IX. THE STUDY OF METABOLIC ACTIVITIES OF SMALL AMOUNTS OF SURVIVING TISSUES

By I. BERENBLUM,[1] E. CHAIN and N. G. HEATLEY

From the Sir William Dunn School of Pathology, University of Oxford

(*Received 29 October 1938*)

THE microrespirometer described in the previous communication is applicable to the measurement of metabolic activities of small amounts of surviving tissues (e.g. for histochemical studies) and of cell suspensions. In the present communication certain features of technique in micro-manometric studies of the metabolism of minute fragments of surviving tissue are described and consideration is given to special difficulties which arise in such studies.

Preparation of tissues for the experiment

The area of tissue slice which can conveniently be used in the microrespirometer is approximately 0·5–8·0 sq. mm. This size depends upon the metabolic activity of the particular tissue used and must be such as to give a gas exchange of not less than 0·3 μl. and not more than 3·0 μl. per hr.

The small slices are obtained by cutting a number of ordinary tissue slices from the fresh organ with a sharp razor in the usual manner. One of these, chosen for its thinness and uniformity, is then placed on a microscope slide and cut into small squares with a sharp razor. These tissue pieces are then washed from the slide into a small dish with the required medium and transferred from the dish to the respiration chamber by means of a very fine platinum spatula. Careful handling of these small tissue slices is essential if damage and consequent decrease in the rate of metabolism is to be avoided. The excess medium is removed with a fine capillary and a known amount of fresh medium is added by means of a micro-pipette.

In the case of suspensions of cells such as bacteria, yeast, blood cells etc., a known amount of the suspension is measured directly into the respiration chamber by means of a micro-pipette.

Cell damage caused by cutting

In tissue slices of very small size the ratio of cut surface to total volume is obviously higher than in large slices such as are ordinarily used in the Warburg apparatus. It might therefore be argued that the higher proportion of dead cells (due to the greater amount of cut surface) would result in lower metabolic values being obtained with small than with large slices.

This problem was investigated by comparing in the Warburg apparatus the rates of O_2 uptake of ordinary tissue slices and of similar slices cut up into small squares (as required for the microrespirometer), using liver, kidney and Jensen rat sarcoma for the purpose. The results summarized in Table I show that there is no significant difference between the two series. (The occasional low results with cut up slices of kidney are attributable to a peculiar clumping effect which sometimes occurs when several small pieces of kidney are shaken in the Warburg apparatus).

[1] Beit Memorial Research Fellow.

Table I. *Comparison between the Q_{O_2} values for whole and cut up slices of different organs (in Warburg apparatus)*

	Q_{O_2}*	
	Whole slices	Cut up slices
Rat liver	8·2	8·0
	9·5	9·3
	8·0	9·0
Rat kidney	17·5	16·0
	16·5	14·0
	18·0	16·3
Jensen rat sarcoma	10·0	9·5

* The figures represent average values in separate experiments, each figure being the mean of two or more determinations.

The unexpectedly good agreement between the values for whole and cut up slices is due to the fact that a certain proportion of the damaged cells on the surface is removed by the gentle agitation during the preliminary washing and that, though the amount of damaged cells is proportionately greater in the small pieces, the amount shaken off is also proportionately greater. Nitrogen determinations on samples of liquid used for the preliminary washing show that in the case of small slices at least 10% of the initial material is lost in the washings.

Diffusion

The rate of respiration of most tissue is known to be within wide limits independent of the oxygen tension. It has been shown in the previous communication [1939] that though no provision is made for a shaking arrangement such as that used in the Warburg apparatus, the values for O_2 uptake of homogeneous extracts in an atmosphere of O_2 are the same in the microrespirometer as in the Warburg apparatus, even with oxidative systems which are strongly dependent on O_2 pressure. In an atmosphere of air, however, low values are obtained. Therefore, as far as diffusion of O_2 through the liquid is concerned, it is evident that provided the tests are carried out in O_2 and not in air, the microrespirometer without any stirring can be used safely for the measurement of oxygen uptake of tissue slices. The limiting thickness of the tissue, however, requires separate consideration.

In the respiration chamber, that part of the tissue which touches the bottom does not come into direct contact with the O_2-containing medium, and under the most unfavourable conditions (i.e. when the whole of one surface is in close contact with the bottom of the cup) almost half of the total surface of the slice will be deprived of direct O_2 supply. It may be assumed, therefore, that the limiting thickness of the tissue which may safely be used in the microrespirometer is, even under the most unfavourable conditions, not less than half of that used in the Warburg apparatus. According to Warburg [1923] in an atmosphere of oxygen the maximal permissible thickness for liver slices is approximately 0·5 mm. and that for kidney slices 0·3 mm. Since it is possible without any special efforts to cut liver slices of 0·25 mm. and kidney slices of 0·15 mm. thickness, no special difficulties need be expected on account of diffusion through the tissue.[1]

[1] The diffusion constant of O_2 through different tissues is not necessarily identical with or even similar to the value for connective tissue of the gut [Krogh, 1919], which has hitherto been generally used in applications of Warburg's formula for the maximal thickness of tissues for metabolic work. For this reason the limiting thickness of any tissue should always be determined by empirical tests.

A comparison between the rates of O_2 uptake of tissue slices measured simultaneously in the Warburg apparatus and in the microrespirometer is given in Table II. The slices were cut without any special precautions, the thinnest and most uniform pieces being chosen for the experiments. (In several cases the thickness of the pieces was estimated from measurement of their areas under the microscope and their weights. No significant differences in the Q_{O_2} values were observed in the microrespirometer experiments with slices of liver varying in thickness from 0·1 to 0·3 mm.)

Table II. *Comparison between the* Q_{O_2} *values for rat liver and kidney obtained in the Warburg apparatus and microrespirometer*

Tissue	Q_{O_2} Warburg apparatus	Q_{O_2} Micro-respirometer	Weight of tissue used in the microrespirometer µg.
Rat liver	8·3	6·5	173
	8·5	7·1	157
	8·3	5·5	187
	9·2	6·5	208
	8·3	7·4	210
	Av. 8·5	Av. 6·6	
Rat liver	5·2	5·0	187
	5·2	5·0	175
	4·9	4·9	207
	5·3	—	—
	5·4	—	—
	Av. 5·2	Av. 5·0	
Rat liver	13·6	9·5	95
	12·6	9·5	101
	10·3	8·2	93
	10·8	9·4	75
	10·5	—	—
	Av. 11·6	Av. 9·1	
Rat kidney	19·7	18·8	33
	15·5	18·1	45
	—	19·8	24
	—	17·3	37
	Av. 17·6	Av. 18·5	
Rat kidney	17·3	17·7	79
	15·5	20·8	78
	—	15·4	89
	—	18·8	120
	Av. 16·4	Av. 18·2	

Table III. *Effect of oxidizable substrates on* Q_{O_2} *values of liver and kidney in the Warburg apparatus and in the microrespirometer*

Tissue	A Q_{O_2} in absence of substrate: Warburg apparatus	A: Micro-respirometer	B Q_{O_2} in presence of substrate: Warburg apparatus	B: Micro-respirometer	Ratio: B/A Warburg apparatus	Ratio: B/A Micro-respirometer
Rat kidney	18·6	16·6	35	27	1·88	1·63
Rat kidney	18·0	18·0	31	29	1·72	1·61
Rat liver	8·0	7·2	13	12·5	1·62	1·73

The substrate used was alanine in the case of kidney and succinate in the case of liver.

Table IV. *Rate of O_2 uptake of yeast suspension expressed as μl. O_2 per ml. suspension per hr.*

Comparison of values obtained in Warburg apparatus and in microrespirometer

Experiment	Warburg apparatus	Microrespirometer
1	170	153
2	200	178
3	213	253
4	136	147

The yeast suspensions were made up as follows: 15 ml. of 1–3% yeast suspension plus 20 ml. of 5% glucose plus 15 ml. of *M*/15 phosphate buffer solution.
Each figure represents the mean of 3–5 determinations carried out simultaneously.

The results show close agreement between the values obtained in the two instruments for the oxygen uptake of kidney slices, but with liver slices in most cases slightly lower values were obtained in the microrespirometer. This is attributable to the fact that liver tissue, being particularly friable, continues to shed dead and damaged cells during the course of the experiment, due to the shaking in the Warburg apparatus, whereas this occurs to a smaller extent with tissues such as kidney. Since these detached cells are not weighed after the end of the experiment, correspondingly high values are obtained with liver slices in the Warburg apparatus as compared with the microrespirometer. The amount of material actually detached was determined by nitrogen estimations of samples from the supernatant fluid in the Warburg vessels taken at different time intervals. It was found to account for approximately 15% of the total weight of tissue. If 15% is added to the Q_{O_2} values for liver obtained in the microrespirometer (Table II), the values approach those obtained in the Warburg apparatus.

For additional evidence in support of the contention that diffusion through the tissue is adequate, experiments were undertaken on measurements of rates of O_2 uptake of liver and kidney slices with and without oxidizable substrates (succinate for liver and alanine for kidney), the experiments being conducted simultaneously in the Warburg apparatus and in the microrespirometer. The percentage increase in the rates of O_2 uptake of the slices in the presence of substrate showed close agreement in the two instruments (Table III).

Experiments were finally undertaken with yeast suspensions, in order to determine whether, even under these stringent conditions (i.e. a marked tendency for the cells to settle and a very high Q_{O_2}), diffusion would still be adequate in the microrespirometer without a shaking system. Once again determinations were made simultaneously in the Warburg apparatus and the microrespirometer, and the results, shown in Table IV, show close agreement in the two instruments.

Determination of amounts of metabolizing material

(1) *Dry weight.* The metabolic values for tissue slices obtained by means of the Warburg apparatus are usually expressed in terms of unit dry weight. The same standard can be used for the small tissue slices required for the microrespirometer, provided the tissue is sufficiently homogeneous. At the end of the experiment the small tissue slice is carefully removed from the respiration chamber by means of the fine platinum spatula, placed in distilled water for a few seconds (to remove the salts of the medium) and transferred to a tared cover slip. This is dried in an oven at 110° for 1 hr. and reweighed. The ordinary Kuhlmann type microchemical balance can be used for determinations of dry weights of 80 μg. or over, but with smaller amounts the weighing error begins

to affect seriously the accuracy of the results. In such cases the Nernst-Donau balance [Donau, 1933] is indicated.

(2) *Nitrogen determination.* As an alternative to dry weight, the N content of the tissue can be used as a standard for expressing metabolic values. Like the dry weight standard, it is only applicable to fairly homogeneous tissues. Since methods of high sensitivity for N determination are now available, the nitrogen standard is applicable to very small amounts of tissue.

(3) *Cell counts.* This has an important theoretical advantage over the previous methods in that it represents a true measure of the respiring elements of the tissue only, so that the variable amounts of non-respiring elements (keratin, collagen, products of secretion etc.) in non-homogeneous tissues will not affect the final results. In practice the cell count standard is applicable to cell suspensions, for which it is probably the ideal method. For tissue slices the method is tedious and troublesome and complicated by the difficulty of making adequate corrections for the cut cells which are counted twice in serial sections. Such a method has, however, been successfully worked out for certain cases by Linderstrom-Lang *et al.* [1935].

(4) *Nucleic acid standard.* When using tissues such as skin, thyroid, brain, necrotic tumour, tissue culture etc., in which large and variable amounts of metabolically inactive material are present, the dry weight and nitrogen standards are unsuitable, since they do not differentiate between the cellular respiring elements and the inactive non-respiring material. The metabolic values, based on such standards, are not only abnormally low but also grossly irregular, since the proportion of living to non-living material in such tissues is liable to vary under different conditions of health and disease and from one animal to another.

The standard required for expressing metabolic activities of such tissues is one which differentiates cellular from non-cellular elements. The nucleic acid content of the tissues appears to answer this purpose.

The method which has been used by the authors consists of the estimation of nucleic acid phosphorus.

Method. After removal from the respiration chamber, the piece of tissue is placed in a small tube of about 5 ml. capacity, containing about 2 ml. of an alcohol-chloroform mixture (3 : 1), and heated for 2 hr. under a small reflex condenser similar to that described by Wasitzky [1932]; this extraction is repeated with fresh alcohol-chloroform mixture for a further 2 hr. The purpose of this is to remove lipoid phosphorus. The tissue is then extracted with $N/10$ HCl in the cold for 3 hr., to remove the inorganic and organic acid-soluble phosphorus. For efficient extraction, continuous agitation is essential, and this is conveniently carried out by mounting the stoppered tubes radially on a vertical disk which is slowly rotated by a motor. The tissue is finally ashed with perchloric acid and the phosphorus estimated by the micro-method of Berenblum & Chain [1938].

The method gives reasonably accurate results with amounts of nucleic acid phosphorus down to 0·2 μg., representing approximately 1·8 μg. of nucleic acid. Table V shows some values for the nucleic acid phosphorus contents of liver, kidney and brain obtained by this method. Fujiwara *et al.* [1937] found the nucleic acid phosphorus content of rat's liver to be 0·434 %, while Javillier & Allaire [1926] give the following figures for horse's tissue: liver = 0·204 %, kidney = 0·169 % and brain = 0·075 %.

Estimations of nucleic acid phosphorus were also carried out on special tissues with the object of examining the suitability of nucleic acid as a measure of cellular material.

Table V. *Nucleic acid phosphorus contents of liver, kidney and brain of the rat*

Tissue	Nucleic acid phosphorus content, expressed as % of dry wt.
Liver	0·27, 0·37, 0·38, 0·36, 0·32 Av. 0·34
Kidney	0·30, 0·29, 0·27, 0·31, 0·29 Av. 0·29
Brain	0·09, 0·14, 0·10, 0·12, 0·11, 0·11 Av. 0·11

The estimations were carried out on small pieces of tissue varying from 216 to 885 μg. in dry wt.

Table VI. *Nucleic acid phosphorus contents of tissues undergoing autolysis*

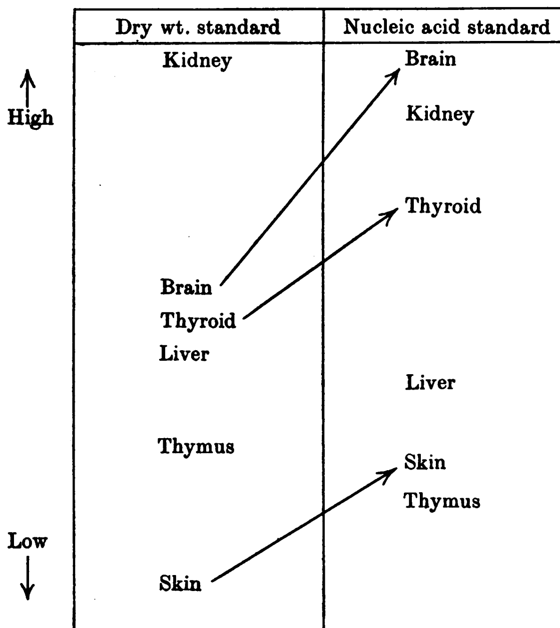
Tissue	Nucleic acid phosphorus content (as % of dry wt.)
Jensen rat sarcoma (non-necrotic portions)	0·70, 0·63, 0·84, 0·52, 0·70, 0·68 Av. 0·68
Jensen rat sarcoma (necrotic portions)	0·15, 0·18, 0·19, 0·24, 0·20 Av. 0·19
Liver (normal)	0·31, 0·35, 0·32 Av. 0·33
Autolysing liver* after 24 hr.	0·19, 0·15, 0·15 Av. 0·16
Autolysing liver* after 2 days	0·19, 0·16, 0·19 Av. 0·18
Autolysing liver* after 5 days	0·20, 0·17, 0·21 Av. 0·19
Tuberculous caseation from spleen of monkey	0·34, 0·37, 0·33, 0·34, 0·37, 0·37 Av. 0·35

* Thin slices of rat's liver floating in saline (with addition of a little toluene) kept at 37°.

Pure connective tissue, as exemplified by tendon of the rat's tail, was found to contain no significant amount of nucleic acid phosphorus. In order to investigate the rate of disappearance of nucleic acid following the death of a cell, three different examples of necrotic tissue were chosen for investigation, namely autolysing liver, the necrotic part of a tumour and tuberculous caseation. The results of these tests (Table VI) can be briefly summarized as follows.

The necrotic part of the tumour contained about one quarter of the nucleic acid of non-necrotic tumour; in autolysing liver the nucleic acid content fell off rapidly to about half of the original content and then remained fairly steady; in caseating material the nucleic acid content was found to be high.

Table VII. *Diagram showing order of the relative metabolic values* (Q_{O_2}) *of different tissues based on dry wt. and nucleic acid standards*



The results may be explained by the fact that the enzymes responsible for the disappearance of nucleic acid are themselves ultimately destroyed during the process of autolysis. In caseation, where coagulation of the whole cell content sets in quickly, the nucleic acid splitting enzymes are inactivated very early, so that the bulk of the nucleic acid remains intact.

The metabolic values, quoted in the literature [cf. Krebs, 1933], for slices of tissue, have hitherto been calculated in terms of dry weight. Since on this standard no allowance is made for inactive elements in the tissue, the values obtained cannot be expected to bear any relation to the true metabolic activity of the cells contained therein. This is borne out by the comparison between the relative metabolic values (Q_{O_2}) for different tissues based on dry weight and on nucleic acid standards (see Table VII).

From general considerations of the physiological functions of these organs and from their respective blood supplies in the intact body, the order of their metabolic activities would seem to correspond much more closely to that given by the nucleic acid standard than that based on dry weight.

Summary

1. Details of the cutting and preparation of small tissue slices, suitable for use in the microrespirometer, are described.

2. Diffusion of O_2 through the tissue was found to be adequate, provided the slices were cut reasonably thin.

3. In the Warburg apparatus tissue slices which had been cut into small pieces gave identical Q_{O_2} values with whole slices.

4. Comparative experiments on the O_2 uptake of liver and kidney slices, carried out simultaneously in the Warburg apparatus and microrespirometer, showed reasonably good agreement between the two, both in the presence and absence of oxidizable substrates. Thus, under suitable conditions, the effects of diffusion and of damage of the tissue due to cutting, are negligible.

5. Various methods for determining the amounts of metabolizing material are discussed. For tissues containing large amounts of inactive material (e.g. skin, thyroid, necrotic tumour, tissue culture etc.), it is suggested that the metabolic values be expressed in terms of nucleic acid phosphorus content. A convenient method for the estimation of nucleic acid phosphorus in small amounts of tissue is described.

Our thanks are due to the Medical Research Council for a personal grant and a grant for apparatus to one of us (N. G. H.), and to the British Empire Cancer Campaign for personal grants to two of us (I. B. and E. C.).

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