CXXVII. LACTIC ACID OXIDATION IN BRAIN WITH REFERENCE TO THE "MEYERHOF CYCLE."

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IN a series of papers Meyerhof [1920, 1, 2 and 3] showed that the chemical events accompanying muscular contraction revolved in a definite cycle, lactic acid being formed anaerobically from carbohydrate, and being restored aerobically, the energy for this restoration being derived from the oxidation of a portion of the lactic acid itself. Following on this work, a large number of important papers by Meyerhof, Warburg, and their collaborators, have seemed to establish the fact that the cycle was no isolated example of biological machinery, but was a general phenomenon, characteristic of most, if not of all, living cells. It will suffice here to mention three of these papers, in which the point is, perhaps, brought out most clearly [Meyerhof, 1925; Meyerhof and Lohmann, 1926; Warburg, Posener and Negelein, 1924].

In the experiments on muscle, the changes in lactic acid and the synthesis of carbohydrate were demonstrated by chemical methods. The chemical findings, moreover, confirmed, and were confirmed by, the experimental heat determinations of Hill [1922] with most striking precision. While this is true of muscle, the occurrence of a carbohydrate-lactic acid cycle in other cells has not been clearly demonstrated. The experimental work was performed, for the most part, by micro-methods, devised in the first place by Warburg, and frequently modified. They involved manometric measurements, and were performed on a scale which surely precluded accurate chemical estimation.

In the case of muscle, the ratio $\frac{\text{Total lactic acid disappearing}}{\text{Lactic acid oxidised}}$, known as the "Meyerhof quotient," has a value from 3 to 6. By the manometric method, it is possible to measure (1) the oxygen used by the tissue, (2) the CO_2 evolved by the tissue, either anaerobically or aerobically. The tissue is bathed in a solution containing bicarbonate, and kept in an atmosphere containing CO_2 . Any production of strong acid, such as lactic acid, will cause the evolution of CO_2 ; any removal of lactic acid will cause its retention in the fluid, as bicarbonate. From the CO_2 measurements, therefore, are deduced the changes taking place in the lactic acid content of the system.

In dealing with these experiments, the authors have been accustomed to express the "Meyerhof quotient" as the ratio

$$\frac{\text{Lactic acid prevented from appearing in oxygen}}{\text{Lactic acid equivalent of oxygen used}},$$

that is, $\frac{\text{Lactic acid produced anaerobically minus lactic acid produced aerobically}}{\text{Lactic acid equivalent of oxygen used}}.$

In the case of most cells, they have obtained a "quotient" greater than unity, indicating a synthesis of carbohydrate under aerobic conditions, and therefore the existence of a "Meyerhof cycle."

The idea that this "cycle" is a fundamental principle, underlying the chemical dynamics of almost all living cells, is very attractive. At the same time, its truth, in the case of tissues other than muscle, seems to lack complete verification, since the actual demonstration of carbohydrate synthesis under experimental conditions, in which a "Meyerhof quotient" is obtainable, is lacking. With this in view, one of us (E.H.) in conjunction with Mrs B. E. Holmes, attempted, in 1926, to demonstrate the existence of a Meyerhof quotient and its relationship to carbohydrate synthesis in the case of brain tissue. Unfortunately, we were forced by circumstances to abandon the project in favour of other work. But the present experiments have fully confirmed the suggestions made by those earlier ones.

GENERAL PRINCIPLES OF METHOD.

It was impossible to perform the experiments on the small scale adopted by Meyerhof, Warburg and their collaborators. The following plan was therefore adopted. Two large "Barcroft" differential manometers, constructed by Flaig and Co., of Hatton Garden, London, with cups of a total capacity of 110 cc. and manometers of working length 230 mm. were calibrated to work with 5 cc. of fluid in each cup, the right hand cup containing, in addition, 2 g. of tissue. The greatest excursion of the oil corresponded to an oxygen uptake of about 1660 mm.³ (depending, of course, on the factor). With 2 g. of tissue, readjustment was necessary after about one hour.

These instruments were shaken in a water thermostat at 37°. Both contained chopped brain tissue, but while one lot of tissue was suspended in plain phosphate-buffered Ringer's solution, the other was suspended in Ringer's solution of the same composition and p_H , but containing in addition 0.5 % lithium lactate. The oxygen uptakes of each were measured: that of the sample in plain Ringer's solution began to fall off, usually in 60–90 minutes, while that in the lactate-Ringer's solution remained linear, or nearly so. At the end of the experiment, the difference in total oxygen uptake was measured, and represented the extra oxygen used by reason of the added lactate. At the commencement of the experiment, another two 2 g. samples of tissue had been weighed out, and immersed in 5 cc. of plain and lactate-Ringer's solution, respectively. When the taps of the manometers were first closed, alcohol was added to these "initial" samples of tissue. At the end of the experiment

the tissue was killed immediately either by the addition of alcohol or trichloroacetic acid, and lactic acid and free sugar, or lactic acid and glycogen were estimated in all four samples. For convenience, the two samples recovered from the Barcroft cups will be designated OP (no added lactate) and OL (added lactate) and, similarly, the other two, IP and IL. Then:

$(IL - OL) =$ Total lactic acid removed from the sample of tissue to which lactate was added,

and $(IP - OP) =$ Amount of preformed lactic acid removed from the tissue.

So that $(IL - OL) - (IP - OP) =$ Amount of added lactic acid removed.

The oxygen consumed by OL minus the oxygen consumed by OP = extra oxygen used, and corresponds to the added lactic acid removed. In respect of this added lactic acid, therefore, the "Meyerhof quotient" is

$$\frac{\text{Added lactic acid removed}}{\text{Lactic acid equivalent of extra oxygen}}$$

If this be greater than 1.0 the fact should, according to the "cycle" theory, indicate a synthesis of carbohydrate; and from the actual figures, the magnitude of this expected synthesis can be calculated.

CHEMICAL METHODS.

In view of certain complications which recent work has brought to light in the estimation of carbohydrates, the methods employed will be described in some detail.

1. *Lactic acid and "free carbohydrate."* The term "free carbohydrate" covers carbohydrate substances soluble in 60 % alcohol, and in acid mercuric chloride (Schenk's reagent), which reduce ferricyanide under the conditions of the Hagedorn and Jensen [1923] blood-sugar method. Sufficient alcohol was added to the suspension of tissue in Ringer's solution to make the alcohol concentration 60 %. The tissue was finely ground in a mortar and washed into a 50 cc. volumetric flask. The flask was filled up to the mark with 60 % alcohol and allowed to stand for 3 hours, or longer. The contents were then centrifuged, and the volume of clear centrifugate noted (x cc.). The final result was multiplied by the factor $50/x$.

The centrifugate was evaporated to dryness in a beaker over a water-bath. The residue was rubbed up with 5 cc. of Schenk's reagent and filtered through asbestos. Three more 2 cc. portions of reagent served to wash beaker, funnel and precipitate. From the filtrate, Hg was removed by H_2S and HgS filtered off through a washed filter-paper. The precipitate was washed with water, and filtrate and washings were aerated to remove H_2S . The acid filtrate was then sometimes (see "hydrolysed values," Table II) heated for 2 hours on a boiling water-bath; whether hydrolysed or not, it was next neutralised and subjected to the "copper-lime" precipitation. The reducing substances were estimated in the precipitate, the lactic acid in the supernatant fluid. This

procedure has been previously described [Holmes and Gerard, 1929]. Two modifications only have been introduced.

(a) The alkalinity of the final filtrate, upon which the sugar estimations were performed, was adjusted by the method described by Kimmelstiel [1929] for the estimation of cerebroside-galactose.

(b) The lactic acid estimations were performed by the method of Friedemann, Cotonio and Shaffer [1927].

2. *Estimation of lactic acid and glycogen.* The difficulties introduced into the estimation of glycogen by the fact of its solubility in 60 % alcohol have already been discussed [Holmes and Gerard, 1929. See also Kerly, 1930]. To circumvent these difficulties, the following procedure was adopted. The tissue and Ringer's solution were transferred to a centrifuge tube, and washed in with absolute alcohol, in all about 30 cc. of alcohol being used. After standing for a short time, the mixture was centrifuged, and the clear fluid transferred to a beaker and evaporated to dryness on the water-bath. Meanwhile, the tissue was transferred to absolute alcohol, ground, and extracted four times, each portion of alcohol being filtered off through a Jena glass filter (mesh 5/7). When the centrifugate had evaporated to dryness, the residue (called, for convenience, residue A) was extracted 5 or 6 times with absolute alcohol, which was filtered off through the glass filter. The combined alcoholic extracts (*i.e.* that from the tissue, and that from residue A) now contained all the lactic acid, while the glycogen, being insoluble in absolute alcohol, was contained, partly in the tissue, partly in residue A. The tissue was transferred to the beaker containing residue A, and the whole was extracted four times with boiling chloroform (to remove cerebrosides) and finally washed completely, with chloroform, into the glass funnel, and there left until every trace of chloroform had evaporated. The dry, powdery, residue was then transferred to a centrifuge tube and heated for 3 hours with 0.5 cc. of 60 % KOH. It was then cooled; 0.5 cc. of water, 4 cc. of alcohol, and a few drops of bromocresol purple [Kerly, 1930] were added, and the fluid was neutralised with trichloroacetic acid. Water was then added, so that the volume of watery solution (0.5 cc. KOH + 0.5 cc. H_2O + x cc. trichloroacetic acid + y cc. bromocresol purple) was brought to 2.0 cc. The addition of a further 4.0 cc. of alcohol then gave a concentration of 80 % alcohol, 4 cc. of alcohol already being present. After standing overnight, the fluid was centrifuged and the residue washed once with 5 cc. of 90 % alcohol, and twice with 5 cc. of absolute alcohol. It was then extracted with four 3 cc. portions of boiling water, to the first of which a drop of HCl had been added. Each portion was filtered successively through the same washed filter paper into another centrifuge tube. HCl was added to make the concentration 2.2 %, and the fluid was hydrolysed for 3 hours on a boiling water-bath. If necessary, the fluid was again filtered. It was then neutralised, and the copper-lime precipitation and Hagedorn and Jensen estimation were performed as for "free carbohydrate."

The lactic acid estimation was performed as follows. The alcoholic solution

of the lactic acid was evaporated to dryness, and the residue extracted three times with 10 % trichloroacetic acid. The filtrate was treated with copper-lime, and the lactic acid estimated by the method of Friedemann, Cotonio and Shaffer [1927].

RESULTS.

The results (Table I) of a short series of preliminary experiments, performed in each case with 0.3 g. of chopped rabbit brain, put up in 3.0 cc. of

Table I. *Disappearance of lactic acid and oxygen uptake for 0.3 g. of tissue in 3 cc. phosphate-buffered Ringer's solution at p_H 7.6 and 37°.*

Exp.	Duration (mins.)	IL Tissue and added lactate. Initial value mg.	OL Tissue and added lactate. Final value mg.	IP Tissue alone. Initial value mg.	OP Tissue alone. Final value mg.	IL-OL mg. (A)	IP-OP mg. (B)	Added lactic disap- pearing (A-B)	O ₂ ab- sorbed by OL (mm. ³)	O ₂ ab- sorbed by OP (mm. ³)	"Extra" O ₂ ab- sorbed	Lactic equiva- lent of extra O ₂ (mg.)	"Meyer- hof quo- tient"	Strength of lac- tate em- ployed %
1	206	7.9	6.58	0.65	0.28	1.32	0.37	0.95	1287	692	595	0.813	1.17	0.25
1a	206	7.72	6.70	0.70	0.35	1.02	0.35	0.67	1276	711	565	0.77	0.87	0.25
2	90	15.8	14.8	0.425	0.298	1.0	0.127	0.87	438	361	77	0.105	8.3	0.5
2a	180	15.8	13.5	0.425	0.143	2.3	0.283	2.02	921	505	416	0.567	3.54	0.5
3	223	5.45	4.02	0.492	0.049	1.43	0.44	0.99	1074	764	310	0.422	2.34	0.2
3a	223	13.8	11.50	0.492	0.049	2.3	0.44	1.86	1161	764	397	0.562	4.09	0.5
3b	223	17.6	15.45	0.492	0.049	2.15	0.44	1.71	1021	764	257	0.352	4.85	0.7

Exp. 1 is by the trichloroacetic extraction method.

Exp. 1a is by the alcohol extraction and Schenk method.

Exps. 2-3b are by the trichloroacetic extraction method.

Ringer's solution in Barcroft manometers of the ordinary type, show that a "Meyerhof quotient" is obtainable with 0.5 % lithium lactate. 0.25 % lactate on one occasion gave a quotient of almost 1.0, and on another, 0.2 % gave one of more than 1.0. The results obtained by working up the tissue (a) by the trichloroacetic acid method and (b) by extraction with 60 % alcohol, followed by Schenk's reagent, agree quite well. It will be noticed that the largest quotient of all (8.3) is obtained in Exp. 2, the duration of which is the shortest of the series. Exp. 2a was done simultaneously upon further samples of tissue from the same brain, but was allowed to run for 180 instead of for 90 minutes. Exps. 3, 3a, and 3b were also carried out simultaneously, on batches of tissue from the same animal; here the quotient rises with the concentration of lactate. The suggestion—which is being tested further—is that the shorter the duration of the experiment, and the higher the concentration of lactate, the larger the quotient is likely to be.

The results of the experiments with the large manometers, in which carbohydrates, as well as lactic acid, were estimated, are shown in Table II. In all cases, a quotient was obtained. The differences in the amounts of lactic acid estimated lie far outside the limits of experimental error, and the results are therefore put forward with confidence. The quantities of oxygen absorbed agree very well with those obtained in the preliminary experiments, if both sets of values are calculated on the basis of mm.³ of oxygen per g. of tissue

Table II. *Lactic acid, free carbohydrate, glycogen and oxygen uptake of brain tissue at p_H 7.6 and 37°. Glycogen calculated as glucose.*

Duration of exp. (mins.)	Lactic acid (mg.)							Oxygen uptake (mm. ³)			Lactic equivalent of extra O ₂ (mg.)	“Meyerhof quotient”
	Tissue and added lactic acid.	Tissue and added lactic acid after shaking	Lactic acid disappearing	Tissue alone.	Tissue alone after shaking	Lactic acid disappearing	Added lactic acid disappearing	Tissue and added lactic acid	Tissue alone	“Extra” oxygen		
	Initial IL	OL	IL-OL=A	Initial IP	OP	IP-OP=B	A-B	OL	OP			
235	19.8	14.45	5.35	2.98	0.825	2.155	3.195	5770	3981	1189	1.62	1.96
276	20.8	14.35	6.45	2.53	0.41	2.12	4.33	5815	4585	1230	1.677	2.58
257	21.0	12.5	8.5	2.09	0.285	1.80	6.7	6690	4485	2205	3.01	2.23
180	22.3	16.0	6.3	2.62	0.322	2.30	4.0	4255	3550	705	0.96	4.16
240	18.8	12.7	6.1	1.60	0.20	1.40	4.7	4565	3505	1060	1.47	3.2
180	17.0	14.7	2.3	1.36	0.33	1.03	1.27	2160	1750	410	0.56	2.3
256	19.85	17.35	2.5	1.045	0.276	0.77	1.73	2720	1990	730	0.995	1.74

Duration of exp. (mins.)	Expected synthesis of carbohydrate (mg.)	Free carb. in IP and IL (mean)		Free carb. in OL		Free carb. in OP		Glycogen in IP and IL (mean)	Glycogen in OL	Glycogen in OP	“Synthesis”	Wt. of tissue (g.)
		unhydrolysed	hydrolysed	unhydrolysed	hydrolysed	unhydrolysed	hydrolysed					
235	1.57	0.712	1.26	0.925	—	0.626	1.066	—	—	—	+0.3	2.0
276	2.65	0.317	0.849	0.375	—	0.345	—	—	—	—	+0.03	2.0
257	3.7	—	0.97	—	0.660	—	0.632	—	—	—	+0.028	2.0
180	3.04	—	0.923	—	0.699	—	0.689	—	—	—	+0.01	2.0
240	3.23	—	—	—	—	—	—	1.095	0.871	0.895	-0.024	1.5
180	0.71	—	—	—	—	—	—	0.449	0.221	0.363	-0.142	1.0
256	0.74	—	—	—	—	—	—	0.466	0.279	0.361	-0.082	1.0

in 3 hours. It is unnecessary to give the complete protocols of the manometer readings; one typical curve only of the oxygen uptake is, therefore, given (Fig. 1). One point, however, must be made quite clear. The experiments

Fig. 1. Typical oxygen uptake of brain tissue, with and without added lactate. The curves are for 2 g. of tissue.

were performed at 37° and in many cases were prolonged beyond 3 hours. The possibility of bacterial infection therefore arises. The manometer cups were always kept in bichromate mixture until just before use; the constituent solutions of the Ringer's solution were always boiled just before mixing and

adjusting the p_H . (The final solutions had the following composition: mammalian Ringer's solution 80 parts, 0.2 M KH_2PO_4 20 parts, and were adjusted to p_H 7.4 with sodium hydroxide.) The growth of bacteria in the cup of a manometer is almost always signalled by a sharp upward bend in the O_2 absorption curves, and this never occurred in our experiments. Finally, the same results were obtained in the experiments carried on for exactly 3 hours, as in those prolonged for a greater period.

It will be seen that, in spite of the fact that a "Meyerhof quotient" was always found, there was never any synthesis of carbohydrate, either as "free carbohydrate" or as glycogen. The "free carbohydrate" fraction contained material, the reducing value of which increased on acid hydrolysis: in spite of this, the hydrolysed and unhydrolysed values were respectively identical whether the tissue had been oxygenated in the presence or absence of lactate, though both were somewhat lower than the "initial values" (*i.e.* those tissues worked up immediately). In the case of the carbohydrate, the "expected synthesis" was several times greater than the amount of carbohydrate actually found, and far beyond the limits of any possible experimental error.

In two experiments, which can only be regarded as preliminary, we have attempted to measure the respiratory quotient of the "extra" oxygen. We used the method of Keilin [1929], that is, small cups were hung on the soda pots in the manometer cups, one containing baryta and the other acid. Small Barcroft apparatus were used containing 3 cc. and 0.3 g. of tissue. In two the cups were upset immediately after closing the taps to give initial values for CO_2 in tissue and Ringer's solution and in the other two this was done after a suitable oxygen uptake had been measured. In the two experiments so far performed the R.Q.'s of the extra oxygen were 0.9 and 0.86. We place very little reliance on these two results but if confirmed they would suggest that the extra oxygen does indeed completely oxidise part of the added lactic acid.

DISCUSSION.

It is possible that exception will be taken to these experiments on the ground that chopped brain is in an unphysiological condition, and could not be expected to behave normally. The question of the relative merits of slicing and chopping is rather a vexed one. It is quite true that finely minced muscle does not display the chemical phenomena which one associates with intact muscle—but the most striking peculiarity of minced muscle is that its power of taking up oxygen is much impaired. We find, on the other hand, that chopped brain takes up oxygen very rapidly indeed. We have found that it makes very little difference to the oxygen uptake of rabbit cortex whether it be sliced or chopped [Holmes, 1930], and Dixon and Elliott [1929] make the same claim with regard to many other tissues. We feel, therefore, that a sufficient answer to any such objection is that oxygen absorption does occur, and that a "quotient" can be demonstrated.

Warburg and Meyerhof do not seem to be agreed as to whether a quotient is actually obtained with brain tissue or not. Both authors employed slices of grey matter (rat brain). Warburg *et al.* [1924] give a single experiment in Ringer's solution in which the quotient was 1.6, while Meyerhof quotes some thirteen experiments, from which he concludes "...so dass auch hier entweder die ganze Atmung auf Oxydation der Milchsäure zu beziehen ist oder eine wenn auch geringe bilanzmässige Zunahme von Kohlehydrat stattfinden könnte."

Takane [1926] states that he estimated carbohydrates in brain; that in some of his earlier experiments there appeared to be an increase in carbohydrate, when they were conducted in oxygen in the presence of lactate, but that later experiments failed to confirm this. He gives, however, no figures. The balance of opinion seems to be that neither synthesis of carbohydrate nor the existence of a quotient can be demonstrated. We have confirmed the first of these suggestions—undoubtedly no synthesis, either of glycogen or free carbohydrate, occurs. But we have also shown that a "quotient" is most certainly obtainable.

The story must remain incomplete until we can trace the fate of that portion of the added lactic acid which disappears, over and above that which can be accounted for by the extra oxygen. Clearly there are many possibilities. For instance "synthesis" may in fact occur, but the product may be one which is not estimated by the methods we have employed. Again, it is possible that partial oxidation of the lactic acid molecule takes place, leaving a large portion of it in a condition in which it is no longer estimated as such, so that although lactic acid disappears, yet the extra oxygen is insufficient to account for its complete oxidation. Suppose for instance, that a formation of pyruvic acid took place by a removal of two atoms of hydrogen: the "quotient" would then be 6.0. This is intended merely as an illustration. It is almost certain that pyruvic acid is not formed, because (1) the very delicate sodium nitroprusside test for pyruvic acid is negative when applied to a colloidal ferric hydroxide filtrate from brain, which had been shaken with lactate in oxygen at 37° for 2 hours, (2) pyruvic acid would be estimated partially by the method for lactic acid, and (3) pyruvic acid rapidly passes to other products in the tissues. Similarly, the piperidine-nitroprusside test for acetaldehyde [Lewin, 1899] was negative when applied to the colloidal ferric hydroxide filtrate. Acetaldehyde would, on the one hand, be driven off during evaporation when the alcohol extraction method was used, and, on the other, would interfere with the lactic acid estimations when the trichloroacetic acid method was used. That substantially the same results were obtained by the two different methods is a fact of considerable importance.

We have demonstrated that a "Meyerhof quotient" is obtainable with brain tissue and added lactic acid¹, and that, in spite of this, there is not that synthesis of carbohydrate postulated by Meyerhof in his "cycle" theory. The

¹ The lithium salt used in these experiments was that of *D*-lactic acid.

fact that this is possible surely demands a suspension of judgment in the cases of those tissues where the existence of a "Meyerhof cycle" is claimed, but in which no actual synthesis has been demonstrated by chemical means.

SUMMARY.

1. Experiments are described in which lactic acid disappearance, oxygen consumption and production of carbohydrate by brain tissue, with and without added lactate, have been determined.

2. It has been shown that, although a "Meyerhof quotient" is readily obtainable under these experimental conditions, there is no synthesis of carbohydrate from that portion of the lactic acid which disappears but is not accounted for by O_2 uptake.

3. The fate of the lactic acid which disappears is not yet clear.

4. These experiments throw doubt on the reality of the alleged "Meyerhof cycle" in the case of cells in which the actual synthesis of carbohydrate has not been demonstrated by chemical estimation.

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