CCXXI. FURTHER OBSERVATIONS ON THE OXIDATION OF LACTIC ACID BY BRAIN TISSUE.

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(*Received October 30th, 1931.*)

In an earlier communication [Holmes and Ashford, 1930], we reported experiments bearing upon the question of the "Meyerhof cycle" in brain tissue. If lactate is added to brain tissue *in vitro*, and the system thoroughly oxygenated by shaking in a Barcroft's apparatus, or otherwise, it is easy to show that the amount of such added lactate which disappears is far greater than can be accounted for by the additional oxygen taken up by reason of the presence of the lactate. The relationship $\frac{\text{extra lactic acid disappearing}}{\text{lactic acid equivalent of extra } O_2}$ (the "Meyerhof quotient") has a value of about 3·0. We did not succeed in demonstrating any synthesis of carbohydrate; and such a synthesis is required by the hypothesis advanced by Meyerhof, Warburg and their collaborators.

In this paper, experiments are described which substantiate the validity of our previous findings and amplify them in certain important particulars. In the first place, the experiments reported in our first communication on this subject were all performed in a medium which was buffered by phosphate. The experiments have been repeated, therefore, using the CO_2-bicarbonate buffer, but avoiding the manometric method of Warburg, which involves working with very small amounts of tissue. We have investigated more fully the respiratory quotient of the tissue, both in the presence and in the absence of added lactate, and we have endeavoured to trace the fate of the added lactate which disappears.

Section I.

Experiments carried out with bicarbonate-CO_2 buffers.

Since we desired to follow the disappearance of lactic acid by chemical, and not by indirect manometric methods, it was necessary for us to work on a fairly large scale, and to employ at least 2 g. of tissue. At the suggestion of Prof. J. B. S. Haldane, we decided to estimate the oxygen consumption by means of the gas analysis technique.

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For this purpose two flasks with wide necks, and of approximately 50 cc. capacity were obtained and arranged as shown in Fig. 1. The flask and attachments were carried on a wooden carriage, which was so shaped that it could be clamped into the mounting of the Barcroft thermostat and shaken like an ordinary Barcroft manometer. The neck of the flask was marked with a diamond at the point reached by the lower end of the rubber bung, and the volume of the whole apparatus, with tubes in place, accurately determined. The apparatus was made and used in duplicate.

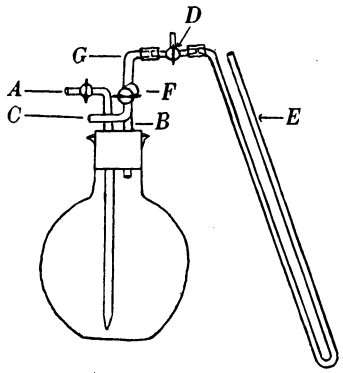

Fig. 1. Diagram (not to scale) of flask with stopper in place. Wooden carriage omitted. *A*. Gas entrance tube. *B*. Tube through which gas escapes during filling, and through which samples are subsequently withdrawn. *C*. Tube through which mercury is expelled during taking of sample. *D*. Three-way tap by means of which flask may be connected to air or manometer. *E*. Mercury manometer. *F*. Two-way tap enabling *B* or *C* to be connected to *G*. All glass tubes and taps are of 1 mm. capillary tubing.

The experimental procedure was as follows.

2 g. of chopped (rabbit's) brain tissue were weighed out, and placed in each flask. 5·0 cc. of Ringer's solution containing 0·025 *M* sodium bicarbonate [Warburg, 1926] was placed in one flask (*OP*) and 5 cc. of a similar bicarbonate Ringer, containing in addition 0·5 % lithium lactate, was added to the other (*OL*). The mass of tissue was broken up with forceps, the stoppers put in place, and the bottles were placed in the carriages. About 4 litres of a CO_2/O_2 (approx. 5 % CO_2 in O_2) gas mixture were bubbled through both bottles, which were arranged in series. Tap *A* was then closed, tap *D* turned so as to connect the bottles to the manometers, and the bottles were placed in the thermostat at 37° and shaken for a few minutes, by which time the manometers had become steady. Tap *D* was then opened to the air for a few moments; taps *D* and *F* were then closed, and the apparatus was shaken at 37° for the required time, usually for 3 hours.

At the end of this period, the bottles were removed, and placed, in their carriages, in a tank of water at room temperature which was stirred by a brisk stream of air bubbles. The manometers were connected to the bottles by turning taps *F* and *D*, and readings of the pressure inside the bottles were taken at intervals, until the manometers became steady. Tap *F* was then closed, and the bottles were removed from their carriages, and immersed in a tall jar of water. They were connected with gas-sampling tubes filled with mercury, and samples withdrawn in the usual way, the dead space in *G* and the connecting tubes being filled with mercury by gravity, the mercury escaping through *C*. The samples were then analysed in a Haldane apparatus [Haldane, 1920], by the standard technique for oxygen-rich mixtures (*i.e.* after preliminary dilution with nitrogen in the gas burette).

In this way sufficient data were obtained to calculate the initial and final volumes of dry gas at N.T.P., and the initial and final percentage composition.

The initial percentage composition was determined in a sample taken direct from the aspirator, where the gas mixture was stored over saturated $CaCl_2$, immediately after the bottles had been filled.

The initial volumes were those of the bottles (already determined) corrected for barometric pressure, temperature (37°), and the tension of aqueous vapour at 37°. The final volumes were those of the bottles, with the following corrections.

(1) Temperature (that of the tank in which they were immersed after removal from the thermostat).

(2) Tension of aqueous vapour at that temperature.

(3) Pressure. This was that of the barometer, *minus* the pressure difference recorded by the manometer. To obtain the latter, the manometer reading had to be multiplied by $\cos\theta$ where θ was the angle of inclination of the manometer to the vertical.

No correction was made for the solubility of O_2 in water at room temperature.

From these data, the amounts of oxygen and CO_2 present in the gas phase in the bottles at the beginning and end of the experiment were calculated, the difference in the values found for oxygen representing the oxygen consumption of the tissues.

Immediately after the final gas samples had been taken, tap *A* was opened, and the stoppers were removed from the bottles. The tubes were washed with the precipitating reagent, and the tissue was worked up for chemical estimation by one of the methods described in our previous paper [Holmes and Ashford, 1930]. For lactic acid only, trichloroacetic acid was employed, for "free sugar," dilute alcohol, for glycogen, absolute alcohol. Two points only need mention. (*a*) During evaporation of the alcohol, CO_2 escapes and the fluid tends to become strongly alkaline, on account of the breakdown of the bicarbonate to carbonate. To guard against this, a few drops of bromocresol purple were added, and the fluid was kept neutral by the addition of dilute HCl in alcohol, drop by drop as required, during evaporation. (*b*) The calculation of the "extra" lactic acid removed in these experiments was made as follows.

If the initial lactic acid of the tissue = IP, and the amount of lactic acid added = L, and if after oxygenation, for tissue alone, lactic acid remaining = OP and for tissue with lactic addition, lactic acid remaining = OL, then lactic removed from plain sample = $IP - OP$.

Lactic acid removed from sample to which lactic acid had been added = $(L + IP - OL)$.

$$\text{Therefore extra lactic removed} = (L + IP - OL) - (IP - OP)$$
$$= L - OL + OP.$$

Hence the estimation of the initial lactic acid of the tissue was unnecessary, and its omission afforded both a saving of time and tissue. L, OP and OL were estimated chemically.

Table I.

Exp.	Ringer	Initial CO_2 %	p_H Initial	p_H Final	Extra lactic acid removed (mg.)	O_2 consumed (mm.³) Plain	O_2 consumed (mm.³) Lactate	Extra O_2	Lactic equiv. of extra O_2	"Q"	Expected synthesis	Observed synth. (mg.) Free carb.	Observed synth. (mg.) Glycogen	Duration of exp. (min.)	Remarks
14. xi. 30	Bicarb.	5·04	7·20	—	8·54	6070	10670	4600	5·95	1·43	3·6	—	—	240	Oxygen
17. xi. 30	,,	4·83	7·21	—	6·11	5670	7990	2320	2·95	2·1	3·2	—	—	245	Oxygen
21. xi. 30	,,	5·51	7·16	—	10·17	5230	10950	4720	6·37	1·6	3·8	—	—	240	Oxygen
24. xi. 30	Phosph.	0	7·6	—	7·51	4080	5080	1000	1·34	5·6	6·7	—	—	225	Air } Parallel exps.
	,,	0	7·6	—	12·71	4580	8700	4120	5·55	2·3	7·2	—	—	225	Oxygen }
26. xi. 30	Bicarb.	5·03	7·2	—	9·40	6020	8380	2360	3·17	2·9	6·2	—	—	180	Oxygen } Parallel
	Phosph.	0	7·7	—	11·11	4000	6720	2720	3·66	3·0	7·4	—	—	180	Oxygen, Barcroft's } exps.
28. xi. 30	Bicarb.	4·45	7·24	7·29	12·14	4970	8310	3340	4·50	2·7	7·6	0·140	—	180	Oxygen
3. xii. 30	,,	6·54	7·11	—	6·68	5920	8420	2500	3·36	2·0	3·3	0·031	—	180	Oxygen } Parallel exps
	Phosph.	0	7·18	—	8·37	3900	6300	1400	1·88	4·4	6·5	0·120	—	180	Oxygen }
6. xii. 30	Bicarb.	5·38	7·22	7·12	7·74	5270	8720	3450	4·65	1·7	3·1	0·190	—	180	Oxygen
12. xii. 30	,,	5·03	7·14	7·09	—	6860	8990	2130	2·87	—	—	—	−0·22	195	Oxygen. Sodium lactate 1 %
18. xii. 30	,,	6·33	7·15	7·20	5·34	5150	8120	2970	4·00	1·5	1·9	—	+0·12	180	Oxygen. Sodium lactate 0·5 %
15. i. 31	,,	7·45	7·01	—	11·75	—	—	—	—	—	—	—	−0·15	180	Oxygen. Sodium lactate 0·5 %
14. iv. 31	Phosph.	—	7·4	—	11·8	3680	6220	2540	3·41	3·5	8·4	—	—	,,	Oxygen, Barcroft's
17. iv. 31	,,	—	7·4	—	10·0	4100	6425	2325	3·13	3·2	6·8	—	—	,,	Oxygen, Barcroft's
24. iv. 31	,,	—	7·4	—	9·15	4040	6590	2550	3·38	2·7	5·8	—	—	,,	Oxygen, Barcroft's
14. v. 31	,,	—	7·4	—	9·75	4145	6675	2530	3·40	2·9	6·3	—	—	,,	Oxygen, Barcroft's
3. vi. 31	,,	—	7·4	—	10·5	4290	6370	2080	2·80	3·76	7·7	—	—	,,	Oxygen, Barcroft's
13. vi. 31	,,	—	7·4	—	8·14	1460	3710	2250	3·00	2·7	5·1	—	—	,,	Oxygen, Barcroft's. Insulin exp., 1 g. tissue
18. vi. 31*	,,	—	7·4	—	5·5	1510	3000	1490	2·00	2·7	3·5	—	—	135	Oxygen, Barcroft's. Insulin exp., 1 g. tissue

* Initial lactic acid content of tissue = 0·47 mg. per g. Except where otherwise stated, 0·5 % lithium lactate was used.
p_H by indicator method in all experiments with phosphate buffer.

Table I shows the results obtained in a number of experiments. The differences between the initial and final amounts of oxygen were large, and the technique, as a whole, proved satisfactory.

Several important points emerge from inspection of Table I. In the first place, it will be seen that the "quotient" is rather lower (average 2) when the Ringer is buffered by bicarbonate than when it is buffered by phosphate (average 3); the oxygen uptake is distinctly larger both in the presence and

Table II.

Average O_2 uptake of 2 g. of tissue in 3 hours at 37°, with different buffers and gas mixtures

Conditions	Oxygen uptake in mm.³		
	Plain	Lactate	Extra
Air/PO_4	3520	4205	685
Air/CO_2/bicarb.	3570	4420	850
O_2/PO_4	3940	6430	2490
O_2/CO_2/bicarb.	5700	8490	2790

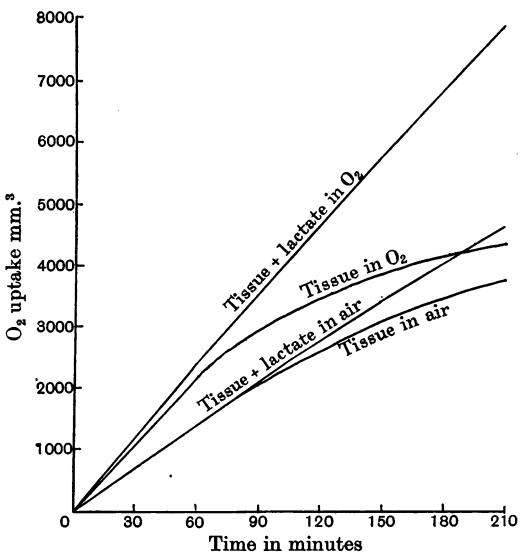

Fig. 2. Effect of oxygen tension on oxygen uptake of brain tissue. All 2 g. tissue. PO_4–Ringer p_H 7·4 at 37°. Lactate 0·5 %.

in the absence of lactate, than when a phosphate buffer is used. Our original experiments were performed in air; and when air is replaced by oxygen, even with phosphate buffer, there is a considerable increase in oxygen consumption (see Fig. 2); nevertheless tissue in bicarbonate buffer and CO_2/O_2 gas mixture shows a still larger uptake than tissue in O_2 and phosphate buffer (see Table II and Fig. 2). In spite of the fall in the quotient in the experiments with O_2/CO_2 and $NaHCO_3$, it will be seen that the net result is that the expected synthesis of carbohydrate is absolutely larger in both sets of experiments carried out in oxygen as compared with those carried out in air. Table II

summarises the differences in oxygen uptake occurring under the different conditions just discussed.

The data given in some cases for "initial" and "final" p_H in Table I, were obtained in order to be sure that, in spite of the large changes in CO_2 percentage which occurred during the experiment (*e.g.* initial 5 %, final 10 %), there was no serious change in p_H.

Table III. *Oxygen uptake of different amounts of chopped brain tissue, in oxygen and Ringer buffered with phosphate* p_H *7·4.*

Amount of tissue taken g.	O_2 uptake per g. in 3 hours mm.³
0·3	2180
0·5	2040
1·0	2025
1·5	2390 (single exp.)
2·0	1970

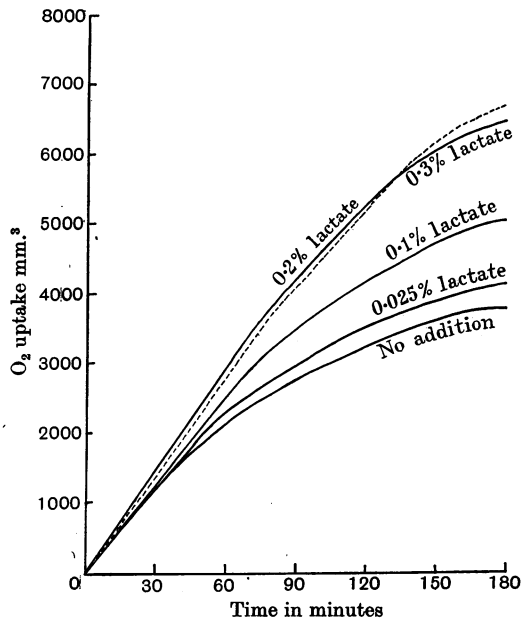

Fig. 3. Effect of varying lactate concentration. 2 g. tissue. p_H 7·4, in O_2 at 37°.

There is no difficulty about determining the initial p_H by means of the quinhydrone electrode. The final p_H was determined in a duplicate flask provided with the necessary electrodes and a device for introducing the quinhydrone at the conclusion of the experiment.

It seems to us that these experiments show that neither the existence of the "quotient" nor the absence of synthesis can be ascribed either to failure of adequate oxygen supply, or to buffering with phosphate, since both phenomena occur equally in air and in oxygen, and in a bicarbonate/CO_2 buffered medium as well as in one buffered with phosphate. Dixon and Elliott [1929]

point out that, if the availability of oxygen is not a limiting factor in experiments with the Barcroft apparatus, then the relationship between oxygen uptake and weight of tissue used will be linear, or, put in another way, the uptake per g. of tissue will be constant, no matter how much tissue is employed in any given experiment. Table III shows that this is the case in our experiments.

One further point should be mentioned here. In most of the earlier experiments, an arbitrary concentration of lithium lactate of 0·5 % was used. Figs. 3 and 4 show the effect of different concentrations of this salt from 0·025 % to 0·8 %. It will be seen that little increase in oxygen uptake is

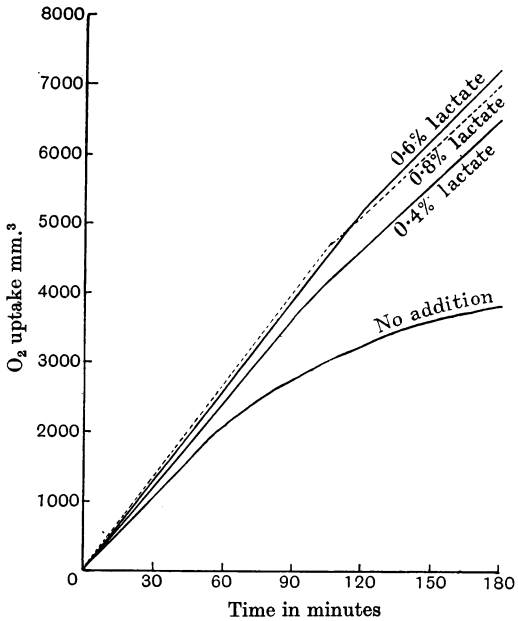

Fig. 4. Varying lactate concentration. 2 g. tissue; p_H 7·4; in O_2 at 37°.

obtained by increasing above 0·4 %. 0·5 % has accordingly been used in all subsequent experiments for convenience of estimation. At lower concentrations, the oxygen uptake curve soon falls off although the initial velocity is in all cases the same. Table IV shows the Meyerhof quotient under different conditions of lactate concentration. It will be seen that at lower concentrations the quotient is somewhat lower, but between 0·3 % and 0·8 % it has an average value of 2·7 to 2·8. These experiments have a further significance in connection with the question of the "sparing" of substrates to be referred to later.

A question of obvious importance is that of the effect of the chopping of brain tissue on its oxidative mechanisms. It has previously been reported by one of us [Holmes, 1930] that the oxygen consumptions of chopped and sliced rabbit brain are about equal. Nevertheless, we wished to try to obtain some

more positive data about the effect of chopping. It was out of the question to use intact mammalian brain (as a control to the chopped tissue), for clearly O_2 could not possibly diffuse quickly enough to supply its needs. On the other hand, with frog's brain in oxygen, and at room temperature, it was to be expected that at the lower temperature the chemical processes of oxidation

Table IV. *Effect of the lactate concentration on the Meyerhof quotient.*

In all experiments 2 g. of tissue were used in O_2 at p_H 7·4 and 37° (Ringer buffered with phosphate).

Concentration of lithium lactate %	Meyerhof quotient
0·025	1·93
0·05	2·26
0·1	1·78
0·2	2·15
0·3	2·54
0·4	2·82
0·5	2·71
0·6	2·96
0·8	2·80

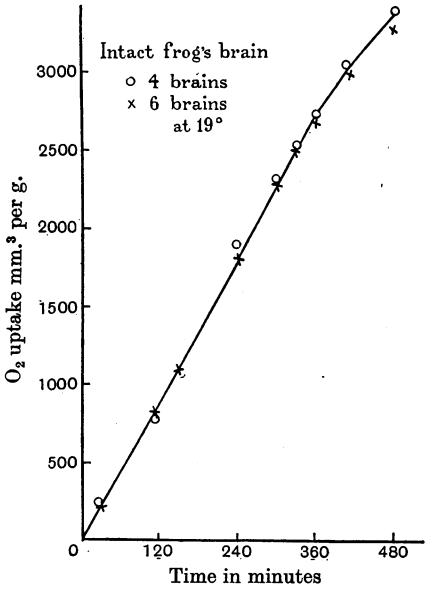

Fig. 5. Oxygen uptake of intact frog's brain at 19°. In O_2 at p_H 7·4.

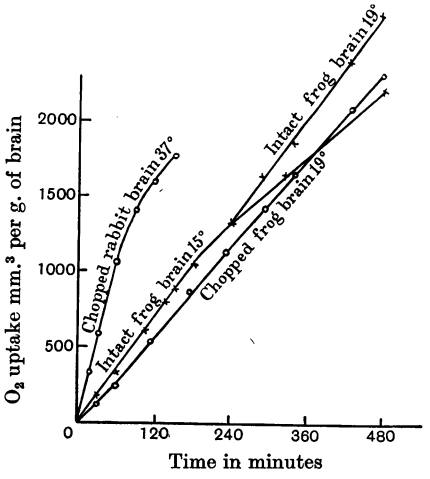

Fig. 6. Oxygen uptake of intact and chopped frog's brain.

would be retarded far more than the physical process of diffusion. We therefore performed some experiments in which the oxygen uptake of intact, freshly dissected frog's brain was determined in the small type of Barcroft apparatus, which is calibrated for 1·0 cc. of fluid. An intact frog's brain is larger than the individual fragments of chopped rabbit's brain used in the other experiments.

Fig. 5 shows the results of two experiments showing that the oxygen uptake per g. is the same when different amounts of tissue are used (4 brains in the one case, 6 in the other).

Fig. 6 shows the effect of chopping on the uptake of frog's brain. The total uptake of the chopped brain is some 16 % less than that of the intact brain, which, in our view, does not suggest that the chopping is likely to have had any very serious deleterious effect upon the oxidative mechanisms. This stands in contrast with the observation of Meyerhof [1919] that chopping of frog's muscle leads to a large increase in the oxygen uptake as compared with the intact muscle.

For purposes of comparison there is added a curve showing the uptake per g. of chopped rabbit brain at 37°.

We think that these experiments tend to show—admittedly, they do not conclusively prove—that our practice of chopping brain tissue is unlikely to interfere with its normal oxidative processes. They do not, of course, throw any light on the possibility of damage to other mechanisms. It must be remembered that less damage is likely to be caused to individual cells in chopping brain, than would be caused by chopping muscle, the long fibres of which would inevitably suffer. It has been shown [Holmes, 1930] that most of the O_2 uptake of brain is due to the cells, and not to the axons.

Section II.

Fate of the lactic acid.

Since the lactic acid which disappeared, over and above that which was accounted for by the "extra" oxygen, was not synthesised to glycogen, or to any form of reducing carbohydrate which could be estimated by the usual methods, we endeavoured, by other means, to trace its fate.

During an informal discussion of our results, it was suggested by Dr B. Woolf, as a possible explanation of them, that all the lactic acid was in reality being oxidised, but that part of it was "sparing" the oxidation of some other substance present in the brain tissue. This would, of course, account for the fact that the "extra" oxygen as measured by the difference between *OL* and *OP* was too small to account for the whole of the lactate which disappeared, simply because other materials were oxidised in *OP* which in *OL* were "spared" and so required no oxygen at all. This suggestion is clearly much to the point; it is also one which it is extremely difficult either to substantiate or to disprove.

It seems clear, however, that it cannot afford the correct explanation, if, in any circumstances, it can be shown that the total lactic acid disappearing would require, if completely oxidised, more oxygen than the total amount of that gas taken up in the manometer containing tissue *plus* added lactate (*OL*). In most of our experiments this is not the case, though, in a good many, there is only a comparatively small margin between the two amounts. If,

however, the experiments are carried out on tissue which has been depleted of its own substrates, either by washing, or by exposure to oxygen, or by the preliminary administration of insulin to the animal, then the oxygen equivalent of the total amount of lactic acid disappearing is very considerably greater than the total O_2 uptake of *OL*. Experiments of this nature are detailed in Table V, and it seems to us therefore unlikely that our results are due simply to the "sparing" of other substrates by lactic acid.

Table V.

Date	O_2 equivalent of total lactic removed mm.³	Total O_2 taken up by *OL* mm.³	Remarks
21. viii. 30	3420	2770	Washed tissue
22. viii. 30	3870	2440	Washed tissue
26. viii. 30	1640	751	Tissue washed and kept 12 hrs. in ice-chest
29. vi. 31	4350	2800	Tissue shaken in O_2 for 3 hrs. before lactate added and readings commenced
1. vii. 31	3185	1564	Tissue shaken in O_2 for 3½ hrs. before lactate added and readings commenced
18. v. 31	4290	3000	Animal rendered hypoglycaemic with insulin before killing
13. v. 31	6360	3710	Animal hypoglycaemic with insulin before killing

If the concentration of lactate is sufficiently low, this relationship does not hold good, at least in the case of washed brain; probably because the enzymes concerned are then no longer saturated with substrate. This point is further discussed at a later stage.

In endeavouring to trace the fate of the lactic acid, it seemed to us to be best to obtain information about the respiratory quotient of the tissue, and particularly about that of the "extra" oxygen. Some preliminary experiments on this point were reported in our first communication [1930]. The technique finally adopted was the one of which the details are given by Dixon and Elliott [1929]. For our purposes, six Barcroft manometers were required, and we have used either the ordinary (3 cc.) or the large type. We may designate them *A*, *B*, *C*, *D*, *E*, *F*. *A* contains tissue and plain Ringer, and the CO_2 is absorbed by sodium hydroxide. *B* is similar to *A*, but contains lactate Ringer. *C* and *E* each contain tissue and plain Ringer, but instead of sodium hydroxide they have a Keilin cup containing saturated oxalic acid solution hung on the sodium hydroxide pot. *D* and *F* are arranged similarly to *C* and *E*, but contain lactate Ringer instead of plain Ringer. All the cups were filled with oxygen.

C and *D* were put into the thermostat and the taps closed as soon as temperature equilibrium was obtained. The acid was immediately spilled into the body of the cup by sharp shaking, and the manometers read until the reading became steady; this took usually 5 to 10 minutes. These readings represented preformed CO_2 in *OL* and *OP* respectively.

The remaining four Barcroft apparatus were then shaken in the thermostat for an appropriate period—2 to 3 hours. The manometers of *A* and *B* were read, and the difference between them gave the "extra" oxygen. At the end of the period, the acid in the cups of *E* and *F* was spilled, and the manometer reading represented the difference between O_2 absorption and total CO_2 in the system at the end of the experiment. Thus, if, for the moment, we take the letters to represent the gas volumes indicated by the readings of the various manometers (*i.e.* the manometer readings × factor)

$A - E = CO_2$ contained in *OP*,
$B - F = CO_2$ contained in *OL*,
$A - E - C = CO_2$ evolved by *OP* (*i.e.* contained in *OP minus* initial CO_2),
$B - F - D = CO_2$ evolved by *OL*.

From the data so obtained, we can calculate the respiratory quotient of the tissue alone, the tissue to which lactate has been added, and the amount of CO_2 corresponding to the "extra" oxygen.

Table VI gives some results obtained by this method. It will be seen that it includes certain experiments performed on the brains of animals with insulin hypoglycaemia. These were performed with the object of discovering whether there was any definite fall in the respiratory quotient as a result of the shortage of lactic acid which has been shown [Holmes and Holmes, 1925] to exist in the brain under these circumstances. No such fall is apparent: evidently, therefore, substrates are still present which maintain the R.Q. at, or close to, unity.

Table VI. *Tables of values found for respiratory quotients.*

Experiments in air				Experiments in O_2			
Date	Tissue only	Extra O_2		Date	Tissue only	Extra O_2	
8. vii. 30	0·935	0·937	Normal animals	8. v. 31	0·924	0·96	Normal animals
11. vii. 30	0·985	0·920	,,	9. v. 31	0·87	—	,,
31. vii. 30	0·758	1·124	,,	12. v. 31	0·87	1·10	,,
5. viii. 30	0·875	1·128	,,	13. v. 31	0·96	0·94	Insulin animals
7. viii. 30	0·909	1·020	,,	18. v. 31	0·89	0·96	,,
20. viii. 30	0·928	0·984	,,				
Mean	0·912	1·018					

The R.Q. of the "extra" O_2 is 1·0 (mean values, 1·018 and 1·03, are certainly within the limits of experimental error) whether the experiments are performed in O_2 or in air. Loebel [1925] gives figures which are somewhat lower than these. This, on the face of it, suggests that the extra oxygen is concerned with the complete oxidation of a corresponding amount of lactic acid. We realise that conclusions from experiments of this kind must be drawn with caution; nevertheless, these findings make it highly improbable that the existence of the Meyerhof quotient depends on a partial oxidation such as the conversion of lactic acid to pyruvic acid, since such a process would give a Meyerhof quotient of 6·0, but R.Q. of the extra oxygen would be 0. The results

obtained in the respiratory quotient experiments could be explained if lactic acid were changed to acetic acid. This would involve the absorption of one molecule of O_2, and the formation of one molecule of CO_2. Apparently, however, this change does not take place, for if it did, either the acetic acid must be further oxidised, or must remain behind unchanged. Brain tissue, however, takes up no extra oxygen with acetate, nor does acetate accelerate the reduction of methylene blue by brain tissue (Thunberg technique); while, again, we were unable to demonstrate an excess of volatile acid in *OL* at the end of the experiment, under conditions in which acetic acid could be quantitatively recovered. Tests for pyruvic acid and acetaldehyde were negative.

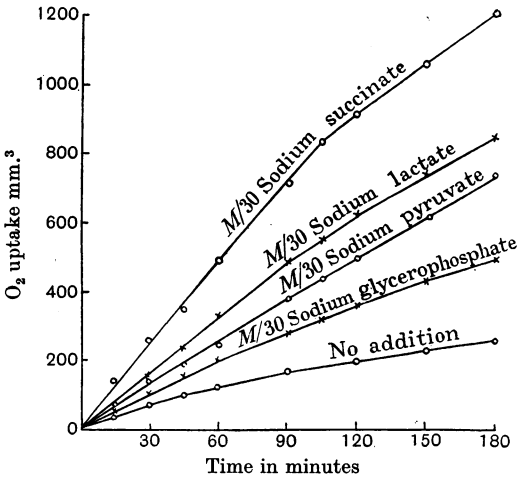

Fig. 7. 0·3 g. tissue in 3 cc. of fluid; p_H 7·4; in air at 37°.

This was again demonstrated by a large scale experiment in which 90 g. of pig's brain were oxygenated, both in the presence and absence of added lactate. The tissues, and also a similar initial sample, were worked up with trichloroacetic acid and filtered. The filtrates were neutralised and concentrated *in vacuo* in the presence of sodium sulphite [*cf.* Neuberg and Kobel, 1930; Case and Cook, 1931]. In no case was a precipitate obtained on adding 2:4-dinitrophenylhydrazine in hydrochloric acid.

In such attempts to follow the course of the disappearance of the lactic acid, the fate of a number of substrates which might possibly act as intermediaries has been investigated, both by the Barcroft aerobic technique and the Thunberg methylene blue reduction technique. Toennissen and Brinkmann [1930] have put forward a scheme involving, for the oxidation of lactic acid by muscle, the formation of pyruvic acid, which in turn gives an unstable 6-carbon compound by the loss of hydrogen. This, in turn, gives succinic and formic acids, which under appropriate conditions may be oxidised. It has already been mentioned that no evidence of pyruvic acid formation has been

obtained in our experiments. In one experiment conducted at room temperature, as opposed to those at 37°, there was very faint and doubtful evidence of the presence of pyruvic acid, as shown by the nitroprusside reaction. In spite of this, we have performed experiments with substrates suggested by Toennissen's work, using equivalent concentrations of each; Fig. 7 shows the oxygen uptake of brain with different substrates. It will be seen that succinate [*cf.* Quastel and Wheatley, 1931], lactate, pyruvate and glycerophosphate are all "oxidised." On the other hand, no evidence of an extra oxygen uptake was obtained with either formate or acetate while mandelate actually caused an inhibition of the order of 50 %. A parallel series of findings is obtained with the methylene blue technique (Table VII).

Table VII. *Effect of various substrates on reduction of methylene blue by brain tissue.*

Hydrogen donators		Not hydrogen donators	
Lactate, Glucose, Succinate, Glycerophosphate	Approx. the same activity	Glycerol, Dihydroxyacetone	No change in reduction time
Pyruvate	Slight change in reduction time	Acetate, Formate, Mandelate	inhibitors

Since our attempts to discover the fate of the lactic acid by qualitative tests for likely substances have given negative results, and since our determinations of the respiratory quotient threw no light on the probable fate of that portion of it which had not been oxidised, we decided, on the advice of Miss M. Stephenson, to perform estimations of total "soluble" carbon. If we estimated the total carbon in protein- and lipoid-free filtrates of *OL* and *OP*, then we should expect that the carbon in *OL* would be equal to that in *OP*, *plus* the carbon added as lactate, *minus* that which had been oxidised to CO_2, and absorbed by the potash during the experiment. If this were not the case, then either the lactate must have been changed to some substance which, though not glycogen, was still not soluble in the precipitating agent used in the preparation of the filtrates, or, alternatively, there must have been some fallacy inherent in our previous experiments or in our reasoning. The method adopted for the estimation of total carbon was the "wet combustion" method of Raistrick [1931]. It was necessary to choose a reagent for precipitating protein and lipoid which was free from carbon; while the presence of halogens in it was undesirable. The acid mercuric sulphate reagent of Hopkins and Cole (10 % mercuric sulphate in 7 % sulphuric acid) was found to be suitable.

2 g. of chopped brain tissue were put into the right-hand cups of each of four large Barcroft manometers, two of which contained plain Ringer, and the other two lactate Ringer. The Ringer in both cases was buffered with phosphate. Each apparatus was filled with oxygen, and shaken for 3 hours in the thermostat, the oxygen uptake being observed as usual. At the end of the experiment the 4 g. of tissue (from the two *OP* Barcroft's) were washed

into a 50 cc. volumetric flask with 10 cc. of the mercuric sulphate reagent, and the necessary amount of water to make up to volume. The 4 g. of *OL* were similarly treated. After standing for not less than 1 hour, the contents of the flasks were transferred to centrifuge-tubes and centrifuged. The supernatant fluid was poured off, and the mercury removed with H_2S. HgS was removed by filtration on a small Büchner funnel, then through an ordinary dry filter-paper, and H_2S by aeration with moist air. 5 cc. of the solution were taken for estimation, and represented one-tenth of the original amount of material.

Table VIII shows the results obtained in these experiments. The figures in the column labelled "calculated" were obtained by adding to the value for *OP* found by estimation the difference between the amount of carbon in the added lactate and that in the CO_2 corresponding to the extra oxygen; in other words, by adding to the value for *OP* the carbon of the "expected synthesis."

Table VIII.

Date	Carbon added as lactate mg.	Carbon equiv. of extra O_2 mg.	Carbon found in *OP* mg.	Carbon calculated in *OL* mg.	Carbon found in *OL* mg.	Percentage difference
25. v. 31	16·16	2·12	13·99	28·03	27·04	3·6
3. vi. 31	16·64	2·24	12·85	27·25	24·65	9·5
14. v. 31	9·91	2·73	12·78	22·69	22·42	1·2
9. vi. 31	16·58	2·78	13·75	27·55	25·89	6·0
					Average	5·07

It will be observed that the values for carbon found in *OL* are less on the average by some 5 % than those "calculated." This may be the result of experimental error; the figures for the calculated value depend upon three separate measurements; (i) the estimation of the added lactate, (ii) the measurement of the "extra" oxygen, and (iii) the estimation of the carbon in *OP*, and some error is likely to creep in. It is, however, possible—since the discrepancy is always in one direction—that it really means that the fate of a small amount of the added lactate is in some way different from that of the remainder.

We feel that these experiments give an assurance that we are dealing with a real phenomenon. We have already stated our reasons for thinking that we are not dealing simply with the sparing of some other substrate by lactic acid. The carbon estimations do not throw any special light on the question of the sparing of substances soluble in mercuric sulphate, as there is clearly no way of identifying the carbon as being derived from lactic acid. But they seem to us to provide some additional evidence against the sparing of protein or of lipoids. If protein were spared by the oxidation of lactic acid, it is surely likely that the molecules would be broken down into smaller and more soluble moieties, and that these would then be oxidised. But in that case, it is unlikely that they would be oxidised so completely as to leave

absolutely no residual soluble carbon, and if any residual carbon were left, it would be estimated in *OP*, and we should find considerable excess of carbon in *OP*. The same argument applies to the sparing of lipoids. We shall return to this point later, when we discuss experiments which deal directly with the question of nitrogen sparing.

We performed a series of experiments to discover whether the missing lactic acid could have been changed into volatile organic acid. Filtrates were prepared, both by precipitation with mercuric sulphate and also with "colloidal iron," using, on several occasions, 16 g. of brain tissue. In the former case, the sulphuric acid was partly neutralised, to avoid risk of charring when the solution became concentrated; in the latter, the solution was acidified with phosphoric acid. The solutions were then distilled *in vacuo*, and the distillate was caught in standard alkali. The hydrochloric acid, some of which (derived from the chlorides of the Ringer) was always present in the distillate, was estimated with silver nitrate, and was deducted from the total volatile acid.

Small amounts of acetic acid were quantitatively recovered in control experiments; nevertheless, in no experiment was there an excess of volatile acid in *OL* over that found in *OP*; though small amounts were present in both samples. Beyond showing, by treatment with mercuric chloride, that this acid was not formic, we have been unable to identify it.

On another occasion, we repeated the experiment, but distilled into bisulphite, instead of into standard NaOH. Traces only of bisulphite-binding substances were present. We have performed experiments in which brain tissue has been shaken in oxygen in the presence of sulphite and lactate. The oxygen uptake is almost entirely inhibited by the sulphite. On applying the nitroprusside test to the tissue and fluid at the end of the experiment, we were unable to satisfy ourselves that pyruvic acid was present. The amount of bound sulphite was in these cases practically zero, and the same in *OL* and *OP*.

Another possibility which suggested itself, was that synthesis of some organic phosphorus compound was taking place which reduced feebly, or not at all, and was therefore not estimated as carbohydrate. We therefore carried out a series of experiments in which we estimated acid-soluble organic phosphorus in the freshly chopped tissue (initial value) and in the tissue which had been shaken in oxygen, both in the presence and absence of added lactate. These experiments were carried out in bicarbonate buffer with O_2/CO_2 atmosphere, to avoid adding phosphates, and estimations of inorganic phosphate, pyrophosphate, and total acid-soluble organic phosphorus (by incineration with sulphuric acid and "perhydrol") were carried out in the trichloroacetic acid filtrates [Briggs, 1924; Lohmann, 1928].

The results of a series of experiments are shown in Table IX. There is consistently slightly more organic acid-soluble phosphorus in *OL* than in *OP*, but *OL* is consistently lower than the initial value. The "expected synthesis" (reckoned as carbohydrate) in all these experiments is large and cannot be

traced to the synthesis of an acid-soluble organic phosphorus compound. Our total carbon experiments make it highly improbable that some insoluble organic phosphorus compound is being formed.

Table IX. *Determinations of acid-soluble phosphorus fractions.*

All figures are mg. P per 2 g. of tissue.

	Initial				Oxygenated without addition (*OP*)				Oxygenated with lactate (*OL*)			
Date	Total	Inorg.	Pyro.	A. s. org.	Total	Inorg.	Pyro.	A. s. org.	Total	Inorg.	Pyro.	A. s. org.
29. ix. 31	1·585	0·965	0·119	0·501	1·85	1·410	0·090	0·350	1·785	1·270	0·155	0·360
30. ix. 31	1·892	1·200	0·031	0·661	1·768	1·270	0·090	0·408	1·745	1·152	0·072	0·521
3. x. 31	1·612	0·907	0·021	0·684	1·870	1·330	0·060	0·480	1·922	1·320	0·044	0·646
9. x. 31	1·594	0·948	0·036	0·609	1·677	1·337	0·023	0·317	0·184	0·356	0·007	0·437
12. x. 31	2·010	1·390	0·160	0·461	2·005	1·470	0·147	0·391	0·960	1·365	0·145	0·445
,,	,,	,,	,,	,,	2·080	1·626	0·140	0·314	0·820	1·294	0·153	0·373
Average	1·738	1·082	0·073	0·583	1·875	1·407	0·091	0·376	1·841	1·293	0·096	0·464

A further significance attaches to these results, in that the consistently slightly greater figure for acid-soluble organic phosphorus in *OL*, than in *OP*, suggests that a certain small amount of sparing of phosphate ester has taken place. The question of "lipoid sparing" will be discussed elsewhere. Loebel [1925] showed that brain was capable to some extent of oxidising hexose-diphosphate, and our results would be in accordance with this finding.

A series of experiments was undertaken, in which total nitrogen and ammonia were estimated in the mercuric sulphate filtrates from the tissue oxygenated both in the presence and in the absence of lactate. Total nitrogen was estimated by that micro-Kjeldahl method in which alcohol is added and the distillation is assisted by aeration, *N*/200 NaOH being used for titration in the presence of de Wesselow's indicator. Ammonia was determined by Watchorn and Holmes's [1927] modification of Stanford's method [1923].

The results given in Table X show that, except for a single experiment, there is always more total nitrogen in *OP* than *OL*, and invariably more ammonia in the former than in the latter.

Table X. *Total soluble nitrogen and ammonia in samples oxygenated without addition (OP) and in samples oxygenated with added lactate (OL).*

All 4 g. of tissue in O_2 at p_H 7·4 for 3 hrs. at 37°.

	Total N (mg.)			Ammonia-N (mg.)		
Date	*OP*	*OL*	Diff.	*OP*	*OL*	Diff.
17. vii. 31	5·35	4·50	0·85	1·054	0·682	0·372
20. vii. 31	6·55	6·60	−0·05	1·256	0·657	0·599
24. vii. 31	6·05	5·29	0·76	1·247	0·772	0·475
30. vii. 31	4·01	3·31	0·70	1·130	0·768	0·362

Loebel [1925] has already shown that less ammonia is formed by brain tissue in oxygen in the presence of glucose or of lactate, and his results are confirmed, with regard to the latter substance, by the evidence which we

present here. The interpretation of these experiments is not at present easy. Clearly, in view of the figures for total carbon, nitrogen which would otherwise be insoluble in mercuric sulphate (*e.g.* protein- or lipoid-nitrogen) cannot have been built with the lactic acid into some nitrogenous compound; but our results would not preclude the possibility of nitrogen already present in some soluble form being transferred to a new compound derived from the lactic acid.

The fact that there is more ammonia in *OP* than in *OL* suggests strongly that the presence of the lactate has inhibited deamination. Pohle [1929] has demonstrated the presence of adenylic acid in brain tissue, and Holmes and Holmes [1925] had earlier stated that pentose was present in brain. At the same time, B. E. Holmes had isolated hypoxanthine, in the form of hypoxanthine silver nitrate, from brain tissue, but as it was subsequently found that Thudichum [1884] had made the same observation at a much earlier date, and as the significance of adenylic acid was not then realised, no mention of this finding was made. In view of this, and of the importance of the deamination of adenine in muscle, it seems very likely that the ammonia is derived, at least partly, from adenine, and that this process is partly inhibited in the presence of lactate. In this case, it is possible that the breakdown of pyrophosphate might also be inhibited. We have, however, found no evidence that this occurs. Table VIII shows that very little pyrophosphate is to be found in the brain under the conditions of our experiments, and that there is no suggestion that its amount is altered by the presence of lactate.

The alternative view—the one which is supported by the change observed in total nitrogen—is that the effect of the lactate is actually to spare protein or lipoid, and that part at least of the N in *OP* represents the deamination of protein or lipoid, or of both; but if such is the case, it must be remembered that the total carbon figures show that the remainder of the molecule must have undergone almost complete combustion.

Loebel [1925] suggests that, if protein is burned, 4–5 molecules of oxygen should be used for every molecule of nitrogen split off. Table IX shows (neglecting the second experiment) that about 0·77 mg. of ammonia-N was apparently "spared" by the lactate. This represents $0{\cdot}77/14 = 0{\cdot}055$ millimoles of ammonia-N, and should, in turn, represent about $0{\cdot}055 \times 4{\cdot}5$ or 0·246 millimoles of oxygen. This oxygen would completely oxidise $\frac{0{\cdot}246}{3} = 0{\cdot}082$ millimoles, or 7·38 mg. of lactic acid.

Lusk [1928] from Loewy's data, states that, in the animal body, 100 g. of (meat) protein would require 138·18 g. of O, and that 8·49 g. of O would be required per g. of urinary N. Thus, 1 mg. of N should correspond to $\frac{138{\cdot}18}{8{\cdot}49} = 16{\cdot}28$ mg. of O and 0·77 mg. would correspond to $\frac{138{\cdot}18}{8{\cdot}49} \times 0{\cdot}77 = 12{\cdot}53$ mg. of O, *i.e.* to $\frac{12{\cdot}53}{32} = 0{\cdot}393$ millimoles of O. This would account for the complete oxidation of $\frac{0{\cdot}393}{3} = 0{\cdot}131$ millimoles, or 11·79 mg. of lactic acid.

Now the average figure which we obtain for the amount of lactic acid

which disappears and is not accounted for by the extra oxygen, is, for 4 g. of brain, 14·68 mg. (mean of 7 experiments in which total carbon is estimated). It will be seen therefore that only about three-fifths of the lactic acid could, on the basis of these figures, have been used for "protein sparing."

If, on the other hand, lipoid (*e.g.* lecithin), instead of protein were spared, and if we assume that the molecular weight of lecithin is 773, then 1 mg. of nitrogen would correspond to 221 mg. of lactic acid. It will be seen, however, from Table IX that the total acid-soluble phosphorus is practically identical in amount both in *OP* and *OL*. It is difficult to picture any manner in which lecithin could be broken up without giving rise to soluble phosphorus compounds, and it seems, therefore, fair to discount the possibility of lipoid sparing having occurred. Table IX shows also that there is only a small liberation of soluble phosphorus in either *OL* or *OP*, the increase being only 0·137 mg. of P in the one case and 0·013 mg. in the other. This is in conformity with the relatively high R.Q. (0·9 and over) which is found even in the absence of lactate. It also falls into line with the experiments of King [1931] who finds that brain tissue does not break down lecithin at all readily.

Table XI.

Date	Lactic acid in *OL* after 1½ hrs. shaking in O_2 mg.	Lactic acid in *OL* after 1½ hrs. in in O_2 followed by 1½ hrs. in N_2 mg.	Lactic acid after 1½ hrs. in O_2 followed by addition of glucose, and 1½ hrs. in N_2 mg.	Lactic acid in *OP*, after shaking 1½ hrs. in O_2, followed by glucose and 1½ hrs. in N_2 mg.
4. viii. 31	5·75	5·75	—	—
6. viii. 31	6·40	6·50	14·6	10·05

In the course of experiments still in progress [unpublished], Sherif and Holmes have observed (in confirmation of previous workers McGinty and Gesell [1925], Kinnersley and Peters [1929], and Jungmann and Kimmelstiel [1929]) that if brain tissue is very rapidly cooled in liquid air (in these experiments mice were used), a comparatively low value for lactic acid is obtained, which rises on incubation at 40°. Simultaneous estimations of total carbohydrate show a fall in the latter, coincident in time with the rise in lactic acid, but sometimes less in amount (compare work of Kerly [1931] on muscle). In other words, lactic acid appears from some source other than substances estimated as carbohydrate. It seemed possible that this unindentified lactic acid precursor might, in fact, be the substance into which lactic acid was changed in our own experiments. To test this, samples of brain with added lactate were shaken for 1½ hours in oxygen. One sample was then analysed for lactic acid. The Barcroft apparatus containing another similar sample was then filled with nitrogen, and shaken for a further period of 1½ hours. Analysis showed that this had exactly the same lactic acid content as the sample taken for estimation after 1½ hours in oxygen—no reappearance of lactic acid had taken place under anaerobic conditions. Control experiments showed that,

after 1½ hours' shaking in oxygen, the tissue in nitrogen could still freely form lactic acid from glucose. These results are illustrated by Table XI.

Evidently the lactic acid is not changed into the unknown precursor, which exists, apparently, in the intact brain.

Discussion.

From the experimental evidence before us, we must conclude either that the larger part of the lactic acid which disappears when brain tissue is shaken with lactate in the presence of oxygen is transformed into some substance which has so far eluded identification, or else that all the lactic acid which disappears is in reality oxidised, but in the process "spares" some other substance or substances from oxidation.

The points in favour of the latter explanation are, first, its inherent plausibility. The principle of the "sparing" of one substrate by another in the animal body is well established, and there is no obvious reason why it should not apply to individual isolated organs. Indeed, the occurrence of nitrogen sparing by sugars in the growing kidney has been demonstrated by Watchorn and B. E. Holmes [1927, 1931]. From our own experiments we know that there is actually more soluble nitrogen in brain tissue that has been shaken in oxygen in the absence of lactate than its presence.

Brain tissue certainly contains oxidisable substances other than lactic acid—Thudichum [1884] demonstrated the presence of succinate, and succinate takes up oxygen even more freely than lactate—and probably there are others. The fact that 95 % of the carbon corresponding to the missing lactic acid can be found as soluble carbon is no bar to this explanation, if the line be taken that some soluble, carbon-containing substrate is spared; there is no means of identifying the carbon as derived especially from lactic acid. But it does bear on the question of protein or lipoid sparing, in the sense that it means that, if such sparing occurs, then practically the whole protein or lipoid molecule must be spared. We picture, after all, that hydrolysis must precede oxidation; and if this is so, all the hydrolysed fragments must be completely oxidised—if any were left unoxidised, they would upset the carbon balance. But this complete oxidation seems improbable. Against this explanation too, is the fact that, in tissue depleted of substrate by various methods, more lactic acid disappears than can be accounted for by the oxygen uptake (Table V). Here an explanation on the lines of a "sparing" effect is clearly impossible. We are thrown back on the assumption that some substance is present which, so far, we have failed to identify.

Still further evidence against the "sparing" hypothesis is afforded by a consideration of Figs. 2 and 3 and of Table IV, which shows the effect of varying concentrations of added lactate. We may assume that we have initially present in the tissue unknown substrates, which we may denote by X, and a certain amount of lactic acid l. When shaken in oxygen, suppose these amounts

become X_0 and l_0 (*i.e.* the value given as *OP* in the Tables). In the case of *OL* an amount L of lactic acid was added to the tissue, giving $(X + l + L)$ initially. After shaking in oxygen, suppose the amounts become $x_0 + l_0 + L_0$.

If now $X_0 = x_0$, then the differences in the amounts of oxygen taken up in the two cases should be due to the added lactic acid. If, however, X_0 is not equal to x_0 and is less than x_0, competitive inhibition or "sparing" has taken place. If this inhibition is of a competitive nature [Haldane, 1930], then an increase of L should cause a diminution in the amount of X oxidised, *i.e.* x_0 should be greater than X_0. In effect more of the oxygen taken up will have been used in the oxidation of the lactic acid than is shown by the difference of the two oxygen uptakes, *i.e.* more lactic acid is disappearing but the amount of "extra oxygen" remains the same. Hence the Meyerhof quotient should increase as we increase the concentration of the added lactate. On the other hand, assuming that, in actual fact, total oxidation of lactic acid takes place as the R.Q. suggests, the ratio

$$\frac{\text{O}_2 \text{ equivalent of total lactic acid disappearing}}{\text{Total oxygen uptake}}$$

should approach unity or fall as the lactic concentration is increased. It should be noted that 2 g. of the chopped tissue would contain about 2·7 mg. of lactic acid, and that this amount is almost maximal, *i.e.* aerobic glycolysis does not take place (as it does in muscle) except to a very small extent, owing to the absence of precursor in the tissue [Holmes and Holmes, 1925]; Ashford

Table XII. *Ratio of* $\frac{\textit{Oxygen equivalent of total lactic acid removed}}{\textit{Total oxygen uptake}}$ *at different initial concentrations of added lactate.*

Approx. conc. Li lactate %	Initial lactic acid g.	Final lactic acid g.	Lactic acid removed g.	O_2 equiv. of lactic acid removed mm.³	Total O_2 uptake mm.³	Ratio
0·025	3·7	0·56	3·14	2,350	4710	0·5
0·05	4·7	0·68	4·02	3,000	4875	0·615
0·1	6·9	1·6	5·31	4,450	5000	0·89
0·2	11·97	2·885	9·1	6,750	6450	1·05
0·3	17·0	4·65	12·35	9,200	6700	1·37
0·4	18·4	7·31	11·1	8,300	6480	1·28
0·5	22·4	10·32	11·1	8,260	6300	1·31
0·6	26·7	13·2	13·5	10,000	7110	1·40
0·8	34·2	20·1	14·1	10,500	7000	1·44

and Holmes, 1929]. At the lowest concentrations employed therefore, the amount of added lactic acid is small, or comparable with the amount already in the tissue. The findings have already been noted, *viz.* that at the lowest concentrations the Meyerhof quotient is smaller but that between 0·3 % and 0·8 % there is little change. This makes it unlikely that competition between substrates is taking place, or in other words, probably no "sparing" occurs. Further evidence of this is given in Table XII. It will be seen that the ratio

$$\frac{\text{O}_2 \text{ equivalent of total lactic acid disappearing}}{\text{Total oxygen uptake}}$$

is less than unity at low concentrations, but afterwards increases above unity, whereas if the suggestion made above were correct, it should decrease with increased concentration of lactate.

Summary.

1. Experiments are described in which it is shown that a Meyerhof quotient can be obtained if brain tissue is shaken with lactate in the presence of a bicarbonate buffer, in an O_2/CO_2 atmosphere. A technique is described by which the use of manometric methods is avoided. In spite of the existence of the "quotient," no carbohydrate synthesis occurs.

2. The O_2 uptake is greater in the presence of a CO_2-bicarbonate buffer than in the presence of phosphate and increases with increased oxygen tension in both cases.

3. There is little difference between the oxygen uptake of intact and chopped frog's brain, measured in O_2 at room temperature, in the presence of phosphate buffer.

4. The respiratory quotients, both of the brain tissue alone and of the "extra oxygen," are close to unity, even in the case of animals rendered hypoglycaemic by insulin injection.

5. With brain tissue, glucose, lactate, succinate and glycerophosphate all act as hydrogen donators to methylene blue. Glycerol and dihydroxyacetone are without effect on the reduction time; formate, acetate and mandelate prolong it.

6. Estimations of total carbon in protein-free filtrates of tissue shaken with and without lactate indicate that the "extra oxygen" gives a true measure of the lactic acid oxidised.

7. There is no evidence of formation of volatile acid, or of any substance capable of binding bisulphite, from lactic acid in the presence of brain tissue.

8. Inorganic, pyro-, and acid-soluble organic phosphorus have been estimated. There is evidence of a small increase both in inorganic and in total phosphorus, at the expense of organic phosphorus, both in the presence and in the absence of lactate; but there is no evidence of the synthesis of any phosphorus compound from the fraction of the lactic acid which disappears and is not accounted for by oxidation.

9. There is more non-protein nitrogen, and more ammonia, in filtrates from brain tissues which have been aerated in the absence of lactic acid, than in its presence.

10. The possibility of accounting for the disappearance of lactate on the lines of a "sparing" effect on some other substrate is discussed, and it is shown that such an explanation is probably not the correct one.

Our thanks are due to Sir F. G. Hopkins, for his interest in this work.

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