Rate of Respiration of Tissues in Contact with Oxygen.* (21022)

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Standard Warburg methods for the *in vitro* study of tissues involve suspension of the tissue slices, or of various types of tissue homogenates, in volumes of suspension fluid greatly in excess of the volume of the tissues. Many different solutions have been used with the aim of approximating the *milieu intérieur* so that the rates of respiration obtained might represent those in the intact animal. Quite different values are obtained with different solutions and it is difficult to know which, if any, represents the *in vivo* condition. Since suspension of tissues in a liquid results in dilution of metabolites to a level below the optimal extracellular fluid level, Krebs(1) has proposed a medium which makes a partial restitution of these metabolites. However, this solution does not compensate for dilution of hormones normally present or other of the many materials to be found in the cells of living tissues. On the other hand, the added material might stimulate the rate of tissue respiration beyond the normal *in vivo* rate. The uncertainties of the physiologic picture complicate the interpretation of *in vitro* pharmacologic investigations. Furthermore, a drug in a quantity which may produce a significant effect on the intact animal may be so diluted when the tissue is placed in a Warburg vessel that little or no effect can be observed. To demonstrate cellular effects the investigator must often resort to the addition to the suspension medium *in vitro* of forms and quantities of material it is hoped will duplicate conditions in the intact animal. There would appear to be need for a method for the quantitative assessment *in vitro* of the tissue actions of a drug administered *in vivo*.

This paper presents a method that meets this requirement by the use of specially-designed vessels in which the tissues are carefully spread out on fiber-glass mats to permit ready contact of gas with both sides of the slice. Such a method is so different from standard technics as to require critical evaluation. One test of the validity of a method of measuring tissue respiration is a comparison of the summated value with the BMR. Field, *et al.*(2) carried out a summation of tissue metabolism in the white rat which accounted for about 66% of the BMR. They felt this result indicated that the rates of tissue respiration *in vitro* bore a reasonable relationship to the rates *in vivo*. In the work that follows we have compared the respiratory rate of slices from the same organ on mats, in Krebs-Ringer-Phosphate (KRP) solution and in Krebs Medium III (KMIII)(1); and have assessed the significance of the results by summation calculations.

Methods. 1. *Apparatus.* When an attempt is made to study tissues directly in a gas phase it is difficult to assure adequate diffusion of gas to the under surface of the tissue slice. If a slice were to be cut thin enough to allow an adequate supply of oxygen from one side only there would be too high a proportion of damaged cells and the slice would be very difficult to handle. A solution to the problem is found by the use of an inert glass-fiber mat which supports the tissue effectively yet permits free access of gas to the under surface. Such a material is available from battery manufacturers under the name of "battery mat". This product is a feltwork about 0.5 mm thick of fine glass fibers bonded together with a small amount of starch. The starch may be removed by treatment with acid but we found no difference in respiratory rate after such treatment and the starch lends useful stiffness to the mats. Thin slices of tissue are spread out without folds or overlapping on tared ovals of mat cut with scissors to suitable size. Thereafter the tissues need not be touched. Such a mat and tissue may be forced into an ordinary Warburg vessel, as was done in preliminary experiments in this

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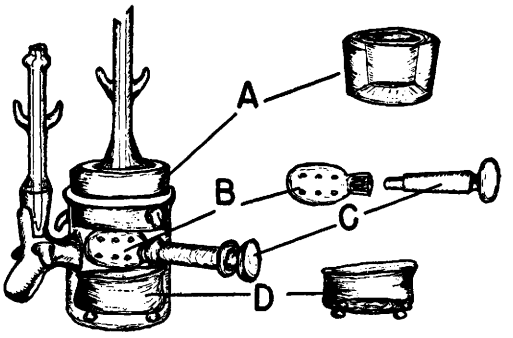

FIG. 1. A. Adapter to attach vessel to standard manometer. B. Paddle. C. Handle of paddle. D. Removable tray.

study. However, since it is practically impossible to keep the tissue spread evenly as is necessary for optimal respiratory exchange, a wide-mouthed vessel† was designed and is briefly described here (Fig. 1). The vessel, of about 19-ml capacity, is attached to a standard manometer by a glass adapter. A removable tray (D) with 4 small legs sets on the bottom of the vessel. A mat bearing the tissue is placed on a rotatable paddle (B) above the tray. When used for studying oxygen uptake, KOH solution is placed on filter paper on the bottom of the vessel and Ringer or other solution is placed in the tray to maintain normal vapor pressure. The apparatus can be used for standard procedure by placing the tissue not on the paddle but in the solution in the tray. The paddle was made rotatable so that the mat and tissue might be dropped into the solution in the tray. This is desirable for 2 types of experiment: (a) for measurement of the R.Q. Here the tissue is dropped into strong acid in the tray and the carbon dioxide which is released is absorbed by alkali formed by the interaction of KI solution from the side arm and $KMnO_4$ solution on the bottom of the flask, after the second method of Dickens and Simer(3); (b) to test the effect of dilution on drug action. Here the effect of a drug administered *in vivo* is determined with the tissue on the paddle and, after an interval, the tissue is dropped into a solution in the tray and the experiment continued but now with shaking. Significant dilution and diffusion of the drug would be detected by changes in the rate of oxygen consumption.

† Obtained from E. Machlett and Co., New York City.

2. *Procedure.* The animals used were male albino rats averaging 263 g body weight. The animals were killed by crushing the cervical vertebrae or exsanguination by heart puncture. The organs to be investigated were immediately removed in a moist cold chamber (0-5°C). Skeletal muscle, except diaphragm, was prepared by teasing out small groups of fibers. Diaphragm was excised *in toto* and pieces cut parallel to the fibers with scissors. Liver, kidney, and spleen were cut with a Martin slicer(4); brain and heart were cut through a template(5) with a razor blade. Pieces of intestine, including both mucosa and muscle layers, were taken from the small intestine beginning about 6 inches from the pylorus. The testes were cut with scissors. The tissues were spread out carefully on the fiber-glass mats, weighed and kept in moist Petri dishes in the cold chamber until all had been weighed. The mats were then placed on the paddles of the vessels which had been warmed to 37°C on a hot plate. Of each organ studied usually 2 samples were placed on mats, 2 in KRP and 2 in KMIII. The tissues not on mats were weighed on tared pieces of waxed paper and removed to the solution in the tray. The oxygen consumption was determined by the direct method of Warburg using a gas phase of oxygen. The determinations were carried out at a temperature of 37.9°C and at a shaking rate of 125 cycles per minute whenever tissues were suspended in solution in the tray, which normally received 1.5 ml of solution. CO_2 was absorbed by 0.2 ml of 5% KOH on a piece of filter paper on the bottom of the flask. The equilibration period in the water bath was approximately 15 minutes during which period it proved essential that all ground glass connections be thoroughly tightened. Readings were taken at 10-minute intervals for the first hour and at 20-minute intervals thereafter.

Results. QO_2. The rate of tissue respiration in artificial media usually declines to some extent with time. Since the tissues we used were kept in a cold chamber until all were ready to be placed in the vessels, the respiratory rate would be low until the vessels

TABLE I. Metabolic Levels *In Vitro*. Figures represent mean QO_2 values in ml O_2 per g wet wt per hr at time of setting and at 60 min. thereafter.

Organ	On mats					In Krebs-Ringer-Phosphate					In Krebs Medium III				
	No. of exps.	0′	s.d.	60′	s.d.	No. of exps.	0′	s.d.	60′	s.d.	No. of exps.	0′	s.d.	60′	s.d.
Brain	35	2.77	.42	1.24	.27	12	1.39	.35	1.04	.14	13	2.21	.56	1.92	.35
Heart	24	2.86	.45	1.82	.43	6	1.55	.17	.94	.34	12	1.81	.34	.85	.37
Intestine	20	1.38	.41	.92	.28	6	1.08	.26	.65	.18	12	1.35	.35	1.13	.24
Kidney	22	4.68	.64	4.08	.47	5	3.72	.49	3.54	.28	10	3.76	.58	2.75	.15
Liver	32	3.06	.48	1.95	.36	5	1.70	.20	1.59	.32	6	3.11	.36	2.43	.17
Skeletal muscle															
Sterno-hyoid, sterno-mastoid, sterno-thyroid	24	1.33	.29	.83	.23	7	.89	.19	.55	.17	15	.92	.19	.68	.15
Ext. oblique, int. oblique, rectus abd.	17	1.01	.22	.57	.26										
Caudofemoralis and semitendinosus	12	.98	.18	.49	.35										
Diaphragm	26	1.62	.32	1.42	.21	14	1.18	.17	1.18	.10	13	1.42	.15	1.34	.15
Spleen	25	1.56	.25	1.46	.15	19	1.34	.13	1.34	.13	8	1.85	.03	1.79	.12
Testis	27	.81	.09	.51	.01	6	.64	.08	.52	.07	14	.89	.16	.87	.19

and tissues were warmed to body temperature by the water bath. The tissues would be expected to return to their maximal respiratory rates at the conclusion of equilibration. The respiratory rates at this time were determined by straight line extrapolation of rates obtained during the experimental period. The results obtained with 9 tissues are recorded in Table I. So that it may be possible to compare rates of decline of tissues studied by the 3 methods we have included in the table the rates of respiration at 60 minutes.

A striking feature of the findings of this investigation is the remarkably high respiratory rates of many tissues on mats. These values are so much higher than those of equivalent tissues suspended in KRP as scarcely to require statistical examination. In comparison with tissues in KMIII the application of Fisher's "t" test demonstrates the following: (a) at zero time on the mats the rates are reliably higher for muscle, brain, kidney, and heart; the rates are the same for liver, intestine and testis; and lower only for spleen. (b) At 60 minutes on the mats the rates are reliably higher for muscle ($p = 0.05$), kidney and heart; and the rates are reliably lower for liver ($p = 0.04$), intestine ($p = 0.05$), brain, testis, and spleen. While the rate of decline of the tissues on mats is not excessive it is apparent that it is somewhat more marked than in KMIII.

pH. When living tissues are studied *in vitro* there is always a possibility of changes in pH. A series of experiments was therefore performed with brain, intestine, kidney, liver, and muscle in which the pH of each tissue was followed during the experiment. Serum exposed to the low CO_2 tension characteristic of the direct Warburg method progressively loses CO_2 to the KOH in the vessel and becomes strongly alkaline. Even though some serum and extracellular fluid accompany the cells of the tissue slice what changes took place were in the acid direction. Muscle and brain showed essentially no change in pH. Intestine and kidney showed a very slight drop, and liver showed the most change with a drop of approximately 1.5 pH units after one hour. The production of acid material by the respiring tissue was noted also when tis-

sues were run in fluid media. When control experiments were undertaken on the media in which no tissue was suspended, the well-buffered KRP showed little change of pH but the weakly-buffered KMIII became more alkaline. When tissues were suspended in the latter medium the pH increased slightly but did not become as alkaline as the controls. Since it has been shown by Canzanelli *et al.* (6) that the respiration rates of tissue slices are influenced by changes of pH it may be that a part of the higher values obtained with KMIII with some tissues is due to an increase in pH.

Summated tissue respiration. In order to apply summated tissue respiration as a criterion of the validity of these 3 modifications of the Warburg technic certain essential computations must be performed. We have followed the same general procedures as Field *et al.*(2); and for those tissues we have not reinvestigated, which account for less than 15% of the total summated respiration, have used their values of QO_2. The mean starved weight of the animals used was 263 g. The weights of the several organs were calculated after Donaldson(7), and Field *et al.*(2). In the case of skeletal muscle the figure of 45.4% of the body weight was used. Since it has been shown(8) that in the rat about 3.4% of the striated muscle consists of relatively inert connective tissue, the weight of skeletal muscle was reduced by this amount. Diaphragm, however, was not corrected for connective tissue. The BMR was calculated from Davis (9) using the values obtained for the sleeping animal. For a 263 g rat the oxygen consumption is 229.3 ml per hour. The QO_2 value used for skeletal muscle on mats is the unweighted mean of the values for neck, abdominal and leg muscles. In the case of skeletal muscle in KRP and KMIII only neck muscles were used. The figure for intestine was used for the entire alimentary canal.

The results of the tissue summations are contained in Table II. The total by our technic is 233.4 ml of oxygen per hour which is 101.8% of the BMR. The value in KRP is 180.4 ml per hour which is 78.7% of the BMR; and in KMIII is 207.9 ml per hour or 90.7% of the BMR.

Discussion. The technic of determining tissue respiration with the tissues on mats in an atmosphere of oxygen would appear to yield reproducible values of general physiologic importance. The observation that the respiratory rate of tissues can be measured without the introduction of the uncertainties of an artificial liquid medium, opens up the possibility of physiologic and pharmacologic investigations not previously practicable.

It is our feeling that the results obtained by the new method may, in many cases, be taken to be the most reliable approximation to *in vivo* conditions yet available since there is a minimal adjustment necessary to a foreign environment. No substances are added and none are lost excepting only CO_2 and some water. The O_2 tension is the same as that employed in the other usual technics. On the positive side there is evidence that the tissues are in a physiologic state. The pH changes are small and in the direction of tissue activity. The decline in respiratory rate is gradual and only slightly more marked than in the best standard technics. Despite the necessity for making certain assumptions the results of the tissue summation calculations are gratifying. Using KRP it was possible to account for 79% of the BMR which is somewhat higher than that reported by Field *et al.*(2). As might be expected the fortified solution proposed by Krebs(1) yielded a higher total which accounted for 91% of the BMR. This appears to us a rather satisfactory equivalency and lends support to the further use of this medium in experimental procedures where it is desirable to have the tissues suspended in a fluid medium. The summated tissue respiration calculated from the values on mats is the highest at 102% of the BMR and, to our minds, helps to complete the proof that the respiratory rate of tissues measured *in vitro* is of the same order of magnitude as when functioning in the intact animal. We do not feel that it is a cause for concern that the value comes to more than 100% of the BMR in view of the estimates necessary in the calculations and the uncertainty of methods of determining the proportion of metabolically active tissue in an organ (for a discussion see Martin and Fuhrman

TABLE II. Summated Tissue Respiration in the Rat.

Organ	Organ wet wt in 263 g rat	Field, *et al.*(2) QO_2	Mean O_2 consumption at time of setting manometers: On mats QO_2	On mats Whole organ	In Krebs-Ringer-Phosphate QO_2	In Krebs-Ringer-Phosphate Whole organ	In Krebs Medium III QO_2	In Krebs Medium III Whole organ
Alimentary canal	12.2		1.38	16.8	1.08	13.2	1.35	16.5
Brain	1.9		2.77	5.1	1.39	2.6	2.21	4.2
Heart	1.0		2.86	2.9	1.55	1.6	1.81	1.8
Kidney	2.2		4.68	10.3	3.72	8.2	3.76	8.3
Liver	12.4		3.06	38.0	1.70	21.1	3.11	38.6
Skeletal muscle	115.3		1.11	128.0	.89	102.7	.92	106.1
Diaphragm	1.7		1.62	2.8	1.18	2.0	1.42	2.4
Spleen	.7		1.56	1.1	1.34	.9	1.85	1.3
Testis	2.5		.81	2.0	.64	1.6	.89	2.2
Blood	15.3	.025		.4		.4		.4
Integument	47.3	.362		17.1		17.1		17.1
Ligaments	13.0	.070		.9		.9		.9
Lungs	1.5	1.15		1.7		1.7		1.7
Skeleton	15.5	.145		2.3		2.3		2.3
Remainder	20.5	.200		4.1		4.1		4.1
Totals	263.0			233.5		180.4		207.9
% of BMR	(229.3 ml/hr)			101.8%		78.7%		90.7%

(10)). The fact that the tissues on mats metabolize at a high rate may be due to more adequate oxygenation and preservation of catalytic substances at all stages of essential metabolic cycles.

It is not surprising that the rate of decline of brain respiration on mats is relatively marked in view of the well known ability of this tissue to exhaust its supply of carbohydrate rapidly. We therefore feel that the high initial value is sound and will be substantiated by other work.

Many investigators in the field of tissue respiration would agree with the comments of Bertalanffy and Pirozynski(11) in their concern over variations introduced by different media amounting to 2- to 3-fold. It may be that our technic offers a solution to some of these problems.

Summary. 1. A method is described for the determination of the respiratory rates of tissues in contact with oxygen by supporting them in the gas phase on fiber-glass mats in a modified wide-mouthed Warburg flask. 2. Investigated in this way the tissues maintained a satisfactory physiologic state as evidenced by slow decline in respiratory rate and negligible changes in pH. 3. For 8 rat tissues studied the respiratory rates on mats were considerably higher than those in Krebs-Ringer-Phosphate; and higher than those in Krebs Medium III in the case of muscle, brain, kidney, and heart, lower only for spleen, and not significantly different for liver, intestine, and testis. 4. Application of the respiratory rates to summated tissue calculations yielded the following values relative to BMR: on mats, 101.8%; in Krebs-Ringer-Phosphate, 78.7%; and in Krebs Medium III, 90.7%. 5. The utility of the technic using mats as a means of avoiding the variables due to different liquid media is discussed.

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