36. A Critical Analysis of the Tissue Slice Method in Manometric Experiments

Effect of Variations in O_2- and CO_2-tension

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Tissue slices are very frequently used in manometric experiments for the determination of a variety of metabolic reactions of tissues. The interpretation of such experiments as physiological, i.e. as giving a true picture of the actual happenings in the intact organ or in the body, has been widespread and often unreserved.

In this paper an attempt is made to analyse the conditions prevailing in experiments with tissue slices *in vitro* and to assess how far one is justified in attributing physiological significance to such experiments. The use of minced tissue as compared with tissue slices is also examined from the same point of view.

1. EXPERIMENTS IN ABSENCE OF CO_2

Method

The experiments described in the first part of the paper were carried out at 38·5° using the ordinary (open) Warburg manometer with conical vessels. The fluid used was Ringer-phosphate (3·0 ml.). The inner cup contained 0·3 ml. 10 % KOH and a slightly protruding filter paper (Whatman's no. 40).

Substances to be added during the course of the experiment were tipped into the main part of the vessel from the side bulb, and were dissolved in Ringer-phosphate solution with adjustment of *p*H to neutrality where necessary.

If the gas had to be changed during the experiment (O_2 after N_2) the gas, 300–400 ml., was passed through the vessels and escaped through their stoppers while the vessels were shaken in the water bath. The gas was taken from a set of aspirator bottles in the same water bath and, therefore, had exactly the required temperature, thus allowing a new reading almost immediately after the vessels had been re-filled with the gas. The tissues used were obtained from the mouse and comprised slices of kidney and muscle, intact portions of diaphragm, or minced diaphragm or abdominal muscles.

Results

A. *Tissue slices and O_2-tension*

It was shown by Warburg [1923] that slices, in order to be evenly provided with O_2, must be thinner than $4{\cdot}7 \times 10^{-2}$ cm. in pure O_2 and thinner than $2{\cdot}1 \times 10^{-2}$ cm. in air. Since the difficulty of preparing slices is greater the thinner they are required to be, the respiration of slices was generally measured by him in pure O_2.

This method was generally adopted by other workers and all aerobic experiments on tissue slices have been carried out either in 100 % O_2 or in a mixture of 95 % O_2 + 5 % CO_2.

It has been shown previously [Laser, 1937] that although the magnitude of respiration of a variety of tissues in Ringer-bicarbonate and in presence of glucose was to a wide extent independent of the O_2-tension, the aerobic glycolysis on the contrary appeared or was increased under low O_2-tension. Stress was laid on the point that experiments carried out in 5 % O_2 represent more the normal O_2-tension of the body than experiments with 100 % O_2.

The rate of O_2-uptake of kidney slices without addition of substrate and in presence of sodium lactate ($M/50$) in 100 % O_2 is shown in Fig. 1. The shape of both curves is characteristic and always more or less the same. Sometimes, however, O_2-uptake in presence of lactate does not remain steady so long as 90 min., but falls off earlier. In absence of any added substrate (curve I) O_2 uptake always decreases rapidly and regularly at the beginning, the relative decrease however becomes less marked with time, i.e. the curve representing the rate of O_2-uptake flattens out. This slope of the O_2-uptake curve may be due to the following reasons.

(1) Tissue slices represent a heterogeneous population of cells, some of which being more differentiated than others may therefore be more labile and die at the beginning of the experiment. Generally however elimination of tissue cells from respiration through death cannot be made responsible for the particular slope of the curve for two reasons. First, if the number of dying cells increases at a uniform rate the curve showing the O_2-uptake of the remaining cells should decline linearly. Secondly, if the rate of dying cells increases with time, as would seem to be more likely, the curve should show an increasing fall with time. The actual shape of the curve does not agree with either of these assumptions.

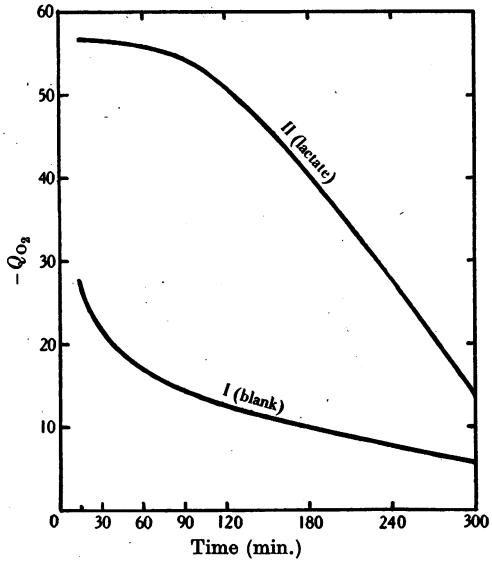

Fig. 1. Mouse kidney slices. Q_{O_2} without addition of substrate (I) and with sodium lactate ($M/50$) (II).

(2) The diffusion out of the cells into the surrounding medium of some substances which cannot be replaced by the cells but which are normally provided within the body. That some such diffusion takes place is obvious from the appearance of the medium even after experiments of comparatively short duration. These substances however have no relation to the rate of the O_2-uptake. If different amounts of tissue are suspended in the same amount of fluid, their rate of respiration is absolutely the same over long periods. It can be proved, however, that some oxidizable substances diffuse out, because fresh tissue slices shaken in a medium in which other slices have previously been kept for some hours start with a higher rate of respiration than their controls, but the relative shape of the curve is not changed.

That diffusion alone is not responsible for the decrease in the rate of O_2-uptake is shown also by the difference in relative decline of tissue respiration in presence and in absence of substrate. The rate of diffusion should be the same in both cases, and both curves, although lying at different levels should run parallel. This is however not the case, as shown by the experiment summarized in Fig. 1. Among other metabolic reactions, the aerobic acid production, however, is affected by diffusion of a substance out of the cells. This explains the results of Elliott & Baker [1935] who have shown that aerobic acid production of retina decreases in proportion to the increase in the amount of tissue suspended in a given volume of fluid.

(3) It is also conceivable that a substance essential for normal cellular metabolism may be used up rapidly at the beginning of the experiment in absence of added substrate. Experimental evidence for this assumption is summarized in Fig. 2. Here the Q_{O_2} of kidney slices is plotted as function of the O_2-tension at different times of the experiment. Q_{O_2} at the beginning of the experiment was gained by extrapolation from the first two

readings at 10 and 20 min. It will be seen that only for the first 7½ min. was the Q_{O_2} higher in 100 % O_2 than in 20 % O_2. Later the value of Q_{O_2} in 100 % O_2 decreased more rapidly than at lower O_2-tension so that after 90 min. it is actually smaller than in 10 % O_2. If each initial rate at the different O_2-tensions is taken as 100, then the % decline of Q_{O_2} increases with increasing O_2-tension (Fig. 3). This observation suggests that some substance is oxidized at the beginning of the experiment *in vitro* and that this oxidation is more rapid in 100 % O_2 than at lower tensions.

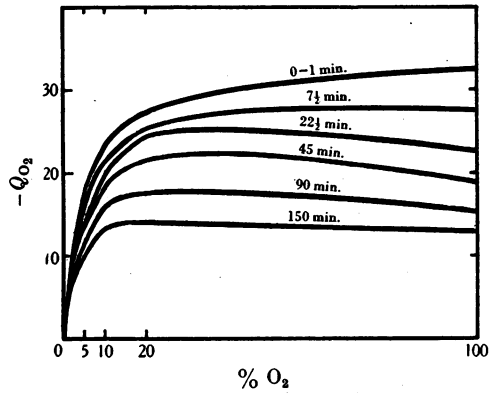

Fig. 2.

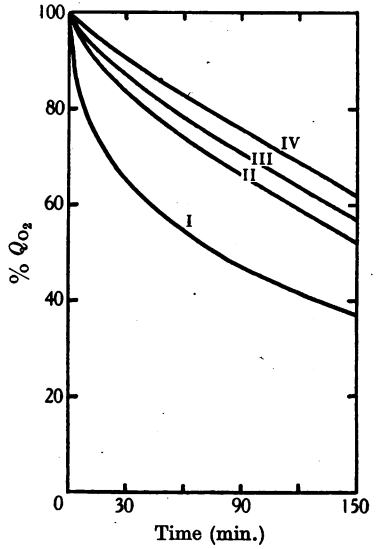

Fig. 3.

Fig. 2. Mouse kidney slices. Q_{O_2} as function of the O_2-tension at different periods of the experiment.

Fig. 3. Mouse kidney slices. Percentage decline of Q_{O_2} with time at varying O_2-tensions. Each *initial* value at the respective O_2-tension = 100. I = 100 % O_2; II = 20 % O_2; III = 10 % O_2; IV = 5 % O_2.

Q_{O_2} curves as function of the O_2-tension have been obtained with succinate and lactate as substrates in the same way as without substrate. To compare the three sets of curves they have been plotted in a different way (Figs. 4 and 5) to show the relative changes of Q_{O_2} which occur with time at varying O_2-tensions. Fig. 4 shows their relation at the beginning of the experiment and Fig. 5 the state at $t = 45$ min. In each case the respective Q_{O_2} in 100 % O_2 has been taken as 100. The different curves, therefore, represent the values of respiration under different O_2-tensions expressed as percentage of their respective Q_{O_2} in 100 % O_2.

At the beginning of the experiment (Fig. 4) the rate of succinic oxidation in 10 % O_2 is already 93 % of that in 100 % O_2, while the rate of lactate oxidation in 20 % O_2 is only 75 % of that in 100 % O_2. The curve representing the oxidation rate of the substances which are present in tissue slices and are being oxidized if no substrate has been added lies somewhere between the two former values.

Fig. 5 describes the corresponding oxidation rates for the period 30–60 min., i.e. it shows the state at $t = 45$ min. The curve involving the succinic system has remained unaltered while the two other curves have changed in opposite directions. The rate of lactate oxidation has now still more declined at lower O_2-tensions, while tissue slices without added substrate show actually a higher rate of respiration between 10 and 20 % O_2 than at 100 % O_2.

The O_2 affinity is a property of the enzyme which combines with O_2. It is conceivable that in the aerobic oxidation of lactate two independent systems may be involved: the cytochrome system and another system which has a much lower affinity for O_2.

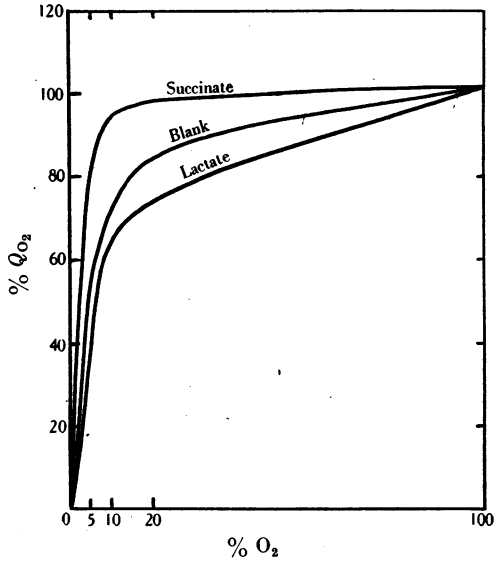

Fig. 4.

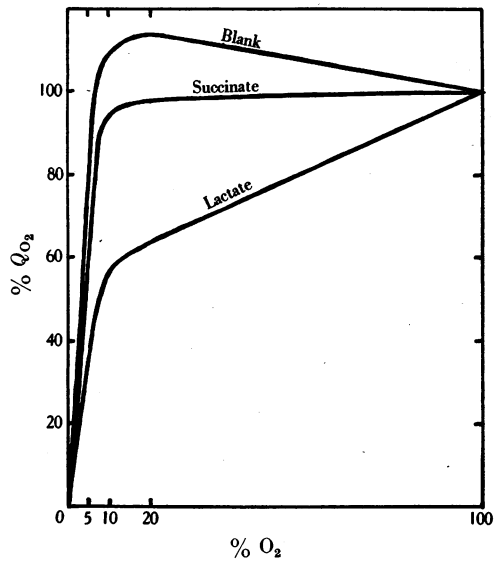

Fig. 5.

Fig. 4. Mouse kidney slices. Q_{O_2} at different O_2-tensions expressed as percentage of the Q_{O_2} in 100% O_2 (=100) without addition of substrate and with sodium succinate and lactate ($M/50$) at the beginning of the experiment.

Fig. 5. Mouse kidney slices. Same as Fig. 4 for the period 30–60 min., i.e. at $t=45$ min.

B. *The addition of substrates and time factor*

The O_2-uptake of tissue slices was also measured when different substrates were added after a preliminary period of respiration in absence of any added substrate. Several substrates and combinations of them have been tried such as glucose, sodium lactate, pyruvate, fumarate, citrate, succinate and boiled yeast extract.

The results of these experiments fall into three groups: (1) the O_2-uptake by the tissue after the addition of the substrate reaches the same rate which it has in presence of the same substrate added at the beginning of the experiment; (2) it reaches the rate of O_2-uptake of the control sample at the time of adding the substrate; (3) it remains lower than the rate of O_2-uptake of the control at the time of adding the substrate.

To the first category belongs only sodium succinate, i.e. the system catalysing its oxidation is not affected either by diffusion away or oxidation of one of the respiratory components during the time that tissue slices respire in absence of succinate (Fig. 6). To the second category belong sodium fumarate (Fig. 7) and pyruvate, citrate and glucose, additions of which give similar curves to those shown in Fig. 7. The activity of the systems catalysing their oxidation is noticeably weakened in tissue slices which have been kept for some time without the respective substrates; their activity declined to the same extent as that of the control samples in presence of substrates. Both oxidation rates are therefore equal after the substrates have been added. In the case of some of these substances however (fumarate and citrate) O_2-uptake in presence of the respective substrates had already fallen off so much by the time the substrates were added to the slices devoid of them that it needed only a slight rise in the oxidation rate

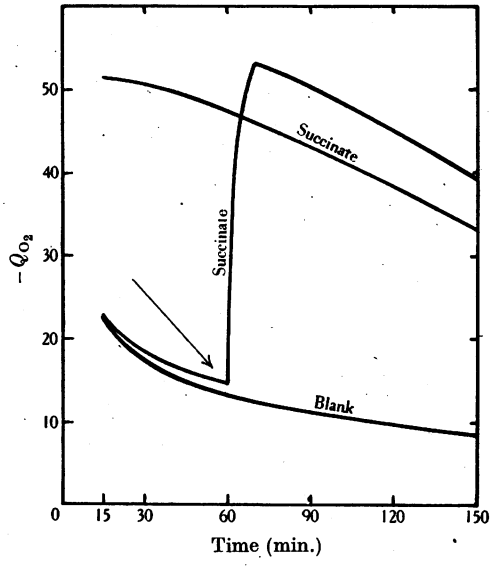

Fig. 6.

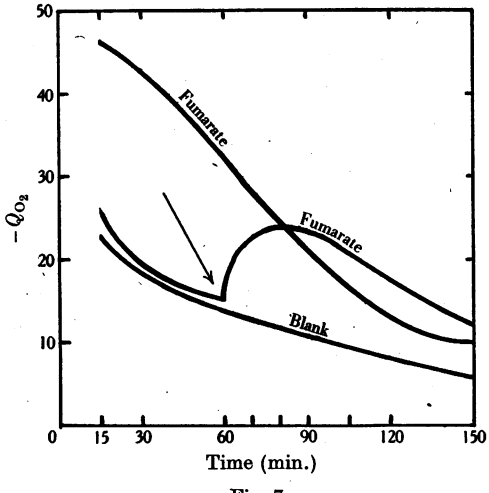

Fig. 7.

Fig. 6. Mouse kidney slices. Q_{O_2} on addition of sodium succinate ($M/50$) after 60 min. incubation in 100 % O_2 without added substrate, and two controls, i.e. without added substrate throughout (blank) and with succinate present from the beginning.

Fig. 7. Mouse kidney slices. Same as Fig. 6 with fumarate ($M/50$) as substrate.

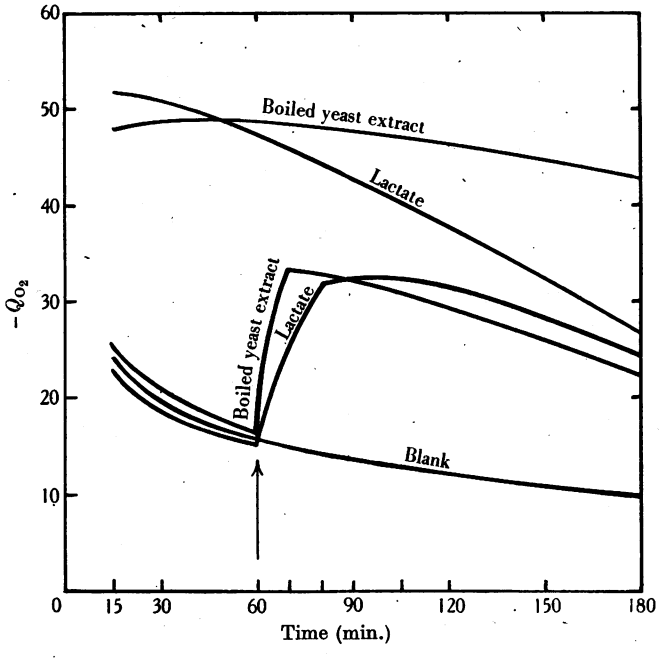

Fig. 8. Mouse kidney slices. Same as Fig. 6 with lactate ($M/50$) and with 0·2 ml. boiled yeast extract as substrates.

to reach that level. To the third category belong sodium lactate and boiled yeast extract (Fig. 8). The activity of the catalytic systems involved in these oxidations has been considerably reduced during the preliminary stage. On addition of these substrates, although the rate of oxidation is increased it does not reach the rate shown by the control slices at the same time. It is remarkable that the addition of boiled yeast extract did not raise the rate of O_2-uptake more than described above, since when added at the beginning of the experiment it not only raises the O_2-uptake considerably but keeps it at a steady rate for a considerable period of time (5 hr.).

Considering that no other substrates have been found to produce this effect, it seems safe to assume that this particular action of boiled yeast extract is not due to the presence of substrates in the extract but to an additional factor. It is not the presence of coenzyme I which causes respiration to remain steady because the addition of coenzyme either alone or in combination with sodium succinate, fumarate and lactate did not stabilize the rate of O_2-uptake. In this respect the tissue slices examined differ from muscle mince, the respiration of which can be stabilized by the addition of fumarate, especially in presence of small amount of tissue extracts [Stare & Baumann, 1937]. That a substance other than substrate or coenzyme is lost by the slices, owing to its oxidation could be proved by the following experiments.

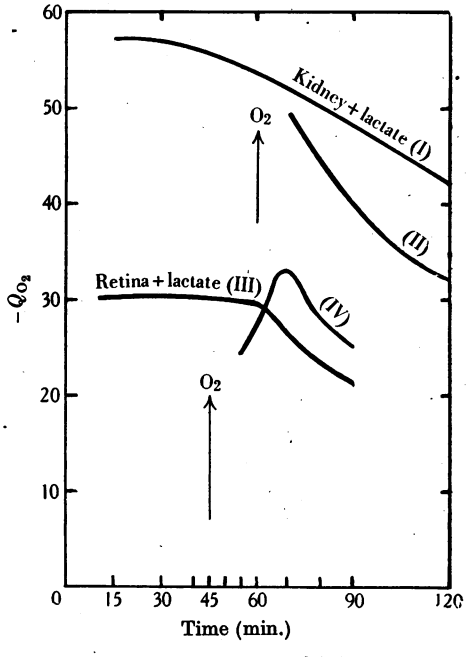

Fig. 9. Mouse kidney slices: I and II. Pig retina: III and IV. I and III: Q_{O_2} in presence of lactate in 100 % O_2. II and IV: preliminary incubation in presence of lactate for 45 and 60 min. respectively in N_2, when 100 % O_2 was admitted. Note higher rate of O_2-uptake after admission of O_2 than after incubation in O_2 and addition of substrate after 60 min. (Fig. 8).

Kidney slices and retina were shaken in the manometer vessels 45–60 min. in presence of sodium lactate in N_2, and at the end of this period O_2 was passed through the vessels (see Methods). It was then found that the O_2-uptake in these experiments was higher than that of tissues to which substrate was added after they had been kept in 100 % O_2 for the same length of time. In most cases under these conditions the type 3 of recovery of respiration due to the addition of substrate reverted to the type 2, e.g. it reached the rate of the O_2-uptake of the control at the time t' (Fig. 9).

C. *O_2 uptake of minced and ground tissues*

Minced tissues are capable of utilizing molecular O_2 without addition of any substrate and of oxidizing a number of substrates. In most cases the Q_{O_2} of minced tissue is considerably smaller than that of tissue slices. Their O_2-uptake is of about the same order of magnitude only if both are measured in phosphate buffer solution. This, however, is not the optimum medium for tissue slices, which require a balanced saline-phosphate solution containing Ca^{++} and Mg^{++} in addition to phosphates. The requirement of minced muscle in this respect has been described differently by different authors. While Stare & Baumann [1937] obtained optimal response on addition of fumarate in phosphate-Ringer solution, Meyerhof [1919; 1930] uses phosphate buffer solution as do Krebs & Eggleston [1938], who state that Ca^{++} and Mg^{++} have a depressing effect on the O_2-uptake of minced muscle.

The discrepancy between slices and mince is also very great in their ability to oxidize added substrates. I have confirmed the results of Krebs & Eggleston [1938] that many substances which are oxidized by tissue slices are not oxidized by mince.

But even with those substances which are oxidized by slices (or intact tissues) and mince there exists a great difference in the rates of their oxidation. Thus sodium succinate increases the O_2-uptake of intact muscle (diaphragm) by 100–150 % but that of muscle pulp by 700–800 %.

The respiratory activity of mince depends furthermore upon the degree of destruction of cellular structure. If the structure of the cells is thoroughly destroyed by grinding (with or without sand) the respiration becomes very low, in the case of muscle reaching only 4 % of that of the intact muscle. This very small oxidation has obviously no longer much in common with the normal mechanism of respiration, because such a strong respiratory inhibitor as HCN affects very little or not at all the O_2-uptake of ground muscle in absence of substrate or in presence of sodium lactate (Table 1). The oxidation

Table 1. *Percentage inhibition of O_2-uptake of intact diaphragm and ground skeletal muscle of the mouse*

Ringer-phosphate, 100 % O_2.

	No substrate added		+Lactate, *M*/50		+Succinate, *M*/50	
Inhibitor	Intact	Ground	Intact	Ground	Intact	Ground
Pyrophosphate, *M*/100	0	40	0	0	10–15	68
HCN, *M*/1000	80	0–20	80	0	80	60
Sodium azide, *M*/500	70	0	0–10	0	12–18	37

of sodium succinate, however, is inhibited by HCN in ground muscle only slightly less than in intact muscle. It must, however, be remembered that ground muscle oxidizes sodium succinate at a much higher rate than intact muscle. As regards other respiratory inhibitors, Dixon & Elliott [1929] showed that pyrophosphate acts as inhibitor of respiration of muscle pulp; Leloir & Dixon [1937] have also demonstrated that pyrophosphate acts as a specific inhibitor of succinic dehydrogenase. The action of pyrophosphate on respiration of intact and ground muscle (Table 1) is in agreement with these earlier reports. It may be added, however, that acting on intact muscle, *M*/50 pyrophosphate *increases* the O_2-uptake by 50–100 %. NaN_3, which inhibits strongly respiration of yeast cells in an acid medium [Keilin, 1937], was tested. In experiments with kidney slices it was found that *M*/500 NaN_3 inhibited respiration by 60–80 % at *p*H 6·8 and by 40–60 % at *p*H 7·3. The change in *p*H did not itself affect the rate of respiration of kidney slices with or without addition of substrate (glucose). The respiration of intact muscle without addition of substrate was 70 % inhibited by NaN_3. In presence of lactate or succinate it was inhibited by 10–20 % only. The O_2-uptake by ground muscle alone or in presence of lactate was not inhibited at all by sodium azide, but with sodium succinate the inhibition was greater in pulp than in the intact muscle. These muscle experiments were all at *p*H 7·3.

D. *The validity of measurements of* R.Q.

The R.Q. represents the sum of many complex reactions. Since, as has been shown above, considerable relative changes of the metabolism of tissue slices occur with time it becomes obvious that the R.Q. can only be determined with any expectation of physiological significance in experiments of comparatively short duration.

Of the commonly used manometric methods for the determination of the R.Q. (Dickens & Šimer [1930; 1931], Dixon & Keilin [1933], Sumerson [1939]), the method of Dickens & Šimer requires by far the longest time. Some of the experiments recorded by Dickens & Šimer took as long as 3–5 hr. This very long duration of the experiments

perhaps explains why their results have not been confirmed by Elliott & Baker [1935]. I too found for rat retina, with the method of Dixon & Keilin in a 45 min. measurement and in presence of glucose, an R.Q. of 0·8 instead of 1·0 as described by Dickens & Šimer.

Discussion

It has been shown in Part 1, above, that several distinct processes occurring in tissue slices show marked differences in their dependence on the O_2-tension at different periods of the experiment. Thus the respiration in absence of added substrate falls off much more rapidly in 100 % O_2 than at lower O_2-tensions. After 60 min. respiration under the respective O_2-tensions the rate of respiration is actually higher at an O_2-tension between 10 and 20 % than in 100 % O_2.

Secondly, the additional oxidations, as of sodium lactate, show also a change of their O_2-dependence with time, e.g. the additional O_2-uptake at the beginning of the experiment in 20 % O_2 is 66 % of that in 100 % O_2. After 60 min. under the respective O_2-tensions the additional O_2-uptake in 20 % is only 27 % of that in 100 % O_2. The dependence of the lactate oxidizing system on the O_2-tension therefore has changed with time in the reverse direction from the oxidation of those substances which are oxidized in absence of any added substrate.

While these are only quantitative differences in the way in which the magnitude of respiration and of additional oxidation differs with the O_2-tension and changes for each oxidative system differently with time, it cannot be excluded that qualitative changes may also occur similarly. In fact it is conceivable that under the influence of the O_2-hypertension (100 %) usually applied *in vitro*, some reactions are set in motion which under the conditions of the low O_2-tension within the body do not normally occur at all.

The next point to be considered is the stability of the different enzymic systems in tissue slices *in vitro*. The usually sharp decline of the O_2-uptake (except when boiled yeast extract has been added) after a variable period cannot be attributed to progressive death of tissue cells from considerations of the curve of O_2-uptake of tissue slices without addition of substrates. It must be attributed either to exhaustion or to an irreversible oxidation or to diffusion of some factor out of the system. Probably all three factors act together. It has been proved, however, that oxidation of some factor is at least partly responsible for the rapid decline, because in the case of sodium lactate oxidation starts at a higher rate after preliminary incubation in N_2 than the oxidation rate of slices which were kept for the same period in O_2. The instability of the different enzymic systems in absence of their specific substrates is also proved by the fact that, with the exception of sodium succinate, the rate of O_2-uptake never reaches that of the control in presence of the substrate at the beginning of the experiment, if the substrate is added at a later time. With sodium lactate and boiled yeast extract it does not even reach the rate of O_2-uptake of the control at the time of adding the substrate.

This fact raises the further question of how different systems in tissue slices are affected by the treatment which preceded the actual experiment, e.g. preparation of slices, time which elapsed before the beginning of the experiment, and the medium used during this period.

Mince shows still greater anomalies in respect of its suitability for the measurement of oxidative metabolism. The optimum oxidative power of tissue cells is dependent on their intact structure because this guarantees the biochemical pattern of the cell and the mutual accessibility and relative concentrations of the different catalysts. The discrepancy between slices and pulp in their ionic requirements necessitated the introduction of two subsidiary hypotheses to explain the conditions in mince: (1) the diffusion of catalysts and coenzymes out of minced cells; and (2) the effect of Ca^{++} and Mg^{++} which may reach intracellular enzymes of mince to which they have normally no access. The inability of muscle mince to oxidize many substances which are oxidized by the intact

muscle required two more assumptions: (1) the possibility that the enzymes in mince are already saturated with the substrates, or (2) that the activity of the enzymes concerned in mince is insignificant [Krebs & Eggleston, 1938].

Furthermore, the degree of cell destruction has to be taken into account, to assess how far the oxidative processes are still to be considered as normal. If the tissue cells are thoroughly destroyed by grinding, their respiration not only decreases very greatly (in the case of muscle to 4 % of that of the intact muscle) but it changes qualitatively as is shown by the fact that this small residual respiration is very little or not at all affected by HCN or NaN_3.

The conditions prevailing in mince become still more complicated when several organic and inorganic substances are allowed to act on it simultaneously and when the reactions occur in 100 % O_2, which alters to an unknown degree the relative velocity of several enzymic processes. The experiments become therefore dependent upon so many additional factors that their physiological significance declines in proportion to the number of variants acting on an already unphysiological material.

All that can be expected from experiments with muscle mince, therefore, is that they show one particular and well-defined effect under the particular conditions of the experiment, e.g. the catalytic action of fumarate [Stare & Baumann, 1937], or the catalytic action of citrate [Krebs & Johnson, 1937]; and only then can it be assumed with some degree of certainty that such might also be the case in the body.

Certain facts, therefore, have been shown which limit the use of muscle mince for the measurement of the oxidative metabolism. These restrictions however do not apply when washed muscle is used for the study of an isolated enzymic system or when muscle extracts are used for the study of carbohydrate breakdown.

The R.Q. is the outcome of a number of complex reactions. Each of these is, or may be expected to be, differently dependent on the O_2-tension and changes its O_2-dependence differently with time. It is obvious therefore that measurements of the R.Q. should be made within a comparatively short time.

2. EXPERIMENTS IN PRESENCE OF CO_2

All the experiments reported so far have been done with CO_2 absorbed, i.e. with a CO_2-tension of practically zero in the reaction vessel. It has long been recognized that under these conditions results are obtained which in some respects differ fundamentally from those which are gained on the same material when a bicarbonate-containing medium is used buffered by CO_2 in the gas phase [Alt, 1930; Ashford & Holmes, 1931; Dixon, 1934; v. Heyningen, 1935; Laser, 1935]. Lately the role of CO_2 has acquired new importance. Gladstone *et al.* [1935] found that certain aerobic bacteria were unable to grow when CO_2 was rigidly excluded from the medium. Wood & Werkmann [1938; 1940, 1, 2] have shown that propionic acid bacteria utilize CO_2 and have assumed a combination with pyruvic acid, forming oxaloacetic acid and, by reduction, succinic acid. Further data supporting this theory have been added by Krebs & Eggleston [1940] and by Evans & Slotin [1940; 1941]. Krebs & Eggleston found that CO_2-utilization was dependent on the CO_2 pressure, as had also been shown by Elsden [1938] for the formation of succinate by *B. coli*. Another task of CO_2 in tissue metabolism *in vitro* is the neutralization of basic metabolites by the formation of bicarbonate. Elliott & Schroeder [1934], Elliott *et al.* [1935; 1937] and Elliott & Greig [1937] have determined for a variety of tissues and substrates the appearance or disappearance of CO_2 according to the formation of either acid or basic intermediaries. They obtained the value Q_A (μl. CO_2 per mg. per hr.) which is positive when CO_2 is expelled, e.g. by glycolysis of tumours, or negative when bicarbonate is formed as with kidney in presence of sodium succinate or lactate.

In the following section experiments are described in which the influence of CO_2 on the metabolism of tissues is studied. The results are compared with those which were obtained in absence of CO_2.

The following terms are used throughout:

$-Q_{O_2}$ = μl. O_2 taken up per 1 mg. dry weight of tissue per hour.

Q_S = μl. CO_2 given out per 1 mg. dry weight of tissue per hour.

Q_A = μl. CO_2 given out by any acid formation (+) or taken up (−) by any acid disappearance per 1 mg. dry weight of tissue per hour. (In my experiments Q_A was not directly measured by bicarbonate determinations.)

$Q_{CO_2} = Q_S - Q_A$ = respiratory CO_2.

$Q_L^{O_2}$ = μl. CO_2 given out by lactic acid formation in O_2 per 1 mg. dry weight of tissue per hour.

R.Q. $= Q_{CO_2}/-Q_{O_2}$.

Method

Warburg's differential method [1924; 1925] was used. It was, however, found, if the Q_{O_2} was followed for several hours, that considerable irregularities often occurred in the values obtained. These could be traced to the dilution factor, e.g. to the fact that two lots of tissue slices, suspended in different amounts of fluid which is equimolar with regard to substrate, are exposed to different absolute amounts of substrate unless the ratio of their weights is the same as the ratio of the volumes of fluid in the two vessels. This was, however, difficult to effect and would, furthermore, have increased the experimental error in the vessel containing the small amount of fluid and tissue. Therefore the modified method, as described by Laser & Rothschild [1939], was applied in which the volumes of the pair of vessels differ (about 27 and 11 ml.) while the volumes of fluid (5 ml.) and amounts of tissue slices in each vessel are the same. It is very easy without the use of a torsion balance to select nearly equal portions of tissue for the two different vessels. In this way consistent results were obtained.

The fluid used was Ringer-bicarbonate made up according to Krebs & Henseleit [1932] and the gas 5 % CO_2 in O_2. Temperature 38·5°.

The tissue was mouse kidney and the substrates applied were sodium lactate, succinate, fumarate and pyruvate in final concentrations of $M/50$–$M/100$.

Results

Fig. 10 represents the O_2 uptake of kidney slices to which no substrate was added and shows the Q_{O_2} and the percentage decline of Q_{O_2} over a period of 4 hr. Both curves agree closely with those obtained when CO_2 was absorbed (Fig. 1, curve I; Fig. 3, curve I). The absence of CO_2 can therefore not be held responsible for the decline of O_2 uptake in absence of added substrate and the reasons discussed above for the particular shape of this curve must be considered to hold good also in presence of CO_2.

The shape of the Q_{O_2} curve was, however, considerably altered when substrates were added.

(1) In all the cases examined the initial magnitude of respiration was about the same as the corresponding one determined with absorption of CO_2 in Ringer-phosphate, but it remained much longer at the high initial level or at least decreased much more slowly (Figs. 11–13).

(2) It has previously been observed with all substrates examined (with the exception of succinate) that the rate of oxidation is much lower when the substrate was added after preliminary incubation without substrate than when the substrate was present from the beginning of the experiment. This effect was abolished in all cases of this series,

i.e. the O_2-uptake rose to the initial level of the control when substrate was added to tissues after preliminary incubation of 1 hr. in absence of substrate.

It must be concluded therefore that the relative absence of CO_2 during the preliminary incubation period, as described in Part 1, alters tissues irreversibly within a comparatively short time in such a way that at a later stage they are no longer able to oxidize different substrates at the same rate as they would have done, had they not been deprived of CO_2 during the preliminary period.

It has been seen that in presence of CO_2 the rate of O_2-uptake remains comparatively steady for some time. It remains now to be ascertained whether the O_2-uptake generally can be taken as an indicator for the 'physiological' behaviour of tissue slices during a certain period *in vitro* and whether a relative constancy of Q_{O_2} justifies the assumption that other reactions (only the end-products of which may have been determined) have occurred with a similarly constant rate over the same period of time.

If this were the case, then, the ratio $Q_S : Q_{O_2}$, and the R.Q., should remain constant at least as long as the Q_{O_2} remains constant.

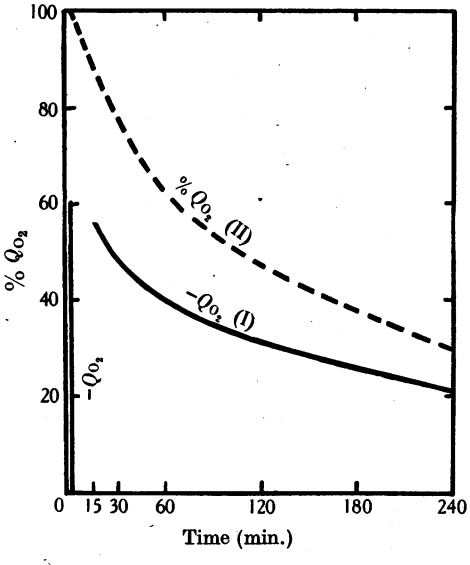

Fig. 10. Mouse kidney slices. $-Q_{O_2}$ (I) and percentage decline of Q_{O_2} with time (II) without addition of substrate.

Although the manometric method employed here can be adapted for the determination of R.Q. and Q_A [Laser & Rothschild, 1939], it has for technical reasons not yet been possible to do the many determinations which

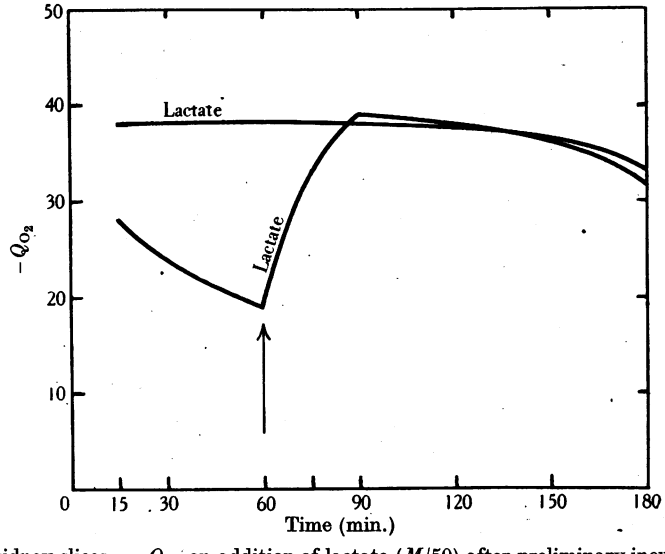

Fig. 11. Mouse kidney slices. $-Q_{O_2}$ on addition of lactate ($M/50$) after preliminary incubation for 60 min. in absence of added substrate and control with lactate present from the beginning.

would have been necessary with either this or one of the other manometric methods for the determination of the R.Q. in presence of CO_2.

The available data, however, for Q_{O_2} and Q_S allow some conclusions to be drawn as to the most likely variations of R.Q. and Q_A, if not as to their absolute values. They

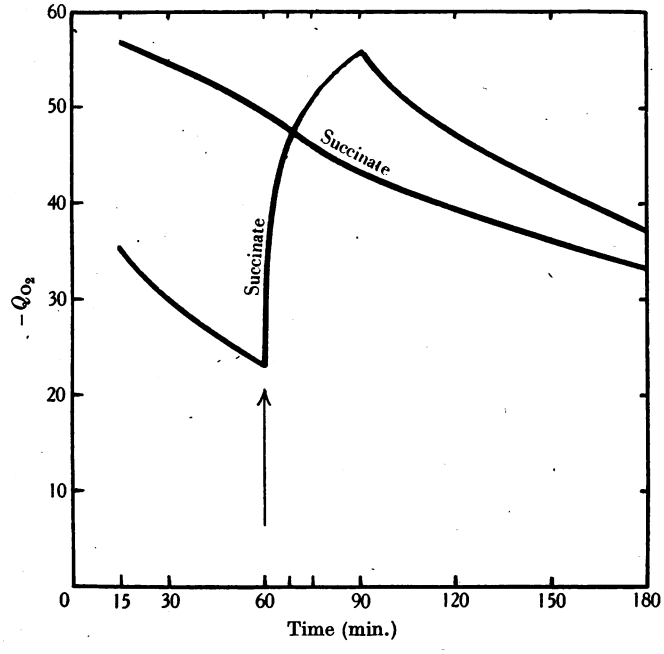

Fig. 12. Mouse kidney slices. Same as Fig. 11 with succinate ($M/50$) as substrate.

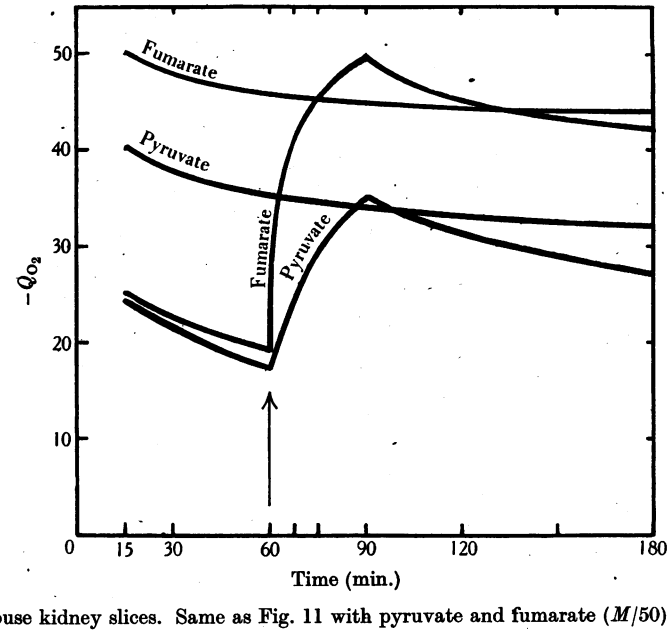

Fig. 13. Mouse kidney slices. Same as Fig. 11 with pyruvate and fumarate ($M/50$) as substrates.

indicate clearly that, in the case of succinate as substrate, of the two values, R.Q. and Q_A, at least one changes considerably with time.

In Fig. 14 Q_{O_2} and Q_S are plotted from the data actually obtained; and Q_A as calculated on the assumption of a steady R.Q. of 0·77 [Elliott *et al.* 1935]. The curves for Q_A and Q_{O_2} (Fig. 14) seem to show some similarity. A considerable divergence, however, becomes clear if they are plotted in a different way (Fig. 15), as percentages of their initial values obtained by extrapolation from the first two readings. The curves then demonstrate that the Q_{O_2} decreased by 52 % in 4 hr. while the Q_A decreased by 91 %. This means that at the end of the experiment while the tissue was still respiring with half its initial rate it had either practically completely lost its power to bind CO_2, for whatever purpose it utilized it at the beginning of the experiment, or the tissue had changed its metabolism in such a way that the participation of CO_2 was longer required.

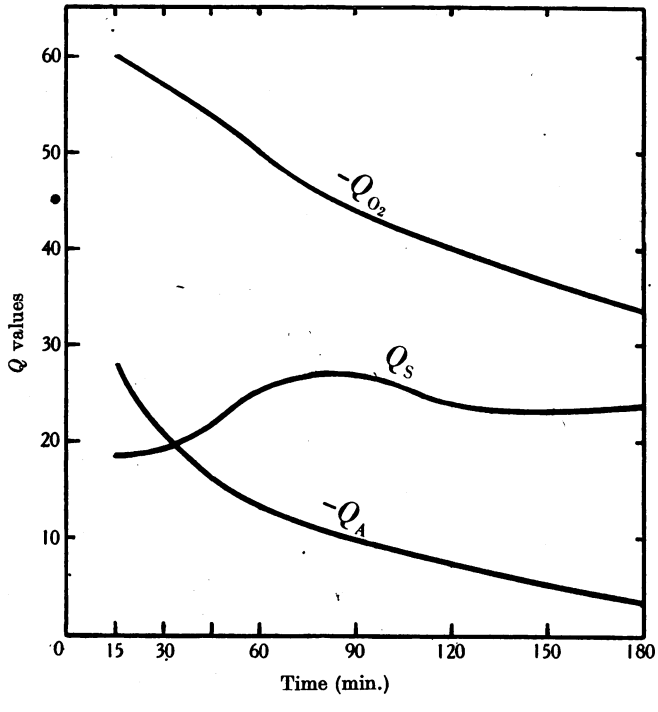

Fig. 14. Mouse kidney slices. Substrate: sodium succinate ($M/50$). $-Q_{O_2}$, Q_S and Q_A. (Q_A, calculated under the assumption of constant R.Q. = 0·77.)

This explanation, however, holds good only if the R.Q. is constant during the whole experimental period, for which there is no proof. The other alternative had therefore to be considered, namely, that Q_A remained constant, in which case $Q_S - Q_A = Q_{CO_2}$. In this way the potential R.Q. can be calculated for different periods. Fig. 16 shows one such hypothetical case where a constant value of $Q_A = -13$ was assumed (the value obtained for Q_A in Fig. 14 at $t = 60$ min.). The curve representing the R.Q. becomes then S-shaped, indicating a change of R.Q. with time. It is irrelevant with regard to the shape of the curve whether this or any other value of those shown for Q_A in Fig. 14 is substituted. The curve for R.Q. will always remain similarly S-shaped provided that Q_A remains constant; thus indicating a change of R.Q. with time: only the position of this curve will be either raised or lowered.

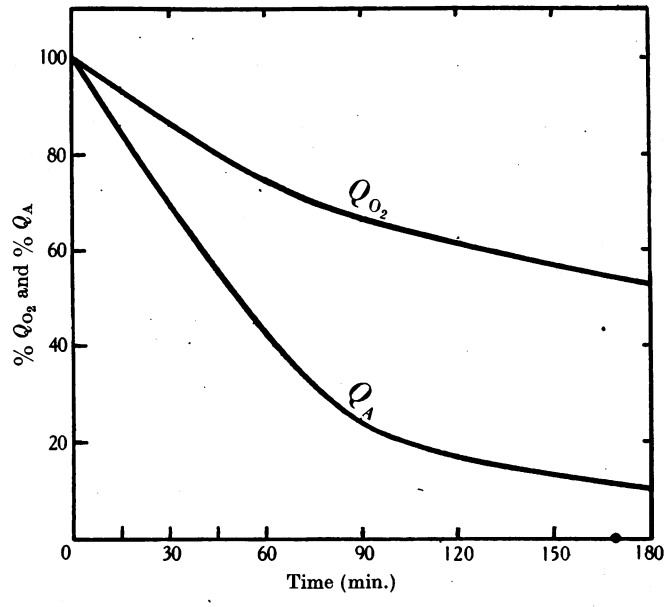

Fig. 15. Mouse kidney slices. Substrate: sodium succinate. Percentage decline of Q_{O_2} and Q_A with time. Q_A calculated as in Fig. 14.

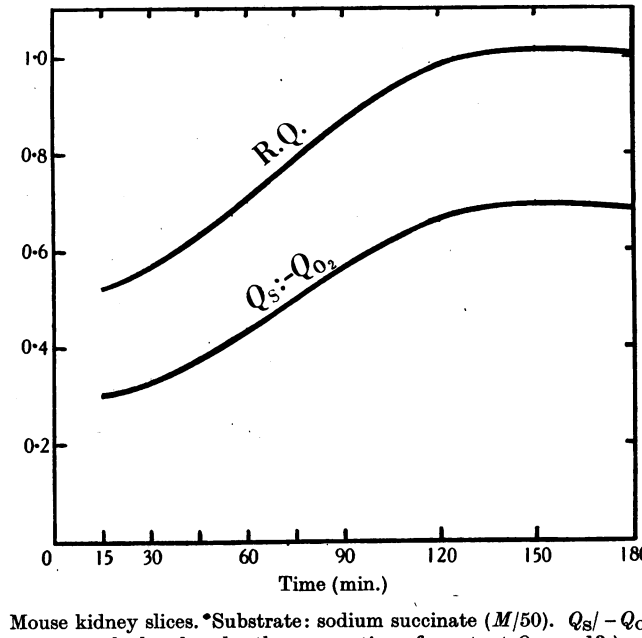

Fig. 16. Mouse kidney slices. Substrate: sodium succinate ($M/50$). $Q_S/-Q_{O_2}$ and R.Q. (R.Q. calculated under the assumption of constant $Q_A = -13$.)

Discussion

The main results of Part 2 of this work, concerning the oxidation of different substrates by kidney slices in presence of CO_2, are: (1) the initial rate of oxidation is much longer maintained or the falling off of its rate is much slower than in absence of CO_2; (2) if substrates are added after a preliminary incubation period of 1 hr. they are oxidized at

the same rate as if they had been present from the beginning. This is not the case if CO_2 is absent during the preliminary incubation period and denotes that a certain CO_2-tension is necessary for the reactivity of tissues to substrates.

It has, furthermore, been proved from the available data that, with succinate as substrate, of the two values, R.Q. and Q_A, at least one undergoes considerable changes during the course of the experiment. A steady R.Q. would result in comparatively great changes in Q_A, during the experimental period, i.e. in the power of the tissue to utilize CO_2. On the other hand, a comparatively steady Q_A would denote a change of R.Q. with time. In fact, it is most likely that the latter is responsible, for the following reasons: (1) added substrate may be oxidized to different end-products as time goes on, (2) the number of other substrates present in the tissues and the rate with which they are oxidized may also vary considerably with time.

There are no data available on the minimum CO_2 tension which would be required for the various processes described by Wood & Werkman, Elsden, Krebs & Eggleston, and Slotin & Evans. It can be assumed, however, that the required tension is comparatively low since in the experiment of Elsden [1938] the formation of succinic acid was only 50 % greater in 100 % CO_2 than in 5 % CO_2/N_2, and complete removal of CO_2 decreased the formation of succinic acid by only 13–40 % according to the different substrates. Similarly, in the experiments of Krebs & Eggleston [1940] the utilization of pyruvate was increased by only 37–94 % if 5 % CO_2 in O_2 was used instead of pure O_2. This indicates that the affinity for CO_2 of the systems in question is very high and that they are able to utilize rapidly CO_2 produced either aerobically by oxidation or anaerobically by fermentation, before it can be effectively removed.

It must be assumed, therefore, that in manometric experiments in which the CO_2 is absorbed, some CO_2 is available to the cells before it can be removed by the absorbent (e.g. KOH). This tension must, however, lie below the minimum required.

With regard to the part played by CO_2 in neutralizing basic metabolites *in vitro* by formation of bicarbonate, comparatively large amounts are required, with some substrates up to 50 % of the respiratory CO_2, and it seems certain that this amount is not readily available under conditions of CO_2-absorption. Even if the use of a phosphate-buffered Ringer solution prevented the medium from becoming alkaline, the *p*H inside the cells might under these conditions be considerably shifted to the alkaline side in the absence of glycolysis.

Since differences corresponding to the presence or absence of CO_2 exist in the metabolic rate of tissue slices, and since qualitative differences may occur depending on very slight differences of CO_2 tension, especially if this is very small, the determination of the R.Q. in tissue slices by the method of Warburg & Yabusoe [1924] may produce misleading results because O_2-uptake is measured in one vessel with KOH and this value is correlated with another obtained in absence of KOH.

The question of using serum instead of Ringer-bicarbonate has not been dealt with in this paper. It is known that some differences of metabolism occur according to which of the two media is used. I found that aerobic glycolysis ($Q_L^{O_2}$) of rat retina is 45 in Ringer-bicarbonate and only 25 in serum. Although it is tempting to assume that serum is a more physiological medium than Ringer-bicarbonate solution, serum differs in many respects from plasma. It is conceivable, therefore, that some of the physiological advantages of serum as compared with Ringer might be offset by some intrinsic effect of serum.

SUMMARY

O_2-uptake and CO_2-production by kidney slices in absence (Part 1) and in presence (Part 2) of CO_2 have been examined for different periods of prolonged experiments (4 hr.).

(1) In absence of CO_2:

(*a*) The shape of the Q_{O_2} curve in absence of added substrate shows a characteristic decline with time. The reasons for this particular shape of curve are discussed.

(*b*) The rate of O_2-uptake without added substrate declines with time more rapidly in 100 % O_2 than at lower O_2-tensions; thus the rate of respiration after 60 min. is higher at O_2-tensions between 10 and 20 % O_2 than in 100 % O_2.

(*c*) The rate of O_2-uptake in presence of added substrate expressed as function of the O_2-tension changes with time in a particular way for each substrate.

(*d*) The rate of O_2-uptake when substrates are added after preliminary incubation for 1 hr. in absence of added substrate is lower (except in the case of succinate) than the Q_O of the control in presence of substrate at the beginning of the experiment and, in some cases, lower than the Q_{O_2} of the control at the time of adding the substrate.

(2) In presence of CO_2:

(*a*) The shape of the Q_{O_2} curve without added substrate is the same as in absence of CO_2.

(*b*) The rate of O_2-uptake in presence of substrates is either constant for several hours or falls off much less than in absence of CO_2.

(*c*) The rate of O_2-uptake when substrates are added after preliminary incubation without substrate for 1 hr. becomes in all cases practically the same as the Q_{O_2} of the corresponding control at the beginning of the experiment.

(*d*) Analysis of the data obtained for Q_{O_2} and Q_S reveals that of the two values, R.Q. and Q_A, at least one undergoes considerable changes with time.

The main conclusions, therefore are:

I. Maximum activity of tissue slices and reactivity to substrates in absence of CO_2 are maintained only for a comparatively short time.

II. The presence of CO_2 stabilizes the rate of O_2-uptake of tissue slices in presence of substrate for several hours and safeguards their ability to oxidize substrates which are added after incubation in absence of substrate.

III. A comparatively steady Q_{O_2} does not indicate a similar steadiness of other reactions (only the end-products or final figures of which have been determined) occurring during the experimental period.

IV. The results summarized under (1) and (2) make it obvious that the R.Q. can only be determined with any expectation of physiological significance in experiments of short duration (e.g. 15–30 min.) and under conditions of equal CO_2-tension in all the vessels necessary for 1 determination.

V. Significant quantitative and qualitative discrepancies exist between the oxidative powers of untreated tissue mince (or pulp) and tissue slices which limit the use of mince as a physiological material in the study of the oxidative metabolism of tissues.

VI. The variations of the catalytic activity of different oxidative systems with time and with alterations in O_2- and CO_2-tension, the decreasing ability of tissue slices with time to utilize CO_2 and the—mostly uncontrollable—variations in interaction of different enzymic systems with time, which result in changes of the R.Q., ought to be considered in each particular case when assessing the degree of physiological significance which can be attached to experiments with tissue slices *in vitro*.

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