CCXC. GLYCOLYSIS IN BRAIN TISSUE.

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A SURVEY of figures in the literature for the values of the anaerobic glycolytic rates of brain and other tissues reported by different workers reveals a wide divergence of values. For rat cortex, Warburg *et al.* [1924] give a value for $Q_M^{N_2}$ of 19 by the manometric technique. For rabbit cortex, Krebs and Rosenhagen [1931] give values of from 10·3 to 20·9, when measured in serum by the manometric method and for white matter values of the order of one-third of these. These large values are only measured over short periods, and the maximum rates are often only obtained by the addition of pyruvic acid [Mendel *et al.*, 1931] the co-ferment T of Bumm *et al.* [1933] and tissue slices have been used. Our own practise [*e.g.* Ashford and Holmes, 1929; Ashford, 1933] has been to use chopped tissue suspended in Ringer solution buffered by phosphate or borate and to estimate the lactic acid by chemical methods. The higher value of $Q_M^{N_2}=20{\cdot}9$ given by Krebs and Rosenhagen for rabbit cortex actually corresponds to a lactic acid production of 84 mg. lactic acid per g. dry tissue per hour or 17 mg. per g. wet tissue, assuming the ratio of wet to dry weight to be 5 to 1. (It is perhaps worthy of note that a mean of 5 determinations of the ratio between dry and wet weight gave the former as 20·95 % of the latter and so a figure of 20 % has been assumed throughout with little error.) The figures given by Ashford [1933] for lactic acid production from glucose are much smaller and in fact only average 7 mg. per g. dry weight. It was therefore considered of interest to investigate this discrepancy more fully. Two factors were of obvious importance: (*a*) the state of the tissue used and (*b*) the incubation medium and atmosphere. The Warburg school has always used tissue slices suspended in Ringer solution buffered by 0·025 *M* $NaHCO_3$ in equilibrium with an atmosphere consisting of 5 % CO_2 and 95 % N_2, and the tissues have been washed free of salts at the conclusion of the experiment and dried and weighed. In the experiments to be reported here, this procedure has been tested against the method of chopping the tissue and incubating in a phosphate-buffered Ringer solution of the same p_H *in vacuo* or in an atmosphere of nitrogen. For convenience the general experimental methods used will be briefly described first.

GENERAL EXPERIMENTAL METHODS.

Rabbits have been used for all experiments. They have been killed by a blow on the back of the neck and bled before the brain was excised. The tissue was washed in Ringer solution and freed from blood clots and membranes. For the incubations, glass bottles similar to those described by Krebs [1933] were used. They were of approximately 100 ml. capacity and were fitted with a ground-in glass stopper carrying two narrow tubes with taps, one tube reaching to the

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bottom of the flask so that gas could be bubbled through the liquid placed there. The total volume of fluid used in each case was 10 ml. and the Ringer solution, bicarbonate and glucose were added separately in the form of 1 ml. of a solution of 10 times their physiological strength. The gas mixture was bubbled through slowly to avoid frothing and splashing for 20 mins. before the bottles were placed in the thermostat at 37°, fitted with an overhead horizontal shaker. The ground glass joints were simply lubricated with distilled water and a strong rubber band was added to hold the stopper firmly in position. At the end of the experiment, the tissues were either removed, washed free from salts and lactic acid, dried and weighed, lactic acid being estimated in the deproteinised fluid and washings, or the tissue and fluid were immediately precipitated by trichloroacetic acid and the tissue washed on the filter-paper before being dried and weighed. In either case the protein-free extract was made up to 50 ml. (except when phosphate was also being estimated, when the volume was 25 ml.) and lactic acid estimated by the method of Friedemann *et al.* [1927]—after copper-lime removal of carbohydrate *etc.*

Experimental.

For a comparison of sliced and chopped tissues and of the relative merits of bicarbonate and phosphate buffers, the following bottles were set up:

A. 1 ml. Ringer (× 10) + 1 ml. $NaHCO_3$ (× 10) + 1 ml. 2 % glucose + 7 ml. H_2O.
B. „ „ „ + 6 ml. H_2O.
C. 1 ml. Ringer (× 10) + 2·5 ml. 0·1 *M* phosphate buffer + 1 ml. 2 % glucose + 5·5 ml. H_2O.
D. „ „ „ + 4·5 ml. H_2O.

Into flasks A and C, about 100 mg. of wet cortex slices and into flasks B and D 1 g. of chopped tissue were placed. A gas mixture of 95 % N_2 and 5 % CO_2 was bubbled through A and B for 20 mins. from a cylinder and pure N_2 was bubbled through C and D for the same time. The p_H of the potassium phosphate buffer had previously been adjusted by addition of NaOH to have the same value as that of the bicarbonate buffer in the 5 % CO_2 atmosphere. The final concentration of bicarbonate and phosphate was 0·025 *M* in each case.

Table I. *Lactic acid production by brain tissue from glucose.*

All values in mg. lactic acid per g. dry tissue per hour.

		Slices		Chopped	
Exp.	Duration hours	N_2/CO_2; $NaHCO_3$	N_2; PO_4	N_2/CO_2; $NaHCO_3$	N_2; PO_4
1	2	57	—	16·0	14·65
2	2	52	32·5	15·5	10·6
3	2	54	33·5	16·0	15·6
4	1	60	—	—	—
5	2	60	—	—	—
6	1	69	—	—	—
	2nd hour	44	—	—	—

The results of this experiment are shown in Table I. It will be seen in the first place that the anaerobic glucolytic rate of the slices is considerably greater than that of the chopped tissue whatever the nature of the buffer. It is again considerably less in the phosphate buffer than in the bicarbonate buffer, whereas the rate of the chopped tissue is little diminished in phosphate buffer. The figure in no case reaches the maximum given by Krebs and Rosenhagen, but

that is understandable for a number of reasons. (i) Manometric measurements are only made for a short time whereas these experiments ran for 2 hours and the rate almost certainly falls off in that time. This is shown in Exp. 4 where the duration was only 1 hour and in Exp. 6 where in the first hour the figure is 69, but in the second hour only 44. This has been confirmed using the Dixon-Keilin manometers [1933] when results with cortex slices were obtained as follows:

$Q_M^{N_2} = 18{\cdot}8$ in the first hour, and $Q_M^{N_2} = 13{\cdot}9$ in the second hour.

(ii) Again, the whole of the experiment had naturally to be carried out on the brain of the same animal. For reliable chemical estimations about 100 mg. of wet tissue slices are required for each pot and it is manifestly difficult to obtain so much pure cortex from one rabbit brain. As the metabolism of the white matter is known from Krebs and Rosenhagen [1931] and Holmes [1932] to be considerably less than that of the grey matter, this adulteration naturally reduces the figure obtained. (iii) This question is even more serious in the case of the chopped tissue, for in this case about 1 g. of tissue is required to lead to reliable chemical estimations. Hence, although every effort is made to obtain pure cortex for these experiments the chopped tissue is probably at least 50 % white matter, and the values obtained would be expected to be significantly lower. In spite of these considerations however the experiments are considered to show the superiority of the slicing technique over chopping and the superiority of the bicarbonate medium over the phosphate buffer—to say nothing of the economy of tissue possible. These observations have a further significance in connection with the nature of the glycolytic mechanism in brain tissue and are again considered later.

It was a natural step to investigate the glycolytic rate of sliced brain tissue in the presence of both bicarbonate and phosphate of the same p_H. Phosphate buffer was added in the form of 0·1 *M* KH_2PO_4 buffer neutralised by NaOH to the same p_H as the $NaHCO_3/CO_2$ buffer in different amounts. The results of two experiments with varying concentrations of phosphate (and of potassium) are shown in Table II. It is again seen that the glycolytic rate is lowered in

Table II. *Lactic acid productions by brain slices in presence of bicarbonate and different concentrations of potassium phosphate.*

All values in mg. lactic acid per g. dry tissue per hour.

Exp.	Phosphate concentration *M*	Bicarbonate concentration *M*	Lactic acid mg.
1	0·00	0·025	41·2*
	0·02	—	51·2
	0·01	0·025	31·7
	0·03	0·025	21·5
	0·05	0·025	19·1
2	0·00	0·025	50·5
	0·03	—	36·3
	0·02	0·025	32·7
	0·04	0·025	26·2
	0·06	0·025	20·6

* This value is abnormally low and is probably an error.

phosphate buffer even in the presence of bicarbonate. At the time when the experiments were performed they were considered to show that not only was the glycolytic rate of brain tissue not increased by the presence of inorganic

phosphate (as is the case with muscle extract) but was actually diminished. Later work shortly to be published [Ashford and Dixon, K. C., 1935] has revealed a different interpretation and discussion is left to the later paper.

In an earlier communication [Ashford and Holmes, 1929] figures were given for the liberation of inorganic phosphate from brain tissue in Ringer solution or in borate buffers of different p_H values (ranging from 8·2 to 9·0) and it was shown that in the presence of glucose the liberation was somewhat less than in its absence, but that concentrations of sodium fluoride which gave 85 % inhibition of glycolysis had practically no effect on phosphate liberation in the presence of glucose. This was regarded as evidence of the absence of synthesis of phosphoric esters, and suggested the view that inorganic phosphate did not play a part in brain glucolysis. The glycolytic rates measured in the 1929 experiments were very low and at a more alkaline reaction (p_H 8·2) and it was therefore felt to be of importance to reinvestigate this phenomenon under the conditions which have now been described and which give a much higher rate of glycolysis. Accordingly bottles were set up as follows:

A. 1 ml. Ringer (×10) + 1 ml. bicarb. (×10) + 8 ml. H_2O.
B. ,, ,, + 8 ml. H_2O.
C. ,, ,, + 1 ml. 2 % glucose + 7 ml. H_2O.
D. ,, ,, + 1 ml. 2 % glucose + 2 ml. 0·1 *M* NaF + 5 ml. H_2O.
Each bottle also contained about 150 mg. of cortex slices.

A was precipitated immediately, B, C and D after incubation for 1 or 2 hours at 37°. The estimation of inorganic phosphate here presented a difficulty as inorganic phosphate notoriously does not diffuse easily out of the cell and it was not possible to grind the tissue as it was required for drying and weighing. The technique adopted was to precipitate the proteins with trichloroacetic acid and to allow the tissue to stand in the acid for ½ hour before filtering off. The tissue remaining on the filter-paper was washed with successive small amounts of acid and water and the filtrates collected in a 25 ml. volumetric flask. The method of Fiske and Subbarow [1925] was used for estimation of inorganic phosphate, and as a control estimations of lactic acid were also carried out. The

Table III. *Liberation of inorganic phosphate in brain slices.*

		Inorganic phosphorus				Lactic acid	
Exp. no.	Time hours	Initial	Final alone	Final +glucose	Final +glucose +fluoride	Glucose alone	Glucose +fluoride
1	1	41·0	76·5	66·5	64·8	12·0 (60)	2·0 (10)
2	2	39·0	76·0	54·7	54·1	12·0 (60)	0·4 (2·0)
3	1	51·5	92·0	75·0	73·0	13·8 (69)	—
	2	51·5	100·5	75·5	74·0	8·8 (2nd hr.) (44)	—

NOTE. The inorganic phosphorus figures are given as mg. per 100 g. wet tissue for the purposes of comparison with the 1929 figures. The lactic acid figures are mg. lactic acid per g. wet tissue per hour. The lower figures in brackets are per g. of dry tissue.

results given in Table III show that compared with the 1929 results the liberation of inorganic phosphate is somewhat larger at p_H 7·35 than at the more alkaline reaction, that it is lowered to about the same extent by the presence of glucose, but that the presence of fluoride affects the latter phenomenon to hardly any appreciable extent although the glucolysis is inhibited. This evidence,

to my mind, argues against the possibility of a participation of inorganic phosphate in brain glucolysis, since the "esterification" is independent of the rate of glucolysis, of the presence or absence of fluoride and of the p_H.

It has been shown in earlier communications that the ability of brain tissue to break down glycogen to lactic acid is very small, being of the order of $\frac{1}{10}$ of its ability to break down glucose (glucolysis) [see Ashford, 1933]. Since the work of Meyerhof and Kiessling [1933] has now revealed the nature of the intermediaries in the breakdown of glycogen by muscle extract, it was decided to see whether brain tissue might not be able to attack these more soluble intermediaries more easily than glycogen. The following bottles were set up to test this point[1]:

A. 2 ml. Ringer-bicarb. (× 10) + 1 ml. 2 % glucose + 7 ml. H_2O.
B. „ „ + 2 ml. Na pyruvate + 6 ml. H_2O.
C. „ „ + 3 ml. K α-glycerophosphate (3·3 %) + 5 ml. H_2O.
D. „ „ + „ „ + 2 ml. pyruvate + 3 ml. H_2O.

The sodium pyruvate used was made by neutralisation of freshly distilled acid and was about 0·2 *M*. The K α-glycerophosphate was prepared from the calcium salt (Merck) by precipitation with potassium oxalate at 0° and tested for freedom from oxalate ions.

Table IV shows the result of such experiments. The ability of the brain slices to produce lactic acid from the muscle intermediaries is evidently small.

Table IV. *Lactic acid production by brain slices from pyruvate and α-glycerophosphate.*

mg. lactic acid per g. dry tissue per hour.

		Substrates			
Exp. no.	Duration hours	Glucose	Pyruvate	α-Glycero-phosphate	Pyruvate + glycero-phosphate
1	4	55	15·0	6·0	9·0
2	2½	60	7·25	5·3	8·1
		25 ml. muscle extract			
3	1	10·25 (starch)	2·8	—	4·6

As a control on the reagents used, a muscle extract was prepared by the method of McCullagh [1928] and it was shown that this system was able to convert pyruvate + α-glycerophosphate into lactic acid, although the extract used was not a very active one.

Discussion.

The results reported in the early part of this paper serve to emphasise the difference in glycolytic mechanisms between brain glycolysis when glucose is the substrate (glucolysis) and glycolysis in a muscle extract. It is made increasingly clear that glucolysis in brain depends on the intact cell-structure and the cells that are least damaged, as by slicing, glucolyse at a much higher rate than the chopped tissue cells which are presumably more damaged in the process. In the author's experience it has never been possible to obtain a cell-free extract from brain tissue; grinding with sand, freezing or drying with acetone [Ashford and Holmes, 1929] all having yielded completely inactive

[1] I have to thank Mr K. C. Dixon for help with these experiments.

preparations. Perhaps too much reliance cannot be placed on this failure in view of the fact that Meyerhof [1932] has succeeded, where many others have failed, in obtaining an active cell-free extract from erythrocytes and has shown that it contains the phosphate system which is typical of muscle extract. On the other hand, Boyland and Mawson [1934], in a paper which appeared whilst this paper was in preparation, have reported their failure to obtain anything but feebly glycolysing extracts from acetone-dried tumour slices, and they review briefly the evidence of other workers that glucolysis in tumour does not involve a phosphate stage. These facts are not surprising in view of the increasing body of evidence that glucolyses in tumour and brain tissue involve the same mechanism, and that the level of the glucolysis depends on the presence of the co-ferment T, which has been shown to be pyruvic acid [Bumm *et al.*, 1933] and has been prepared from both tumour and brain [Kraut and Borkowsky, 1933].

The suggestion of the presence of two mechanisms for glycolysis in brain tissue which was first put forward in 1929 by Ashford and Holmes and has received further support from Ashford [1933] and others, whose evidence was reviewed in 1933, has not gone quite unchallenged. Lundsgaard [1933] studied the action of phlorrhizin on brain glucolysis and from his results expresses doubt as to its non-phosphate nature. He showed that glucose breakdown by brain tissue at p_H 8·3 was not inhibited by phlorrhizin although at p_H 7·2–7·3 it was inhibited to the extent of 60–70 %. The inference is that the glucolysis is non-phosphate in character at p_H 8·3 but not at the more physiological reaction. It is difficult however to see exactly what is the certain evidence that phlorhidzin is a specific inhibitor of phosphorylation and dephosphorylation alone as Lundsgaard claims and in any case it is not by any means unknown in biological systems for an inhibitor to be active at one p_H but not at another [see, for instance, Haldane, 1930]. Such an assumption would explain Lundsgaard's results with brain.

Ashford [1933] reviewed and amplified the evidence for a dual mechanism of glycolysis in brain tissue, but since then a most important piece of evidence had come to light in the form of the action of *dl*-glyceraldehyde. Mendel [1929] had shown that *dl*-glyceraldehyde inhibits glucolysis in brain and tumour tissue in exceedingly low concentrations and the author has verified this in the case of brain (Table V). Holmes [1934] has made the very pertinent observation

Table V. *Inhibition of glucolysis in brain slices by* dl-*glyceraldehyde.*

mg. lactic acid per g. dry tissue per hour.

Glucose alone	Glucose + glyceraldehyde
52	6·5 (0·0055 *M*)
55	3·8 (0·0031 *M*)

that while this substance inhibits glucolysis in chopped brain tissue it is without effect on lactic acid formation by muscle extract when starch or glucose + hexokinase is the substrate. This observation is of obvious importance since *dl*-glyceraldehyde is the only inhibitor so far described which does not inhibit both systems (as does, *e.g.*, fluoride or iodoacetic acid), although the mechanism of inhibition must be different in each case.

Two points are brought out in this paper to support the non-phosphate theory, *viz.* (i) the lack of correlation between disappearance of inorganic phosphate (inhibition of appearance) in the presence of fluoride and the glycolytic rate, and (ii) the small formation of lactic acid from pyruvate + α-glycerophosphate. The existence of two mechanisms for lactic acid formation is not

confined to brain tissue since Bumm and Fehrenbach [1930] have brought forward evidence for this view in the case of the white muscle of the hen and in the case of the kidney, while Gaddie and Stewart [1934] have recently brought out very interesting evidence for the existence of two mechanisms in the ventricle of the frog.

Unfortunately there is still no evidence of the nature of the intermediate steps in glucolysis in brain and allied tissues and, although all such tissues contain a strong glyoxalase, the position of methylglyoxal as an intermediary must now be viewed with suspicion since Meyerhof and Lohmann [1934] have reported that it arises under certain circumstances from the triosephosphate, and is in fact in the nature of a stabilisation product. The nature of the glucose breakdown and of the part played by coferment T (pyruvic acid) in it, still remains obscure but it is perhaps pertinent to note that Holmes (unpublished) has shown that *dl*-glyceraldehyde does not inhibit the production of lactic acid from methylglyoxal in the presence of brain tissue.

SUMMARY.

1. A comparison has been made by chemical methods of the relative glycolytic rates of sliced rabbit brain and chopped rabbit brain. It is shown that the glycolytic rate is much higher (3 to 4 times) in the sliced tissue and the rate is reduced when bicarbonate buffer and 5 % CO_2 are replaced by phosphate buffer of the same p_H.

2. Glucolysis in slices in $NaHCO_3/CO_2$ buffer is partially lowered in the presence of physiological concentrations of potassium phosphate buffer of the same p_H.

3. Inorganic phosphate is liberated rapidly from sliced tissue in the bicarbonate buffer. This liberation is less in the presence of glucose than in its absence, but is hardly affected by concentrations of fluoride which inhibit glycolysis.

4. Brain slices are able to produce lactic acid from pyruvate + α-glycerophosphate only to a very small extent.

5. Inhibition of glucolysis in brain tissue by small concentrations of *dl*-glyceraldehyde is confirmed.

6. The bearing of these and other results on the two-mechanism theory of glycolysis in brain is discussed.

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