A MODIFIED METHOD FOR THE STUDY OF TISSUE OXIDATIONS*

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There are two common methods for preparing animal tissues for oxygen uptake studies; namely, the "slice technique" and the "mince technique." In the former method the tissues are cut into thin slices about 0.3 mm. thick, care being taken to damage the tissues as little as possible. In the latter method no precautions are taken to avoid damage to the tissues, which are either run through a mechanical mincer (1), cut into fine pieces with scissors (2), or even mashed with a bone spatula (3). Dixon and Elliott (1) stated that the same results were obtained by both the slice and the mince technique, but Elliott and coworkers (4) have since adopted extreme precautions to prevent damage to the tissue, and recommend the slice technique. These workers showed that in a majority of cases the oxygen uptake of minced tissue suspensions was much inferior to that of tissue slices.

The present communication describes a new method for the study of tissue oxidations, which involves (a) the rapid preparation of homogenized tissue suspensions by means of a simple apparatus, and (b) the measurement of the oxygen uptake of the suspension in various dilutions. The new technique for the preparation of the tissue suspension differs from mince techniques in that the tissues are broken down while in a buffer medium, and without contamination, inasmuch as the homogenizer is made entirely of glass. It is also possible to have no loss in temperature from the time the animal is killed until the suspensions are in the apparatus.

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EXPERIMENTAL

The Homogenizer\(^1\)—This device consists of a motor-driven pestle which fits into an ordinary Pyrex test-tube (150 × 16 mm.). The pestle is made from a piece of 6 mm. capillary tubing about 220 mm. in length. One end is sealed off and blown into a thick walled cylindrical bulb, whose longitudinal axis is about 20 mm., and which is rounded at the tip to fit the bottom of the test-tube. The sides of the bulb are straight and parallel to the sides of the test-tube for a space of 6 to 7 mm. The bulb can be shaped by placing it in the test-tube while it is still soft and blowing it out to fit the inside dimensions of the test-tube. While the bulb is still hot, about twelve small beads are fused into the tip by applying the tip of a molten glass rod. These small protrusions soon lose their rounded proportions and aid materially in cutting the tissue which is placed in the test-tube. A number of tubes can usually be found to fit the pestle. The fit can sometimes be improved with a little grinding, a fine grade of carborundum and some water being used, and the pestle being driven at a slow speed. The pestle is driven by a cone-drive stirring motor at a speed of 1100 to 1200 R.P.M. The amount of clearance between the pestle and the test-tube in one of our better homogenizers was determined. It was found that the difference between the diameter of the inside of the tube and the diameter of the pestle was 0.23 mm.

To prepare tissue for study, a measured volume of the desired buffer is placed in the homogenizer tube, which is then weighed. The animal whose tissue is to be studied is then killed by decapitation; the desired tissue is quickly excised and a small piece (1 to 2 gm.) is dropped into the buffer in the test-tube. The exact weight of the tissue is then obtained by again weighing the tube. By adding an appropriate amount of buffer, a suspension of any desired concentration can be obtained. In practise, the tissue is partially homogenized in about 4 cc. of buffer, the desired amount of buffer is then run in, and the homogenization is completed.

\(^1\) The homogenizer is a modification of a device used by Dr. Joseph Semb for the preparation of tissues for certain analytical procedures. The authors are also indebted to Dr. L. E. Clifcorn for his suggestions and assistance in the necessary glass-blowing. A similar device for the preparation of fine suspensions of tubercle bacilli has been recently described by Corper and Cohn (5).
During homogenization the test-tube is moved up and down, while the pestle is revolving at high speed. The tissue is torn apart by the protrusions on the end of the pestle and the fragments are kept below the pestle until reduced in size enough so that they can pass by the straight sides of the pestle, where additional grinding takes place. The suspension thus formed settles out very slowly and can be pipetted without difficulty. There are some small bits of connective tissue which tend to clog an ordinary pipette so that pipettes for this purpose are made with slightly enlarged tips. They are also recalibrated for blowout delivery. The apparatus has been used in the study of rat liver and brain, and chick brain, liver, and kidney tissue.

The oxygen uptake was measured in a Barcroft differential respirometer (2). Total volumes of both 3 cc. and 1 cc. were used. The latter volume is to be preferred when rapid uptakes are to be measured. CO₂ was absorbed by filter paper 3 cm. square, frayed at the end, and placed in the center cups in 0.4 cc. of 10 per cent KOH.

The suspension medium was an ~/30 phosphate buffer at pH 7.4, containing equal moles of sodium and potassium. The contents of the right and left flasks were identical with the exception of the tissue suspension which was placed in the right-hand flask, while a corresponding amount of buffer was placed in the left-hand flask.

**Dilution Effect**—A number of experiments have been carried out with tissue suspensions in concentrations varying from 20 to 200 mg. of tissue per cc. At concentrations over 180 to 200 mg. per cc. the viscosity of the suspension limits the rate of oxygen uptake.

It has been found that when a tissue suspension is diluted, in the absence of added substrate, there is a rapid fall in the oxygen uptake (per gm. of tissue) which is greater than the decrease in tissue concentration (see Fig. 1). This “dilution effect” has also been observed by Krebs (6) in deamination studies on kidney tissue. He observed that in brei which was diluted 4-fold (apparently 125 mg. per cc.) the oxygen uptake was still of the same magnitude as in slices, and that in higher concentrations the uptake sometimes surpassed that of slices. In lower concentrations the uptake decreased rapidly. We have observed the dilution effect in every tissue studied thus far. The relation between oxygen
uptake and tissue concentration is shown in Fig. 1. Representative dilution curves are given for rat liver and brain, and for chick liver and kidney. The oxygen uptake is given on the gm. basis. Thus, the dilution curve is a straight line when the uptake is pro-
portional to tissue concentration. The dilution of a tissue suspension cannot be regarded simply as a decrease in the concentration of a one component (i.e. tissue) system. If this were true, the oxygen uptake would, of course, be proportional to the amount of tissue present. A suspension of tissue is in reality a complex mixture of systems whose reaction orders are of the first, second, and probably higher order. It seems altogether probable that the dilution effect is a purely physical phenomenon; that is, a natural consequence of the dilution of systems involving two or more reactants. It is only when the reactions of higher order have been eliminated by dilution that one would expect the oxygen uptake to be proportional to the tissue concentration. This appears actually to be the case. When the tissue concentration has been diluted greatly, the only reactions of any consequence which remain are the first order reactions, whose initial rates are proportional to the concentrations of the decomposing compounds. When a suspension has been diluted to the point that oxygen uptake is proportional to tissue concentration, the uptake must represent the oxidations of autoxidizable compounds, and possibly oxidations which do not require activated oxygen and in addition are caused by enzymes which have an extremely high substrate affinity (such as xanthine oxidase).

A reaction of the second order can be reduced to a reaction of the first order by greatly increasing the concentration of one reactant. Likewise, a reaction of the third order can be reduced by increasing the concentration of one or two of the components. In the living cell, “vital phenomena” may be interpreted to some extent as a reduction of the reaction orders by increasing the concentration of various components in the system. In a tissue suspension without added substrate, increasing the tissue concentration causes the oxygen uptake due to binary and ternary reactions to increase exponentially. This increase would presumably continue until each enzyme was in combination with its substrate the greater part of the time, when the decomposition of the enzyme-substrate complex would become a pseudomonomolecular reaction, and become proportional to tissue concentration.

It can be seen from Fig. 1 that there are marked variations in the types of dilution curves for different tissues and there is also considerable variation between samples of the same tissue. There is a definite tendency toward proportionality at low concentrations in
rat liver and chick kidney and there seems to be a tendency toward proportionality in rat brain and chick kidney at the higher concentrations, as discussed above. A number of experiments with rat liver paralleled the experiment in which uptake was proportional to concentration at all levels studied. This may have been caused by a deficient storage of substrate in the tissue.

Fig. 2. The oxygen uptake of 44 mg. of chick kidney in 1 cc., with various amounts of succinate substrate.

By diluting the cytoplasmic constituents, the oxygen uptake can be decreased to the point where only the monomolecular reactions are occurring. This uptake is ordinarily quite low. If a particular substrate is now added to the suspension, it should be possible to determine the oxygen uptake due to the reaction between the substrate and the particular enzyme which activates it.
If the enzyme under consideration requires a coenzyme, the mere addition of substrate (without coenzyme) would not be expected to bring about the maximum reaction rate.

**Substrate Experiments**—Since succinoxidase does not appear to require a coenzyme (7), succinic acid was the first substrate to be used with the tissue suspensions. As can be seen in Fig. 1, the oxygen uptake of chick kidney is cut down to a marked degree at concentrations below 50 mg. per cc. Experiments with 0.11 per cent succinic acid showed marked decreases in rate of uptake for successive periods of time. Increasing the amount of substrate to 0.44 per cent gave virtually a constant rate of uptake, as is shown in Fig. 2. The oxidation in the absence of substrate was only 800 c.mm. of O₂ per gm. per hour (Qₒ₂ = 4), while the oxidation with succinate substrate was 6800 c.mm. of O₂ per gm. per hour (Qₒ₂ = 34). It should be borne in mind that the 800 c.mm. per hour do not represent the true oxygen uptake of the tissue in the absence of substrate, inasmuch as the tissue was at a high dilution. It is quite apparent that the tissue suspension represented a succinoxidase preparation of high activity. At the higher concentrations of substrate there is a tendency for the particles of tissue to clump and for this reason the substrate is generally used at concentrations of 0.3 to 0.6 per cent.

In the presence of an excess of succinic acid the dilution effect could not be demonstrated in any concentrations which were studied (see Fig. 3). The oxidation of succinic acid is apparently proportional to the amount of tissue present, as long as sufficient substrate is present. This is in accord with the concept that the rate of oxidation may be governed by the “decomposition” of one compound (succinoxidase-succinic acid complex). The fact that the oxidation of succinic acid appears to be proportional to the tissue concentration, while the oxidation in the absence of added substrate is not proportional to tissue concentration, leads to the obvious conclusion that substrate differences might be observed at one concentration and not at another. This fact would have considerable bearing on results obtained with the Thunberg technique and is being studied further in that connection.

Elliott and coworkers (4) have reported that minced rabbit kidney showed great diminution in respiration and was almost completely unable to oxidize substrates other than succinate. These
workers did not use various concentrations of tissue and consequently did not observe the dilution effect, although they did remark that the loss of the activity of the enzymes might be due to the dilution of the coenzymes by diffusion. Their results with

![Graph showing oxygen uptake of various amounts of tissue with different substrates. S = succinic acid, L = lactic acid, G = glucose.]

**Fig. 3.** The oxygen uptake of various amounts of tissue with different substrates. *S* = succinic acid, *L* = lactic acid, *G* = glucose.
minced tissue on no substrate seem unusually low in comparison with their results with slices, inasmuch as their minced tissue was used in fairly high concentration. This may be partly explained by the fact that their tissues were not strictly comparable, since cortex was sliced and whole kidney was minced.

Glucose and lactic acid were used as substrates in a few experiments. The uptake due to these substrates with various tissue concentrations is shown for typical experiments in Fig. 3. Although neither substrate is oxidized as rapidly as succinic acid, the lactic acid appears to be oxidized more rapidly than the glucose. It thus appears that in tissue suspensions glycolysis is the limiting factor in the oxidation of glucose. The coenzymes for glycolysis (adenyl pyrophosphate and Mg++) are probably in too high a dilution to be effective under the conditions of these experiments. In view of the fact that lactic acid dehydrogenase requires a coenzyme it is interesting to note that the uptake with lactic acid is as high as it is. The dilution effect was found with both glucose and lactic acid.

It seems entirely possible that the oxidation of glucose and lactate could be brought up to a maximum rate by the addition of appropriate amounts of the required coenzymes. In this way the tissue suspension could be used to study the limiting factors in the reactions which are brought about by the enzyme systems in the tissues, keeping the majority of the other components of the tissues inactive by dilution.

Boyland and Boyland (8) have recently pointed out that results with tissue slices represent at best a balance of conflicting factors. They state that, "The usual method of preparing tissue slices... not only produces mechanical injury but also facilitates diffusion from the cells of essential substances such as oxygen carriers, which may thus become diluted beyond their effective or optimum concentration. The smaller the pieces of tissue, the more likely this is. Yet if the tissue slices are not sufficiently thin it is impossible for them to have enough oxygen for their needs when suspended in vitro." The experimenter is thus faced with the task of preparing slices thin enough so that oxygen can diffuse in freely, while at the same time having the least possible outward diffusion of necessary respiratory catalysts, chiefly coenzymes. The new

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2 The L(+)-lactic acid was a gift from Dr. E. Tatum, Department of Agricultural Bacteriology, University of Wisconsin.
method of tissue preparation unquestionably permits free access to oxygen and results in a uniform distribution of the coenzymes, which appear to dialyze away from tissues to some extent in all methods of tissue preparation. It seems quite probable that previous low results with minced tissue have not been due to destruction of catalytic systems, but rather due to a dilution of the cell contents.

The new method is not proposed as a substitute for the slice technique, which is essentially an attempt to study tissues that are surviving in vitro and in which the concentration of the cytoplasmic constituents approaches that found in vivo. In surviving tissue slices, however, it is apparent that the reactions observed will be the sum total of a number of reactions all occurring simultaneously. It is felt that at this point the new method can be used to supplement the information given by the slice technique, since it facilitates the study of single enzyme systems, under greatly simplified conditions.

SUMMARY

1. A new method for the study of tissue respiration is described in which the tissues are homogenized in a buffer medium by a high speed glass pestle and studied at various dilutions by means of the Barcroft respirometer.
2. The "dilution effect," i.e. the lowering of the $Q_{O_2}$ which occurs when tissue suspensions are diluted, is shown in the case of rat liver and brain, and chick liver and kidney.
3. The applicability of the method to the study of succinoxidase is shown.
4. The possibility of studying other oxidizing systems by adding appropriate coenzymes to tissue suspensions is discussed.

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