CXVII. THE GLYCOLYTIC MECHANISMS OF BRAIN.

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In an earlier communication [Ashford and Holmes, 1929] evidence was brought forward of the existence in brain tissue of two glycolytic mechanisms, one concerned with the breakdown of glycogen, and requiring the presence of inorganic phosphate, the other with the breakdown of glucose and taking place without the participation of inorganic phosphate. This view of the non-participation of inorganic phosphate in glucose breakdown to lactic acid has received confirmation from later work by Bumm and Fehrenbach [1930; 1931]. They have studied the effect of the three factors, (i) *co-zymase*, which according to Meyerhof [1926, 1, 2; 1927] is concerned with the esterification and breakdown of the active phosphoric esters in the glycolytic mechanism of muscle, (ii) *hexokinase*, which Meyerhof [1927] had extracted from yeast, and shown to be concerned with an activation of glucose so that esterification, and breakdown of glucose took place in the presence of the muscle-enzymes, and (iii) the heat-labile *co-ferment T*, which they extracted from tumour tissue [Kraut and Bumm, 1928; 1929], and showed to accelerate the glucose breakdown by kidney, brain, white muscle and spleen, independently of the phosphate concentration. In the case of brain, Bumm and Fehrenbach [1931] showed that the rate of glucose breakdown was unaffected by addition of co-zymase, or by hexokinase, both of which are concerned with preliminary esterification of inorganic phosphate, but was increased by addition of co-ferment T. Furthermore, addition of arsenate, which is known to accelerate the rate of breakdown of phosphoric esters in muscle and yeast-juice, and hence to increase the velocity of carbohydrate breakdown, had no effect on the rate of glucose breakdown by brain tissue. Their experiments did not cover the glycogen mechanism, but they consider that their findings confirm our own with regard to the glucose mechanism in brain.

The experiments to be reported here are designed to amplify the evidence of the dual nature of the glycolytic mechanisms of brain tissue. There has not been entire agreement among workers as to a production of lactic acid from glycogen by brain tissue. Loebel [1925] did not consider that lactic acid was produced from glycogen, but he showed a production of small magnitude from hexosediphosphate. Tanko [1931] did not consider that his results showed a significant production even in the presence of added co-zymase. Haarmann [1932, 1] has results which indicate a production, on an average of the order of 24 mg. per 100 g. tissue in 3 hours, and this figure is in good agreement with those of Ashford and Holmes [1929] and with the figures reported here. Haarmann [1932, 1] also showed a production of 3 times that amount from hexosediphosphoric acid. From our point of view, a somewhat disconcerting

observation is one of Haarmann and Stratmann [1932] that in the presence of glycogen, glucose breakdown is partially depressed, since this finding would not be in agreement with a theory of the independent nature of the two glycolytic mechanisms of brain. If such is the case the amount of glycolysis in the presence of both substrates should be equal to the sum of the amounts found when each substrate is present alone. Haarmann's results are rather puzzling, as the inhibition seems to be greater with increasing concentrations of glucose, and is actually greatest at concentrations of glucose which give maximum velocities of lactic acid production. If a common enzyme were involved, and it were a question of competitive inhibition by substrates, the contrary result would have been expected. His experiments have not been repeated in their entirety here, but no evidence of inhibition of glucose breakdown in the presence of glycogen has been detected, when the two are present in equivalent and maximum concentrations. On the contrary the observed production has never been less than the sum of the productions from the two substrates alone and in two experiments was practically equal to the sum of the two independent productions (see Table I). Further evidence on these lines is brought forward in this communication.

Table I. *Lactic acid productions from glucose, glycogen and glucose + glycogen.*

All values in mg. per 100 g. chopped brain tissue.

Exp.	Time in hours	Glucose	Glycogen	Glucose + Glycogen	
				Found	Calculated
1	3	443	33	626	476
2	3	406	34	540	442
3	2¼	262	11	296	273
4	3	380	27	417	417

Anaerobic incubation at 37° in phosphate buffer p_H 7·4.

General experimental methods.

In all experiments, 1 g. of chopped brain tissue of the rabbit has been used. This tissue has been weighed out into a pyrex boiling-tube, and the buffered Ringer solution p_H 7·4 (5 cc.) and the substrate (1 cc.) have been added. The tubes were closed by a rubber stopper carrying a bent glass tube and pressure tubing, which after evacuation was closed by a stout screw-clip. The tubes were evacuated at the water-pump for a period of 4 minutes and sometimes filled with nitrogen and re-evacuated, although the latter procedure is unnecessary with a good pump and was later abandoned. The tubes were fitted into a wire frame and connected to the shaking arm of a Barcroft thermostat at 37°. This shaking ensures more complete mixing of the tissue and substrate during anaerobiosis, and more consistent results are obtained if this procedure is followed than if the tubes are allowed to remain at rest in the thermostat during incubation. After the incubation period, the proteins were precipitated by addition of 10 cc. of 20 % trichloroacetic acid, and after standing for ½ to 1 hour, the mixture was filtered into a 50 cc. volumetric flask. The tissue was subsequently ground with further small quantities of 20 % trichloroacetic acid and filtered. The combined filtrate was made up to 50 cc., and 30 cc. were usually taken for the removal of sugar by the $CuSO_4$ and $Ca(OH)_2$ method. Lactic acid was then estimated on 5, 10 or 15 cc. of the filtrate by the aeration method of Friedemann, Cotonio and Shaffer [1927], blank estimations being regularly performed.

Experimental.

In the first place, an attempt was made to investigate the production of lactic acid in the presence of both glucose and glycogen. Tubes were set up as follows:

A. 1 g. tissue + 5 cc. phosphate Ringer + 2 cc. H_2O.
B. ,, ,, + 1 cc. 2·8 % glucose + 1 cc. H_2O.
C. ,, ,, + 1 cc. 2·8 % glycogen + 1 cc. H_2O.
D. ,, ,, + 1 cc. 2·8 % glycogen + 1 cc. 2·8 % glucose.

The p_H of the Ringer solution throughout these experiments was 7·4.

After incubation the protein precipitation and lactic acid estimations were carried out. The results of several experiments are shown in Table I. For the sake of clarity, only the actual lactic acid productions during the incubation period are given. In this and all subsequent tables the column labelled "calculated" is obtained by adding the observed productions from each substrate separately, *i.e.* the results obtained from tubes B and C. The first two experiments show, if anything, an increased production of lactic acid from glucose in the presence of added glycogen, and there is certainly no suggestion of inhibition of glucose breakdown. (At that time Haarmann and Stratmann's results were not to hand.) It should be noted that it was known from previous work that the concentrations of glucose and glycogen were sufficient to give a maximum and linear glycolytic rate over a period of 3 hours, and Haarmann and Stratmann agree with this in the case of glucose. Exps. 3 and 4 are in very good agreement with the theory that the lactic acid productions from glucose and glycogen proceed independently of one another, since the "calculated" and "found" values are in very good agreement with one another. It was felt, however, that owing to the fact that the lactic acid production from glycogen is quantitatively very small compared with that from glucose, it would be difficult to demonstrate accurately a summation of glycolysis in the simultaneous presence of both substrates, in view of the experimental error which is inherent in investigations of this type. It was known that considerably more lactic acid is produced from added hexosediphosphate than from glycogen (4 or 5 times, in fact), and, since the evidence was that a hexosephosphate stage was part of the glycogen mechanism, it was decided to try the result of competition between glucose and hexosediphosphate, and at a later stage, competition between glucose and hexosemonophosphate ("Robison ester"). The hexosediphosphate used was a solution of the potassium salt obtained by grinding the calcium salt (Messrs Baeyer's "Candiolin") with the calculated amount of potassium oxalate, and centrifuging the precipitated calcium oxalate. The solution was tested for freedom from oxalate ions. The solution was 7·9 %, *i.e.* equivalent to 2·8 % hexose. Tubes were set up as follows:

A. 1 g. tissue + 5 cc. phosphate Ringer + 2 cc. H_2O.
B. ,, ,, + 1 cc. 2·8 % glucose + 1 cc. H_2O.
C. ,, ,, + 1 cc. K hexosediphosphate + 1 cc. H_2O.
D. ,, ,, + 1 cc. K hexosediphosphate + 1 cc. 2·8 % glucose.

The results of several experiments are shown in Table II. It will be seen that in the main (in fact, in 5 out of the 6 experiments) the results found and the results calculated are in agreement with a theory of the dual nature of the modes of lactic acid formation. The average values of the "found" and "calculated" quantities are, in fact, in very good agreement with such a view.

To extend this idea further, the experiments were repeated using hexosemonophosphate instead of the diphosphate, since this substance probably

Table II. *Lactic acid productions from glucose, hexosediphosphate, and glucose + hexosediphosphate.*

All values in mg. per 100 g. chopped brain tissue.
Anaerobic incubation at 37° in phosphate buffer p_H 7·4.

Exp.	Time in hours	Glucose	Hexosedi-phosphate	Glucose + Hexosediphosphate Found	Glucose + Hexosediphosphate Calculated
1	3	454	174	674	628
2	3½	493	108	605	601
3	3	453	147	487	600
4	3	284	157	469	441
5	3	327	167	473	494
6	3	340	162	507	502
			Average	536	544

approximates more closely to the "active" ester concerned in glycogen breakdown by muscle than does the diphosphate, if we accept Meyerhof's [1926, 2] view. The monophosphates used in these experiments were (i) a sample prepared by the action of maceration juice on glucose as described by Harden [1932], and was therefore a mixture of the glucose, mannose and fructose phosphates, and (ii) a sample of the barium salt obtained from British Drug Houses. The potassium salt was prepared by treatment with the calculated amount of potassium sulphate and was made equal to 5·1 %, *i.e.* equal to 2·8 % hexose. Tubes were set up as in the previous experiment except that the monophosphate took the place of the diphosphate. The results are shown in Table III, and it will be seen again that the quantities observed and those calculated are in very good

Table III. *Lactic acid productions from glucose, hexosemonophosphate, and glucose + hexosemonophosphate.*

All values in mg. per 100 g. chopped brain tissue.
Anaerobic incubation in phosphate buffer p_H 7·4.

Exp.	Time in hours	Glucose	Hexosemono-phosphate	Glucose + Hexosemonophosphate Found	Glucose + Hexosemonophosphate Calculated
1	2½	322	71	381	393
2	3½	493	109	618	602
3	3	453	91	352*	544*
4	3	284	92	311	376
5	3	300	97	432	397
6	3	338	82	396	420
7	3	419	110	513	519
			Average	458	449

* Omitted from average.

Table IV. *Lactic acid productions from different concentrations of hexosemono- and hexosedi-phosphates.*

Concentrations of substrate as in text.
All values in mg. lactic acid per 100 g. tissue.
Anaerobic incubation at 37° for 2 hours.

Amount of substrate added cc.	Hexosemono-phosphate	Hexosedi-phosphate
1	63	91
0·5	58	101
0·25	1	54

agreement with the theory that the two modes of breakdown involve different enzyme systems. It is further shown in Table IV that the concentrations of phosphoric esters employed were sufficient to maintain the maximum rate of glycolysis over a 2-hourly period. By comparison with Table V, where the results for a 3-hourly period are given, it will be seen that, at any rate up to 3 hours, the rate of lactic acid production is approximately linear.

Table V. *Lactic acid productions from different sugars by brain tissue.*

All values in mg. lactic acid per 100 g. tissue.
Anaerobic incubation at 37° for 3 hours. The concentration of substrate was 0·4 % or its equivalent.

Exp.	Glucose	Mannose	Fructose	Galactose	Hexosemono-phosphate	Hexosedi-phosphate
1	416	340	38	58	91	147
2	385	475	35	93	92	157
3	350	436	23	79	97	167
4	428	472	—	—	82	162
Averages	395	430	32	77	91	158

As a confirmatory point the effect of the addition of other hexoses to the brain + glucose was tried. The relative rates of glycolysis of other hexoses were first of all investigated, and the findings are shown in Table V. It will be noted that the lactic acid production is considerably less from fructose than Haarmann [1932, 1] gives, and is greater in the case of hexosediphosphate. For the former substrate Haarmann gives 90 mg./100 g. in 3 hours, and for the latter 76 mg./100 g. It is to be noted that both Kahlbaum and B.D.H. hexoses were used and gave the same result. It was quite obvious that mannose was the best choice for these experiments as it is glycolysed at a very similar rate to glucose. Tubes were therefore set up as follows:

A. 1 g. tissue + 5 cc. phosphate Ringer + 2 cc. water.
B. „ „ + 1 cc. 2·8 % glucose + 1 cc. water.
C. „ „ + 1 cc. 2·8 % mannose + 1 cc. water.
D. „ „ + 1 cc. 2·8 % mannose + 1 cc. 2·8 % glucose.

The results of four experiments are shown in Table VI. It will be seen that with the possible exception of Exp. 2, where, in any case, the lactic acid pro-

Table VI. *Lactic acid productions from glucose, mannose, and glucose + mannose.*

All values in mg. lactic acid per 100 g. tissue.
Anaerobic incubation at 37° in phosphate Ringer solution p_H 7·4.

Exp.	Time in hours	Glucose	Mannose	Glucose + Mannose	
				Found	Calculated
1	2	170	158	178	328
2	2	110*	140	148	250
3	2	287	253	277	540
4	2	240	295	317	535
	Averages	202	212	230	413

* Bad lactic estimations.

duction from glucose is abnormally low, the lactic acid production in the presence of the two sugars together is never significantly greater than in the presence of glucose alone, and never in any case approaches the sum of the two ("calculated"

column). In this respect it differs markedly from the two cases of glucose + hexosemonophosphate, and of glucose + hexosediphosphate, and makes it reasonably certain that the same enzyme system is responsible for the breakdown of glucose and mannose. As a final point, the effect of the addition of both mono- and di-phosphates together was tried. Tubes were set up as follows:

A. 1 g. tissue + 5 cc. phosphate Ringer + 2 cc. water.
B. ,, ,, + 1 cc. hexosemonophosphate + 1 cc. water.
C. ,, ,, + 1 cc. hexosediphosphate + 1 cc. water.
D. ,, ,, + 1 cc. hexosediphosphate + 1 cc. hexosemonophosphate.

The results are shown in Table VII, and are a little surprising in so far that the observed value is never so large as the value calculated from the sum of the separate productions, but is consistently higher than the production from the

Table VII. *Lactic acid productions from hexosemonophosphate, hexosediphosphate, and hexosemonophosphate + hexosediphosphate.*

All values in mg. per 100 g. tissue.

Exp.	Time in hours	Hexosemonophosphate	Hexosediphosphate	Monophosphate + Diphosphate: Found	Monophosphate + Diphosphate: Calculated
1	3½	109	108	193	217
2	3	91	147	152	238
3	3	92	157	180	269
4	3	97	151	201	248
	Averages	97	141	181·5	243

most rapidly glycolysed substrate (the diphosphate). This effect cannot be due to the absence of sufficient substrate when one only is present, since it is shown (see Tables IV and V) that half the concentration of the substrates actually used will give a maximum velocity of glycolysis up to 2 hours, at least, and that in the concentrations employed in the experiments summarised in Table VII, the lactic acid production is linear up to 3 hours. We are therefore thrown back on the assumption that the mechanisms responsible are alike in many respects, but have at some point a different path. This point is briefly discussed later.

Discussion.

The experiments described here amplify the evidence that brain tissue contains two mechanisms for the production of lactic acid. The limited ability of brain tissue to produce lactic acid from glycogen may, with a certain measure of probability, be regarded as being due to its relative inability to synthesise active phosphoric esters, since it is shown that hexosemono- and hexosedi-phosphates are converted into lactic acid to a much greater extent than is glycogen. This relative inability may be due to the low concentration of the "co-enzyme" factor, for, in the case of muscle, Meyerhof [1927] has shown that lactic acid is produced only from the phosphoric esters and not from glycogen in presence of a low concentration of the co-enzyme. It is now generally agreed that "co-zymase" consists of adenyl pyrophosphate, and that in the presence of magnesium ions [Lohmann, 1931], this substance will activate the "apozymase" of muscle. In the case of brain, Pohle [1929] has demonstrated the presence of adenylic acid and the author has shown the presence of pyrophosphate [Ashford and Holmes, 1929; 1931], while Sym, Nilsson and Euler [1931], from fermentation experiments with extracts of different organs, claim that brain, and more particularly grey

matter, contains a fairly large amount of "co-zymase." On the other hand, Tanko [1931] was unable to demonstrate any significantly increased lactic acid production from glycogen by brain tissue, even in the presence of added co-zymase, so that in fact, the presence or absence of this factor is probably not the only reason for such findings. Nevertheless, the view has been taken in this work, that the systems responsible for the breakdown of glycogen and of the phosphoric esters in brain are almost entirely the same. The results shown in Table VII suggest that the mechanisms responsible for the breakdown of the hexosemono- and hexosedi-phosphoric esters follow some slightly different paths, but involve on the whole a common enzyme system. It is somewhat unexpected that the diphosphate is more easily broken down than the monophosphate, as the contrary result holds for muscle. It is unlikely that the mode of ester breakdown merely involves preliminary hydrolysis to the hexoses, since we have found that increased concentration of glucose above that used in these experiments does not increase the rate of breakdown. This fact was also shown by Haarmann and Stratmann [1932], using much larger amounts of tissue (2·5 g. instead of 1 g. in fact). Again, it is known from the work of Morgan and Robison [1928] that the hexosediphosphoric ester is constitutionally 2:5-fructose-1:6-diphosphate, and fructose has been shown to be very little attacked anaerobically by brain tissue. We do not of course know, however, how the unstable γ-fructose will behave in the presence of brain tissue.

Of the actual intermediary steps of the lactic acid production in brain we are extremely ignorant, nor indeed is there much certain information in the case of muscle. Haarmann [1932, 2] in the case of the dog, and Holmes [1933] in the case of the frog, have shown that methylglyoxal is rapidly converted into lactic acid by brain tissue, as is also pyruvic acid to a much less extent [Haarmann, 1932, 3]. In the first case the production of lactic acid is of the order of 763 mg./100 g. tissue in 3 hours, while in the second case, Haarmann gives figures of 149 mg./100 g. and 253 mg./100 g. in 3 hours. There seems little doubt of the probable occurrence of methylglyoxal in some stage in brain glycolysis. Glycolysis, for instance, is inhibited by iodoacetic acid, which is known to inhibit glyoxalase activity, and Dakin and Dudley [1913] and Meyerhof [1925] have shown the presence of glyoxalase in brain tissue. Meyerhof also showed that the rate of lactic acid production from methylglyoxal is 2 to 3 times as great as the rate when glucose is the substrate, suggesting that the limiting factor is the ability of the tissue enzymes to produce methylglyoxal from the carbohydrate. Case and Cook [1931] showed that anaerobic glycolysis in muscle was accompanied by a production of pyruvic acid, and that conditions which favoured lactic acid production, also favoured pyruvic acid production. It is tempting to speculate whether the glucose mechanism in brain may not give rise to methylglyoxal as intermediary, while the glycogen mechanism gives rise to pyruvic acid. So far, it must be admitted, there is no real evidence of the intermediary steps in brain glycolysis. Further work is obviously required in this field.

SUMMARY.

1. Glucose breakdown to lactic acid by brain tissue is not inhibited by the presence of glycogen, and the figures indeed suggest that the two mechanisms are quite independent.

2. When glucose and hexosemonophosphate or hexosediphosphate are present together, the glycolysis observed is the sum of the glycolyses produced from each substrate separately, showing that the enzyme systems responsible for the two effects are independent.

3. When glucose and mannose are present together, the results show that the same enzyme is responsible for their breakdown.

4. When hexosediphosphate and hexosemonophosphate are present together, the results suggest, in the main, a common enzyme system.

5. Figures are given for lactic acid production from glucose, mannose, fructose, galactose and the hexosemono- and hexosedi-phosphates by brain tissue.

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