated from the alteration of conductivity. The oxygen is determined by Thunberg's principle.

Leaving aside method 2, which is unsuitable and, in fact, was not designed for the measurement of true respiratory quotient, a fundamental objection is found to apply to the above methods. This objection is that no allowance is made for the variations in the $CO_2$ chemically bound in the tissue or the surrounding solution. Any change of the acid-base equilibrium of the whole system, due to production or consumption of acids or bases by the tissue, leads to the diminution or increase of the amount of $CO_2$ chemically bound. Such changes are included in the figure for $CO_2$ production obtained by the methods 1, 4 and 5. In practice, quantitatively the most important part of the error caused by neglecting the chemically bound $CO_2$ is due to the lactic acid produced by glycolysing tissues. In many cases this error is so large that it makes the figure for $CO_2$ valueless. Many figures for respiratory quotient much above unity which may be found in the literature are obviously due to the glycolysis of the tissues examined.

From the methods described, only that of Crabtree is theoretically free from this objection. Here, however, as was mentioned above, other practical considerations limit the accuracy.

For the measurement of true respiratory quotient the only remaining principle is that in which buffer solutions almost free from $CO_2$ are used. The bound $CO_2$ present in the tissue and solution at the beginning and end of the experiment is liberated by addition of strong acid and is included in the calculation. On account of this important advantage the method has been used by many workers [Warburg *et al.*, 1914, 1921, 1924; Meyerhof, 1919; Gerard, 1927; Schorr, Loebel and Richardson, 1930; Richardson, Schorr and Loebel, 1930]. A detailed description of an experiment is given by Richardson [1929]. The solution used is buffered by phosphate instead of bicarbonate. The figures for $CO_2$ are obtained from manometric measurement on two pieces of tissue in two separate manometers, one with KOH, the other with acid in a side-bulb, so that the latter can be mixed with the solution and tissue in the

main part of the vessel at the end of the experiment. The vessel with KOH gives the oxygen respiration, and this, together with the change of pressure occurring in the second vessel, permits the calculation of the $CO_2$ produced. A third manometer vessel is used to determine by acidification the initial $CO_2$ bound by the tissue and solution. The $CO_2$ content of the solution itself is determined in yet another vessel.

Valuable as the method is in certain selected cases, it suffers from an important defect which applies to all the methods where the $CO_2$ is determined from different pieces of tissue, already described under headings 1, 2 and 3. This is the implicit assumption that the extent of respiration, *i.e.* the volume of $O_2$ consumed per hour per unit weight of tissue, is exactly the same in two slices of the same tissue. Although the effect of such a difference on the respiratory quotient is not usually very serious, it is, nevertheless, desirable to remove the source of error. This is particularly the case when tissues with small respiration are concerned, and prolonged experiments are, therefore, necessary.

Another difficulty is due to the uncertainty of the amount of $CO_2$ retained by the tissue and phosphate during the period of temperature-equalisation preceding the experiment. During this period a portion of the respiration $CO_2$ is retained by the phosphate. In addition to this objection, in the case of glycolysing tissues an uncertain amount of $CO_2$ is driven off by the lactic acid formed. The most serious point is that all of these errors concern only the $CO_2$ without compensatory influence on the oxygen values.

With the above points in view, a method has been worked out so that the actual measurement could be made on a single piece of tissue[1], only one correction—a simple determination of preformed $CO_2$—being necessary. The accuracy of the results is not affected by glycolysis.

## Method.

*Principle.* Both oxygen consumed and $CO_2$ produced are determined by readings on the same manometer. The measurement is made on thin slices of tissue [Warburg, 1926] suspended in $CO_2$-free Ringer solution, buffered by means of phosphate and in equilibrium with pure oxygen. The $CO_2$ formed in respiration is absorbed by barium hydroxide solution. The diminution in pressure which occurs is used for the calculation of respired oxygen. After a definite time, an excess of strong acid is added, and the whole of the $CO_2$ is given up from the barium hydroxide, phosphate and tissue. The resulting positive pressure is used for the calculation of the total $CO_2$ at the end of the experiment.

This figure, corrected for the $CO_2$ content at the beginning of the experiment, gives the amount of $CO_2$ formed in respiration during the experimental period.

[1] The method described was worked out before that of Meyerhof and Schmitt [1929] came to our notice. Since their method, though based on similar principles, is solely concerned with muscle and nerve, the practical details in the two methods differ in many important points.

*Apparatus*[1]. The vessel (Fig. 1) used is attached by means of the ground joint to a Barcroft manometer. The total volume is about 20 cc. and is determined by weighing with mercury. The central portion *A* contains a piece of tissue suspended in 2 cc. phosphate-Ringer solution. The annular trough *B* contains 0·5 cc. of a cold-saturated solution of barium hydroxide. The side-bulb *C* contains 0·3 cc. of 2·5 *N* HCl, which can be tipped into the main compartments and the whole contents mixed when desired.

*Solutions. Stock sodium phosphate solution.* An isotonic solution of sodium dihydrogen phosphate and disodium hydrogen phosphate at $p_H$ 7·4 is prepared

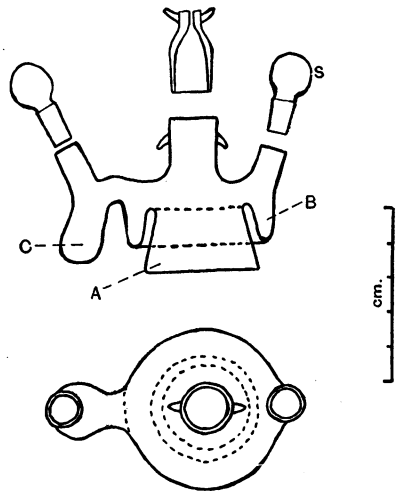


Fig. 1.

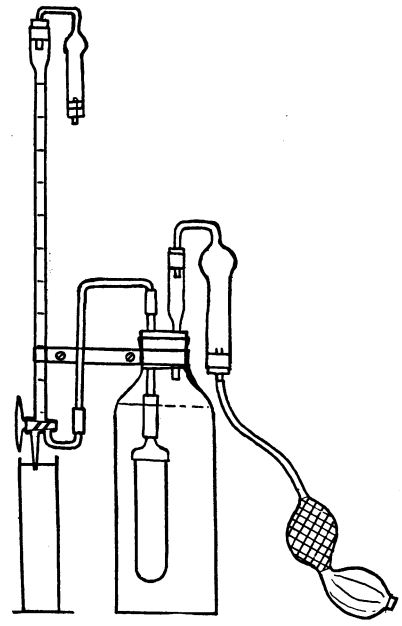

Fig. 2.

with the usual precautions to exclude $CO_2$. With the samples of dried phosphate used, the weights were 2·43 g. $NaH_2PO_4$ and 12·68 g. $Na_2HPO_4$ to 1 litre. Such a solution is about 0·109 *M* in total phosphate. This solution is stored in a burette protected from $CO_2$.

*Phosphate-Ringer solution.* The above solution is diluted for use by means of a suitable salt solution consisting of isotonic solutions of NaCl, $CaCl_2$ and KCl in the proportion 100: 2: 2 to which is added 10 % glucose solution to make the final concentration 0·2 %. The solution is boiled for 15 minutes and, after cooling in a stream of pure oxygen, is diluted to the original volume with $CO_2$-free water. The phosphate solution described above is then added. For tissues with little or no glycolysis 10 cc. phosphate per 100 cc. salt solution is

[1] The apparatus was made for us by Messrs C. Dixon & Co., 27 Devonshire Street, London, W.C. 1.

sufficient. For tumours, retina and other highly glycolysing tissues a more strongly buffered solution should be used. A mixture of 40 parts to 100 salt solution has a concentration of secondary phosphate equivalent to that of bicarbonate in the solution used by Warburg. It is particularly necessary to cool the solution very thoroughly before adding the phosphate, since the mixture is supersaturated with calcium phosphate. If the vessels are perfectly clean the solution usually remains quite clear.

*Barium hydroxide solution.* A cold saturated solution of barium hydroxide (*ca.* 0·3 *N*) is prepared and poured into the bottle of an automatic burette (10 cc.), fitted with a filtration candle (Berkefeld V) as in Fig. 2, $CO_2$-free air having been previously drawn through the whole system. The capacity of the bottle is *ca.* 500 cc. and the whole arrangement must be easily and quickly brought to the vessels when required.

*Procedure.* Three vessels are used in the example described (more if duplicate estimations are required. See protocol, p. 913). Another vessel, without tissue and containing the solutions only, is used as a thermobarometer to correct the readings for variations of temperature and barometric pressure during the experiment.

*Preparation of the tissue.* Thin slices are cut from the fresh tissue with the razor. As Warburg [1926] has shown, the thickness must not exceed a certain limiting value, in order to allow the adequate diffusion of oxygen, carbon dioxide and lactic acid in the tissue. The thin sections are rinsed for 5 to 10 minutes before the experiment by suspending in phosphate-Ringer solution through which a rapid stream of oxygen is passed. This precaution is found to give a greater uniformity in the amount of preformed $CO_2$ in the different slices. The oxygen used is freed from traces of $CO_2$ by passing through a long tube filled with soda-lime.

*Preparation of the vessels.* The vessels must be thoroughly cleaned overnight with strong chromic acid mixture and before use they must be washed very thoroughly to remove all traces of acid, and afterwards dried at 100°. 0·3 cc. of 2·5 *N* HCl is pipetted into the side-bulbs and 2 cc. of phosphate-Ringer solution are quickly added (the solution being run in from a pipette with two graduations) to the central portion of the first vessel. This vessel is immediately connected with its manometer and a rapid stream of $CO_2$-free oxygen is passed through and escapes around the loosely inserted stopper. The remaining vessels are similarly connected up, one at a time, and each of these in addition to the solutions contains an approximately equal piece of tissue. While the gas is rapidly passing, 0·5 cc. of barium hydroxide solution is measured into each vessel, the stopper *S* (Fig. 1) being momentarily removed for this purpose. The tip of the self-filling burette (Fig. 2) is protected by immersion in *N*/20 HCl until the moment of inserting into the vessel. If this operation is carried out with reasonable rapidity a constant and very low blank-value for the $CO_2$ content of the barium hydroxide solution is consistently obtained. The $CO_2$ content of the 0·5 cc. as determined by the control experiment should not

exceed 6 mm.$^3$ and there is no difficulty in reducing the correction to this figure or less. The 2 cc. of boiled phosphate-Ringer solution contain nearly the same volume of $CO_2$, this value also being very consistently maintained throughout our experiments. Since the total volumes of oxygen and $CO_2$ measured are of the order of 400 mm.$^3$, the error introduced by slight variations in the correction for the solutions is negligible, provided that, as a check, their values are freshly determined for each experiment. Hence the use of more elaborate precautions in order to reduce this correction further is in general unnecessary, and would much reduce the simplicity of the technique.

The stoppers of the vessels are now pushed home, and the gas-stream is at the same moment diverted by turning the upper stop-cocks of the manometers.

The manometers and vessels are now attached to the shaking-apparatus by means of which the vessels are moved backwards and forwards in the thermostat [Warburg, 1926]. It is important that the vessels II and III, containing the tissue, should be put into the bath together so that the respiration in both follows the same course. When the vessels are shaken the barium hydroxide solution moves freely in the annular trough without any tendency to splash over into the central portion. This point is important, as the shaking must be sufficiently vigorous (excursion 5 cm.; 100 oscillations to the minute), in order rapidly to establish equilibrium of absorption of gas. Owing to the large surface presented by the barium hydroxide and the rapid movement, which entirely prevents the formation of any superficial crust of carbonate, the absorption of $CO_2$ is extremely efficient and control experiments have shown that it is superior to that of the usual conical vessels containing potash as absorbent.

Ten minutes after inserting the vessels into the bath the first reading of the pressure in all vessels is taken. This period is sufficient for complete equilibrium and should not be prolonged. After taking this reading, vessel II is at once removed from the bath and the acid contained in the side-bulb is thoroughly mixed with the contents by repeated tipping. The vessel is then replaced in the bath. After a further 15 minutes another reading is taken, and from the observed small increase of pressure the total preformed $CO_2$ in solutions and tissue is calculated. Readings of pressure in vessel III are taken at intervals of 15 or 30 minutes throughout the experiment and so the oxygen consumption is followed. After a definite time, measured from the tipping of vessel II, the acid is tipped into vessel III immediately after a reading has been taken. The large positive pressure set up is used for the calculation of the total $CO_2$ in vessel III at the end of the experiment.

As a check that all the bound $CO_2$ has been liberated the mixing of the contents of both vessels is repeated. There should be no further increase of pressure as a result of this second mixing.

At a convenient time during the above manipulations, the acid is tipped into vessel I, containing the solutions without the tissue. The slight positive

pressure set up (a few mm. only) gives the correction for the $CO_2$ content of the solutions.

It is important that the amount of tissue used and the duration of the experiment should be so chosen that nearly the whole range of the manometer capillary (300 mm.) is used, if the greatest accuracy is to be obtained. With most tissues a period of 2–3 hours is sufficient.

At the end of the experiment the weights of the pieces of tissue are determined by removing from the vessels, thoroughly rinsing in a large volume of water, and drying to constant weight at 110°.

*Calculation.* From the above readings of pressure the following quantities are directly determined by simple multiplication by the vessel constants (see below). The actual exchange is determined from vessel III:

(*a*) total oxygen consumption;

(*b*) total $CO_2$ present at the end of the experiment;

readings (*b*) have to be corrected for the following:

(*c*) from vessel II the total $CO_2$ present at the beginning of the experiment;

(*d*) from vessel I, the $CO_2$ contained in the solutions used.

By subtracting (*d*) from (*c*) the amount of $CO_2$ originally present in the tissue, together with that formed by respiration during the initial period of 10 minutes, is given. The weights of the tissues being known, this correction can at once be applied to (*b*), its amount being directly proportional to the weights of the tissues. It is advisable that this correction should not exceed about 10 % of the whole volume of $CO_2$ in vessel III—this is always the case if the experimental period and weights of tissues are suitably selected.

The corrected value of (*b*) is the respiratory $CO_2$ during the period of the experiment. This value divided by (*a*) gives at once the respiratory quotient. The method of calculation for a typical experiment is given in detail in the protocol at the end of the paper.

*Notes.*

*Vessel constants.* The actual volume of gas given off or consumed (mm.$^3$) is calculated by multiplication of the pressure change observed (mm. Brodie fluid) by a constant whose value depends on the volume of the vessel and the nature of the gas. The calculation of the vessel constant ($k$) from the volumes of the liquid and gas ($V_F$ and $V_G$ mm.$^3$) at a temperature $T°$ (absolute) is [Warburg, 1926]

$$k = \frac{V_G \frac{273}{T} + V_F \cdot \alpha}{10{,}000},$$

where $\alpha$ is the Bunsen solubility coefficient of the gas given off or consumed.

The solubility of oxygen, which is in any case small, may be considered as being the same in Ringer solution as in the solutions used in the present method. The same is not true of the $CO_2$, the solubility of which is diminished by the

acidity and salt concentration of the solution obtained after mixing with acid. Since no value was available in the literature, the solubility was determined for this solution by the "first saturation method" of Austin *et al.* [1922], using the manometric Van Slyke apparatus for the estimation. The value found ($\alpha_{CO_2} = 0{\cdot}517$ at 38°: mean of four determinations) is inserted in the above equation for calculation of the vessel constants.

*Accuracy.* As both oxygen and carbon dioxide are determined manometrically on the same piece of tissue, the error in the actual readings (± 1 mm.) is negligible when the pressure is of the order of 250 mm. of Brodie's fluid. The error is, therefore, determined by the error in the corrections for preformed $CO_2$ due to tissue and solutions. The error due to variation in the $CO_2$ content of the solutions themselves has already been discussed. Provided the stated precautions in pipetting are observed, this error does not exceed 1 %, as we have satisfied ourselves by a large number of duplicate estimations extending over several months. In practice, therefore, the accuracy depends on the agreement between the preformed $CO_2$ per unit weight in the duplicate pieces of tissue (vessels II and III). The process of oxygenation of the tissue before the experiment tends to make this correction more uniform, but it is evident that the $CO_2$ present in the tissue and that formed by respiration in the first 10 minutes of temperature equalisation cannot be controlled as accurately as can the amount of $CO_2$ present in the solutions. On account of the removal of the more serious errors of previous methods, this correction becomes the determining factor in the accuracy of the present technique. However, it must be remembered that this quantity is itself a correction term the magnitude of which should not exceed about 10 % of the total $CO_2$. Consequently, if the error in the correction is as high as 20 %, the final error introduced will be only 2 %.

From the above considerations, the greatest error to be expected in the value of R.Q. is 0·02. A better agreement has been observed in a large number of experiments on the same tissue. These observations are included in a forthcoming paper (Part II). It is customary, in the literature of the methods discussed in the introduction to the present paper, to find the R.Q. expressed to the third decimal place. The above considerations show that such a procedure is entirely unjustified by any of these methods.

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## *Protocol.*

*Rat-brain cortex.* Adult female rat fed 24 hours before experiment.

*Solution.* Ringer containing 0·2 % glucose and 11 m.-mols per litre phosphate-buffer $p_H$ 7·47. Slices of tissue rinsed for 5 mins. in oxygenated solution. Duration of experiment: 3 hrs. Temp. 38°.

| Vessel no. | I | II | III | IV | V | VI |
|---|---|---|---|---|---|---|
| Total vol. (cc.) | 21·25 | 22·02 | 20·27 | 22·70 | 20·07 | 22·00 |
| Side-bulb | | 0·3 cc. of 2·5 *N* HCl in all | | | | |
| Main part | | 2 cc. of phosphate-Ringer in all | | | | |
| Tissue | – | + | + | + | + | + |
| Annular trough | | 0·5 cc. $Ba(OH)_2$ solution in all | | | | |

*Readings.* After 10 mins. in bath, readings of all manometers taken.

| Acid tipped in ... | Tip | Tip | No | No | Tip | No |
|---|---|---|---|---|---|---|
| Pressure changes at 30′ intervals corrected for | + 6 | + 14·0 Tip again | – 34·0 | – 25·0 | + 14·5 Tip again | – 44·0 |
| thermobarometer (mm. | — | + 0·5 | – 36·5 | – 24·0 | — | – 42·0 |
| Brodie fluid) | — | — | – 36·5 | – 25·0 | — | – 41·0 |
| | — | — | – 34·0 | – 23·5 | — | – 42·5 |
| | — | — | – 33·5 | – 22·5 | — | – 41·5 |
| | — | — | – 35·0 | – 24·0 | — | – 43·5 |
| | — | — | Tip | Tip | — | Tip |
| | — | — | + 206·5 | + 145·5 | — | + 248·5 |
| | — | — | Tip again | | — | |
| | — | — | — | — | — | + 0·5 |
| Dry weights of tissue (mg.) | — | 11·79 | 8·01 | 6·16 | 7·19 | 12·54 |
| Area of slices (mm.²) | — | 130 | 130 | 130 | 90 | 90 |
| Thickness (mm.) | — | 0·45 | 0·3 | 0·25 | 0·4 | 0·65* |
| *Calculation:* | | | | | | |
| $h_{O_2}$ | — | — | – 209·5 | – 144·0 | — | – 234·5 |
| $k_{O_2}$ | — | — | 1·54 | 1·754 | — | 1·693 |
| Total oxygen consumed (mm.³) | — | — | **323·0** | **253·0** | — | **431·0** |
| $h_{CO_2}$ | + 6·0 | + 14·5 | + 206·5 | + 145·5 | + 14·5 | + 249·0 |
| $k_{CO_2}$ | 1·674 | 1·832 | 1·678 | 1·892 | 1·66 | 1·831 |
| Total $CO_2$ (mm.³) | 10·6 | 26·6 | 346·5 | 275·5 | 24·1 | 456·0 |
| Correction for solutions | — | 10·6 | 10·6 | 10·6 | 10·6 | 10·6 |
| Preformed $CO_2$ in tissue (mm.³) | — | 16 or 1·36 per mg. | — | — | 13·5 or 1·88 per mg. | — |
| Correction for tissue (mean of II and V multiplied by weight) | — | — | 13·0 | 10·0 | — | 20·4 |
| Total $CO_2$ produced in mm.³ | — | — | **322·9** | **254·9** | — | **425·0** |
| R.Q. | — | — | 1·00 | 1·01 | — | 0·99 |
| $Q_{O_2}$ (oxygen: mm.³ respired per hr. per mg. dry weight) | — | — | – 13·4 | – 13·7 | — | – 11·4 |

* Although this piece of tissue was too thick, the value of R.Q. is unaffected.