I. CHANGES IN PHOSPHATE DISTRIBUTION DURING ANAEROBIC GLYCOLYSIS IN BRAIN SLICES

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IT has been shown that the changes in phosphate distribution during fermentation in the living yeast cell are so consistent with the theory, developed from experiments with cell-free extracts, that anaerobic glycolysis takes place by intermediate phosphorylation that there can be little doubt that the processes are analogous [Macfarlane, 1939]. It was pointed out at the same time that in cells the normal glycolysis may be maintained by a cycle of P transference which involves such small amounts of P that the isolation or detection of phosphorylated intermediate products may be extremely difficult. There has been considerable argument over the existence in certain tissues, e.g. brain, tumour, retina, embryo etc., of a non-phosphorylating type of glycolysis, though the preparation of cell-free phosphorylating extracts from these tissues [Geiger, 1940; Boyland & Boyland, 1935; Kerly & Bourne, 1940; Meyerhof & Perdigon, 1939] supports the view that an analogous phosphorylation takes place in the cells. The fact that glucose can act as a phosphate acceptor in dialysed brain dispersions [Ochoa, 1940] is even more convincing evidence of the correctness of this theory. We have examined the changes in P distribution which take place during anaerobic glycolysis and autolysis in slices of rabbit brain and find that they are consistent with the view that the glycolysis is dependent on phosphorylation.

Methods. The rabbits were killed by a blow on the neck and the brain removed immediately into Ringer solution. Slices were cut in the manner usual for manometric experiments and kept aerobically in Ringer solution at room temperature until sufficient material was obtained. Roughly equal amounts of tissue were drained and placed in tared flasks containing the medium (0·5 g. to 5 ml. medium) and the wet weight of tissue obtained by reweighing. The flasks were immersed in a thermostat at 37° and shaken mechanically. A stream of N_2, containing 5% CO_2, was passed through for 3 min.; the outlet was then closed and the flasks were left connected to the gas cylinder. Enzyme action was stopped by the addition of trichloroacetic acid in final concentration 3–5%. Approximately 20–25 min. elapsed between the death of the animal and the start of anaerobic incubation, at which point the zero value was taken. It was anticipated that this method of weighing, though it causes the minimum injury to the slices, would be subject to considerable error owing to occlusion of liquid in bunches of slices; it was found, however, that though there were occasional anomalous values the results of manifold experiments were on the whole in good agreement. The average dry weight was 12·5% of the wet weight.

The medium used was a phosphate-free modification of Krebs & Henseleit [1932] Ringer solution, saturated with the N_2/CO_2 mixture, the K_2HPO_4 being replaced by an amount of KCl sufficient to give the same ionic concentration of K; for glycolysis it contained in addition 0·2% glucose. An experiment was

performed in which the medium used was horse serum which had been dialysed against P-free and bicarbonate-free Ringer solution. Before use the serum was inactivated by heating for 2 hr. at 56°; bicarbonate was then added in the required amount and the serum saturated with the gas mixture. Contrary to expectation the serum did not decrease the autolysis of the tissue, as manifested by the liberation of phosphate, and its use therefore offered no advantage over Ringer solution. Estimations were made on aliquots of the trichloroacetic acid filtrate as follows: total P after ashing, and inorganic plus creatine P by direct estimation, by Briggs's method; pyrophosphate by calculation from the hydrolysis curve in *N* HCl at 100°, pyro-P=Δ (7 min.–0 min.)—Δ (30 min.–7 min.); hexosediphosphate-P by the amount of alkali-labile P formed by incubation with zymohexase (dialysed muscle extract) in presence of 0·1 *N* KCN at *p*H 6·8 and 37° for 15 min.; lactic acid by oxidation with ceric sulphate [Gordon & Quastel, 1939] and iodimetric titration of the acetaldehyde-bisulphite compound. Values are expressed in mg./g. dry weight.

Changes in inorganic and pyrophosphate-P during anaerobic glycolysis. Table 1 shows that the inorganic P content of slices incubated anaerobically with glucose for 30 min. was much less than that of slices incubated without glucose, both absolutely and also relatively to the total acid-soluble P, which increased noticeably during autolysis. There was, however, no absolute decrease in inorganic P compared with the initial value and therefore no direct proof of

Table 1. *Anaerobic incubation of brain slices at 37°*

The values obtained for the three experimental conditions, A, B, C, show the extent of variation in aliquots of the same brain or in similar aliquots from different brains. The horizontal line of figures is not necessarily from the same animal in A, B and C.

A Initial				B 30 min. glycolysis				C 30 min. autolysis			
	Inorganic P				Inorganic P				Inorganic P		
Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.	Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.	Total P mg./g.	mg./g.	% of total	Lactic acid mg./g.
2·80	1·52	54	3·52	3·12	1·56	50	37·2	3·36	2·24	67	2·6
2·96	1·60	54	3·76	2·96	1·54	52	32·5	2·80	1·84	66	2·9
2·72	1·60	59	—	2·56	1·48	58	33·1	3·20	2·24	70	—
2·72	1·52	56	3·20	2·56	1·36	53	37·2	2·96	2·16	73	2·7
2·32	1·28	55	—	2·88	1·68	58	38·8	3·36	2·16	74	—
Average: 2·7	1·5	55·5	3·5	2·8	1·5	54	36	3·1	2·1	68	2·7

esterification. The observed values were nevertheless quite consistent with the idea that the whole of the P necessary for the glycolytic cycle may be derived from adenylpyrophosphate, and that in the absence of glucose the labile P is broken down with liberation of inorganic phosphate; the fact that after short anaerobiosis brain slices lose their capacity to break down glucose [Dickens & Greville, 1933] affords some support for this view. It was thought possible that re-esterification of inorganic P might be demonstrated by allowing a partial breakdown of the combined P to take place before the addition of glucose. It was found, however, that in the absence of glucose the breakdown of the pyrophosphate-P initially present was so rapid, even at 20°, that it was complete in 3 min. (Table 2), little more than the time considered necessary to secure anaerobic conditions, and that after this time the slices had also lost the ability to form lactic acid (Table 3). In the presence of glucose (Table 2) the initial level of

Table 2. *Changes in P fractions during incubation at 37°*

Incubation time min.	Glucolysis					Autolysis		
	Total P	Inorganic P	Pyro-P	Lactic acid	$Q_L^{N_2}$	Total P	Inorganic P	Pyro-P
0	2·12	0·98	0·16	3·4	—	2·83	1·39	0·14
3	2·74	1·44	0·17	13·7	51	3·10	1·79	0
10	2·77	1·58	0·06	16·1	19	3·13	1·83	0·01
30	2·88	1·69	0·07	37·2	17	3·34	2·18	0
90	2·74	1·81	0·06	80·8	13	3·38	2·46	0·01

Table 3. *Addition of glucose after preliminary anaerobic incubation for varying times*

	Total time min.	37°				20°			
		Total P mg./g.	Inorganic P		Lactic acid mg./g.	Total P mg./g.	Inorganic P		Lactic acid mg./g.
			mg./g.	% of total			mg./g.	% of total	
No glucose	0	2·69	1·54	57	3·2	2·56	1·38	54	2·2
,,	3	2·94	1·87	64	3·2	2·77	1·76	63	2·6
,,	15	2·85	1·91	67	3·2	2·58	1·76	68	2·2
,,	30	2·99	2·18	73	2·4	2·74	1·90	69	2·8
Glucose added:									
Initially	30	2·59	1·48	57	32·8	2·61	1·41	54	16·9
After 3 min.	30	2·83	1·95	69	4·8	3·14	1·87	60	5·2
After 15 min.	30	3·02	2·10	70	3·0	2·88	2·13	74	2·6

pyrophosphate-P was maintained for a few minutes and then decreased to a fairly constant level approximately one-third of the original value. There was a concomitant decrease in the rate of lactic acid production, the $Q_L^{N_2}$ falling from a value of 50 over the first few minutes to about 15 over the next hour. The latter is that commonly reported for brain slices, but the equilibration period of 10–20 min. necessary in the manometric technique precludes the observation of a rapid initial glycolysis. The abnormally low initial value of total and inorganic phosphate in this experiment is probably due to the fact that the brain slices were immersed immediately after cutting in glucose-Ringer solution, instead of glucose-free Ringer; it was found on several occasions that this procedure prevented the liberation of some acid-insoluble compound during the aerobic storage of the slices. On the presumption that the pyrophosphate-P is derived from adenyl-pyrophosphate, the amount of pyro-P available for the formation of hexose-phosphoric esters, etc. is of the order of 0·1–0·2 mg. P per g. dry tissue, roughly only 5 % of the total acid-soluble P.

Estimation of hexosediphosphate. From previous experiments with yeast it seemed probable that the ester present in largest amount would be hexose-diphosphate and attempts were made to identify this ester using larger quantities of tissue. The experiments were made with brain slices from three or more rabbits (10–20 g. tissue), the trichloroacetic acid filtrates from the individual experiments being pooled for fractionation. The filtrate was adjusted to *p*H 8 and treated with barium acetate and then with alcohol to 10 % final concentration. The crude Ba precipitate was dissolved in HCl and the Ba removed as sulphate. In most of the experiments attempts were made to remove the inorganic P by addition of $MgCl_2$ and NH_3, but a considerable amount of organic P was carried down with the precipitate; in the third glycolysis experiment (cf. Table 4) the estimation of hexosediphosphate was made without

further purification. The accuracy of the method used—enzymic formation of alkali-labile P—was in this case seriously limited by the tenfold excess of inorganic P over organic P; the method is, however, the most specific available and the positive results quoted are outside the experimental error. There was only a poor recovery (30–70 %) of the organic P calculated to be present in the crude Ba precipitates, probably owing to adsorption on the $BaSO_4$ precipitate. Table 4 shows that in two out of the three glycolysis experiments hexosediphosphate was detected; the amounts present, though absolutely very small, were of the order expected from the amount of pyrophosphate available. No hexosediphosphate was found in the autolysed slices and only a trace in the unincubated slices.

Table 4. *Fractionation of acid-soluble P compounds in brain slices*

	mg. P/g.				
			Glycolysis		
	Initial	Autolysis 20 min.	20 min.	20 min.	15 min.
Total P	2·90	3·33	3·25	3·28	3·28
Inorganic P	1·56	2·16	1·66	1·75	1·84
Pyro-P	0·24	—	—	0·20	0·11
Hexosediphosphate-P	0·01	0	0·03	0·09	0
P in soluble Ba salts,	0·80	0·93	0·89	1·02	0·88

The fractions of acid-soluble organic P hydrolysed between the 7th and 180th min., and that not hydrolysed in 180 min., remained almost the same during anaerobic incubation with or without glucose. The amount of P present in compounds with soluble Ba salts was also of the same order in all experiments. It therefore seems unlikely that these compounds are precursors of lactic acid but their nature was not investigated. According to Tschalisow [1932] and Palladin [1934] hexosemonophosphate is present in brain tissue, but the identification of this "lactacidogen" was very unspecific. Booth [1935] has isolated from both sheep and horse brain a compound, identified as a choline ester of sphingosinephosphoric acid, which accounted for at least 50 % of the soluble organic P present in the tissue. The soluble fraction probably includes the nucleotide-P of the coenzymes.

Discussion

It was shown by Engel & Gerard [1936], working with lobster and crab nerve, that during anaerobiosis there is an extensive hydrolysis of both phosphoarginine and adenylpyrophosphate with a corresponding rise in inorganic phosphate; both these compounds were resynthesized by subsequent exposure to O_2. A rapid breakdown of phosphocreatine takes place in mammalian brain [Kerr, 1935]. We have shown in the present experiments that on anaerobic incubation of rabbit brain slices there is a very rapid breakdown of pyrophosphate-P, followed by a further steady liberation of inorganic P from a source previously insoluble; in the presence of glucose there is only a partial breakdown of pyrophosphate and a very slight increase in inorganic P. It is clear that the maintenance of the inorganic phosphate at its original level during glycolysis is not in itself proof of a phosphorylating glycolysis, since the continuous liberation of P is presumably only one aspect of a tissue autolysis which would be largely prevented by the utilization of glucose, irrespective of the mechanism of glycolysis. The correlation

however between the rapid loss of glycolytic capacity, observed first by Dickens & Greville [1933], and the rapid breakdown of pyrophosphate, and also between the aerobic restoration of this capacity [Quastel & Wheatley, 1937] and the aerobic resynthesis of adenylpyrophosphate in similar tissue [Engel & Gerard, 1936], affords considerable support for the view that the anaerobic glycolysis in brain tissue takes place through the intermediate formation of phosphoric esters by transference of P from adenylpyrophosphate. This view is strengthened by the detection of hexosediphosphate in the glycolysing tissue. The amount of P involved in the glycolytic cycle in brain tissue with a $Q_L^{N_2}$ about 15 is apparently of the order 0·1–0·2 mg. P per g. dry tissue; in living yeast the corresponding figures are of the order 1–2 mg. P per g. and $Q_{CO_2}^{N_2}$ 100 to 200. The glycolytic rate may therefore afford a rough guide to the amount of P engaged in the cycle.

It appears from the high rate of glycolysis in the first few minutes that the values of $Q_L^{N_2}$ generally obtained in manometric experiments are considerably lower than the maximum glycolytic capacity of the tissue. This is consistent with the occasional observation of an aerobic glycolysis which exceeds the normal anaerobic glycolysis, as in certain experiments with NH_4^+ or maleic acid [Weil-Malherbe, 1938] and with guanidines [Dickens, 1939]. This fact bears on the significance of the Pasteur-Meyerhof quotient, since it is obviously difficult to correlate the aerobic and anaerobic activities of a tissue if the enzyme system is so greatly affected by anaerobiosis.

Geiger [1940] has shown that during glycolysis in brain extracts inorganic phosphate is esterified with formation of hexosephosphoric esters, while adenylpyrophosphate, cozymase and magnesium are essential coenzymes for glycolysis. His main objection to the view that hexosephosphoric esters are intermediaries in the glycolysis is the fact that the rate of lactic acid formation from these esters by brain extract is much lower than that from glucose, and is not accelerated by arsenate. The imbalance between the rates of formation and breakdown of hexosephosphoric esters in cell-free extracts clearly does not exclude phosphorylation as the main path of glycolysis; it is a phenomenon which is characteristic of alcoholic fermentation by yeast juice and one to which we owe the isolation of hexosediphosphate by Harden & Young. It has been shown that the rapid fermentation of phosphopyruvic acid, and therefore of its precursor hexosediphosphate, is dependent on the presence both of glucose (or some other acceptor) and inorganic phosphate [Meyerhof & Kiessling, 1935]. The accelerating action of arsenate is apparently an even more complex process for which a number of explanations have been offered [Needham & Pillai, 1937; Meyerhof *et al.* 1937; Negelein & Brömel, 1939]; it seems that it may be influenced in certain cases by factors not yet understood, for Pillai [1938] observed that, contrary to the usual experience, lactic acid formation from glycogen and hexosediphosphate by his fresh muscle extracts was not accelerated by arsenate, though the rate of dismutation of triosephosphate and pyruvate was increased.

Summary

During anaerobic glycolysis by rabbit brain slices the inorganic phosphate is maintained at its initial level while the content of pyrophosphate-P falls to about one-third of the original. Hexosediphosphate was present in small amounts in the glycolysing tissue. Anaerobic incubation without glucose led to a very rapid disappearance of pyrophosphate-P, with a simultaneous loss of glycolytic capacity. These results are considered to support the view that glycolysis is

maintained by a cycle of P transference involving approximately 0·1–0·2 mg. P per g. dry brain tissue.

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