LXX. THE PHOSPHORYLATION OF CARBOHYDRATE IN LIVING CELLS

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(Received 1 March 1939)

It has been shown previously that the fermentation of sugar by living yeast is accompanied by a decrease in the amount of orthophosphate present in the cell as compared with the resting value, and that this decrease persists until the sugar added has been fermented, at which stage the orthophosphate tends to resume its original value [Macfarlane, 1936]. This coincidence of phosphorylation with fermentation provided evidence of what had hitherto been an assumption, namely, that the fermentation of sugar by living yeast takes place in a manner analogous to that found in yeast juice and other cell-free fermentations; through the intermediate formation and decomposition of hexosephosphoric esters. The scale and technique of the previous experiments were not suitable for the identification of any phosphoric esters formed; by working with larger quantities it has now been established that hexosediphosphate is formed during the normal fermentation of sugar by living yeast and that phosphoglyceric acid is also present when the normal fermentation has been inhibited by the addition of NaF. The experiments to be described also illustrate several points of difference between the fermentation of the living cell and that of the dead or cell-free preparation which are interesting in the general question of glycolysis by living cells.

Phosphoric esters formed in living yeast during fermentation

Mild ale yeast was obtained fresh from the brewery vats, washed three times with water and well drained on Büchner funnels. The yeast was used without further pressing, and the water content therefore varied somewhat in different batches. The fermentations were carried out at room temperature in large bottles attached to 500 ml. nitrometers filled with brine. Weighed amounts (100–400 g.) of yeast were mixed with twice their weight of water and the contents of the bottle saturated with CO₂ before connexion to the nitrometers. After an interval of 10 min. to allow equilibrium to be established, the following additions were made to different bottles: (a) water only; (b) NaF, in final concentration 0.05 *M*; (c) sugar, 10 g. per 100 ml. water; (d) sugar, followed by NaF in final concentration 0.05 *M* after the maximum fermentation rate had been established. Enzyme action was stopped by addition of 50 ml. 20 % trichloroacetic acid per 100 g. yeast. The precipitate was separated after $\frac{1}{2}$ hr. by filtration and washed with 5 % trichloroacetic acid. From the combined filtrate and washings a sample was removed for P estimations by Briggs's colorimetric method. The total acid-soluble P was determined after ashing, and the content of "pyro" and organic P was calculated from the hydrolysis curve in *N* HCl at 100° according to the equations:

$$\text{"Pyro" P} = \Delta (7 \text{ min.} - 0 \text{ min.}) - \Delta (30 \text{ min.} - 7 \text{ min.}),$$

$$\text{Organic P} = \text{total P} - (\text{ortho P} + \text{"pyro" P}),$$

$$\text{Hexosediphosphate P} = \Delta (180 \text{ min.} - 7 \text{ min.}) \times 1.5.$$

Alkali-labile P was estimated as the increase of ortho-P after 15 min. in *N* NaOH at 22°.

The bulk of the filtrate was treated with an amount of Ba acetate equivalent to the total P present, followed by Ba(OH)₂ to pH 8.0 and one-tenth volume of alcohol. The precipitate was removed by filtration; the filtrate was treated with basic Pb acetate, and the Pb precipitate removed, decomposed with H₂SO₄ and reconverted into the Ba salt. From P estimations in the successive filtrates the amount of P present in (a) sparingly soluble Ba salts, (b) soluble Ba salts, (c) soluble Ba salts with insoluble Pb salts was calculated by difference.

Identification of hexosediphosphate. The sparingly soluble Ba salt consists to a large extent of inorganic (ortho-, meta- and pyro-) P, together with any hexosediphosphate, phosphoglycerate etc. The crude salt was purified by repeated solution in 10 parts H₂O acidified with HCl to pH 3.6 and reprecipitation, at first as the acid Ba salt by addition of 3 vol. alcohol (the inorganic P tending to remain in solution) and finally by neutralization of the aqueous solution with Ba(OH)₂. The purification was such as to remove any trace of hexosemonophosphate but owing to the overlapping solubilities and the large excess of pyrophosphate it was exceedingly difficult to obtain a sample of hexosediphosphate free from inorganic P. Proof of the presence of this ester was therefore obtained by the preparation from these fractions of the crystalline phenylhydrazine salt of 6-phosphoglucosazone, which is formed from hexosediphosphate with elimination of one P atom.

Protocol, Yeast 31. Fermentation of fructose by 650 g. yeast. The sparingly soluble Ba precipitate, wt. 7.8 g., contained 786 mg. total P, of which 133 mg. were organic. The acid Ba salt was reprecipitated five times with alcohol and finally precipitated by neutralization of the aqueous solution. Wt. 0.81 g. Total P 9.3 %, ortho-P nil. Reducing power (Hagedorn and Jensen) 7.6 %; ketose (Seliwanoff) 5 %. $[\alpha]_{5461} + 1.8^\circ$. Ba hexosediphosphate has a reducing power (Hagedorn and Jensen) of 12 % and a Seliwanoff value of 10 %. Calculated from the hydrolysis curve in *N* HCl at 100°, 72 % of the total P content was hexosediphosphate-P.

Formation of 6-phosphoglucosazone. 0.2 g. pure Ba hexosediphosphate was converted into the free acid, vol. 5 ml., heated with 0.5 ml. phenylhydrazine and 1.0 ml. glacial acetic acid at 100° for 15 min. and cooled in ice for 20 min. The yield of 6-phosphoglucosazone (phenylhydrazine salt) was 70 % of the theory, with 43 % recovery on recrystallization from hot alcohol and chloroform; m.p. 153–4°. 0.33 g. of the Ba salt described above (31 mg. P), treated similarly, gave 151 mg. osazone, which was recrystallized with 45 % recovery; m.p. 153.6°; P = 5.71 %; required for C₂₄H₃₁O₇N₆P, P 5.68 %. Calculated on the basis of a 70 % yield of phosphosazone, 74 % of the total P in the salt was present as hexosediphosphate, which is in good agreement with the other analytical figures.

Estimation of hexosediphosphate. The purification of the hexosediphosphate fraction outlined above entails a considerable loss of organic P and is therefore not suitable for quantitative work, while calculation from the hydrolysis curve of a crude salt containing much labile P involves a considerable error. Attempts to prepare the osazone directly from the crude salt were unsatisfactory. For the routine estimation of hexosediphosphate, therefore, the crude Ba salt was purified once as the acid salt and precipitated as the neutral salt from aqueous solution. The crystalline phospho-osazone was then prepared from an aliquot sample of the salt under standard conditions, and the amount of P as hexosediphosphate was calculated from the weight of osazone obtained on the basis of a 70 % yield.

Table I. *Distribution of acid-soluble phosphorus in yeast*

Exp. no. Additions*	Autofermentation					Sugar fermentation										Sugar fermentation inhibited by NaF				
	29	32	39	39	NaF	29	30	31	32	37	29	39	30	32	29	29	39	30	32	29
Total duration of exp. (min.)	—	—	—	—	—	F	F	F	G	M	F	F	F	G	F	F	F	F	G	F
Duration of exp. after NaF	0	20	20	30	30	25	20	25	20	35	30	30	40	50	65	30	30	40	50	65
CO ₂ evolution, ml./5 min./100 g. yeast	—	—	—	15	0	180	150	135	170	150	10	10	20	30	45	<12	<5	<12	0	0
mg. P per 100 g. yeast:																				
Total acid-soluble P	178	230	205	202	—	175	164	190	224	153	178	191	174	220	190	178	191	174	220	190
Ortho-P	76	81	80	70	—	35	38	45	43	25	22	37	21	23	7	22	37	21	23	7
"Pyro"-P	63	111	82	73	—	64	—	92	115	68	58	75	—	102	77	58	75	—	102	77
Organic P	39	39	43	60	—	76	73	53	67	60	98	78	98	94	105	98	78	98	94	105
(a) Insoluble Ba ppt.	13	13	15	30	—	37	35	22	28	23	46	31	48	54	35	46	31	48	54	35
Hexosediphosphate P†	—	1	—	—	—	—	—	9.6	7.8	11	—	—	—	3	—	—	—	—	3	—
Phosphoglyceric P†	0	—	0	0	—	0	0	—	0	—	6	5	7	—	7	6	5	7	—	7
(b) Soluble Ba salts	26	25	28	30	—	39	38	31	29	36	52	47	50	40	57	52	47	50	40	57
P pptd. by basic Pb acetate	—	6	—	—	—	—	12	3	11	—	—	—	17	22	—	—	—	17	22	—
(c) Alkali-labile P	—	0.7	—	—	—	—	—	—	0.9	—	—	—	—	1.4	—	—	—	—	—	—

* F = fructose; G = glucose; M = mannose.

† Calc. from weight of crystalline osazone obtained from the purified salt on basis of a 70% yield.

‡ Calc. from weight of crystalline BaH phosphoglycerate.

Identification of phosphoglyceric acid. Phosphoglyceric acid was isolated quantitatively from the crude sparingly soluble Ba salts as the crystalline acid Ba salt. It was found advantageous to heat the salt first in *N* HCl at 100° for $\frac{1}{2}$ hr. to decompose labile P compounds. The pH of the solution was then brought to 3.0 with Ba(OH)₂ and 2 vol. alcohol added. The precipitate was dissolved in dilute HCl and 2 vol. alcohol were cautiously added; if any phosphoglyceric acid was present the characteristic crystalline BaH salt was rapidly deposited.

Other phosphoric esters. It was anticipated that the fraction with soluble Ba salts would contain some hexosemonophosphate, together with phosphopyruvate etc. Only a small part of the P present, however, was precipitated with basic Pb acetate and this fraction, after reconversion into the Ba salt, contained only 1–3 % P. In spite of repeated attempts at fractionation by Hg acetate, crystallization of brucine salts or alcohol fractionation, the constituents of this mixture could not be separated sufficiently for the identification of any phosphoric ester and no crystalline phenylhydrazine derivative could be obtained.

The values obtained for the distribution of the acid-soluble phosphorus during different types of fermentation are shown in Table I.

Effect of sugar fermentation on P distribution. During autofermentation the greater part of the organic P is in the fraction with very soluble Ba and Pb salts, probably of nucleotide nature, but a trace of hexosediphosphate is present. During the fermentation of sugar, whether fructose, glucose or mannose, the organic P is increased in amount, the increase being distributed almost equally between the insoluble and soluble Ba salts. In the insoluble fraction only 50 % of the increase was accounted for by the estimated increase in hexosediphosphate-P (10 mg. P per 100 g. yeast); the method of estimation of this ester however almost certainly gives too low values, since no allowance was made for loss in purification or by the hexosediphosphate carried into the soluble fractions. No phosphoglyceric acid could be isolated either from the resting or fermenting yeast and the amount of alkali-labile P was only slightly raised during fermentation.

Effect of NaF on distribution of P. When the fermentation of sugar by living yeast was inhibited by the addition of NaF the presence of phosphoglyceric acid was readily established; the amount found in different batches of the same yeast was constant, c. 7 mg. P per 100 g. yeast, and was not appreciably raised by prolonging the experiment with the NaF-poisoned yeast, e.g. from 10 to 45 min. The ortho-P content however slowly decreased in such yeast (Table I, Yeast 39); this appears to be due to the continued formation of phosphoric esters from glycogen present in the cell, since it was also observed in autofermentation in presence of NaF.

Inhibition of glycolysis with varying concentrations of fluoride

In the foregoing experiments 0.05 *M* NaF was used, since it was found that with large quantities of yeast this concentration was necessary to produce complete inhibition: even so the inhibition was rarely complete in less than 10 min. The effect of various concentrations of NaF on the fermentation rate is shown in Table II. *M*/200 fluoride caused only 41 % inhibition of glycolysis in the cell, though in yeast juice or muscle extract more than 90 % inhibition is produced by this, or even smaller, concentrations. The sensitivity of glycolysis in different tissues to NaF was shown by Dickens & Šimer [1929] to be extremely variable; in view of the disparity between yeast cells and cell-free extracts the sensitivity may be interpreted as a characteristic of the tissue rather than of the glycolytic enzyme.

Table II. *Percentage inhibition with varying concentrations of fluoride*

2 g. fresh yeast + 9 ml. 10% glucose were fermented 15 min. at 25°; 1 ml. NaF was added and the average fermentation rate measured during the next hour.

NaF molarity	ml. CO ₂ /5 min.	% inhibition
0	6.3	—
0.004	4.2	33
0.005	3.7	41
0.006	3.0	52
0.008	1.6	74
0.01	0.6	91
0.02	0	100

Time relationship between esterification and fermentation in living yeast

The coincidence of phosphorylation and fermentation has been studied more closely, particularly in the initial and final stages of fermentation of a limited quantity of sugar.

A suspension was made by mixing one part washed fresh yeast with 5 parts water, some of which (A) was used immediately and some (B) after 24 hr. at room temperature. 10 ml. samples of the suspension were placed in fermentation flasks, saturated with CO₂ and connected to nitrometers. After 10 min. incubation, 1 ml. 10% glucose was added and the fermentation rate measured over short intervals. The fermentation was stopped in individual flasks at short intervals by the addition of 3 ml. 20% trichloroacetic acid, and the orthophosphate content of the filtrate estimated. The complete fermentation of 100 mg. glucose would cause the evolution of 25 ml. CO₂; in the experiment quoted in Table III the rate of fermentation fell, from the maximum of 1.1 ml. CO₂/2 min.

Table III. *Time relationship between esterification of inorganic P and CO₂ evolution in living yeast*

Yeasts: A, fresh; B, after 24 hr.

Time after addition of glucose	CO ₂ rate ml./2 min.		Ortho-P mg./2 g. yeast		Time after addition of glucose min.	CO ₂ rate ml./2 min.		Ortho-P mg./2 g. yeast	
	A	B	A	B		A	B	A	B
0	—	—	1.35	1.66	40	0.7	0.9	—	0.92
15 sec.	0	—	1.34	—	46	0.7	0.8	—	0.86
30 „	0	—	1.30	—	48	0.6	0.7	1.01	0.84
1 min.	0	0	1.27	—	50	0.2	0.6	—	0.92
2 „	0.1	0	1.10	—	54	0.2	0.4	—	1.18
3 „	0.2	0	0.93	—	58	0.1	0.2	—	1.12
5 „	0.5	0.3	0.80	—	60	<0.1	0.1	1.09	—
10 „	1.1	0.7	0.75	—	62	—	<0.1	—	1.12
20 „	1.1	1.0	0.83	—	70	—	—	—	1.17
30 „	0.9	0.9	—	0.88					

to the autofermentation rate of less than 0.1 ml. CO₂/2 min. when approximately 80% of the sugar had been fermented. It will be seen that 3 min. after the addition of glucose 0.42 mg. inorganic P (equivalent to 2.4 mg. glucose as hexosemonophosphate) had disappeared, but that the fermentation did not reach its maximum rate until after the esterification was maximal. The marked lag in the attainment of the maximum rate of CO₂ evolution (Table III) is reminiscent of the Gärasteig period in fermentation by maceration extracts [Meyerhof, 1918] during which hexosediphosphate is formed. The decrease in orthophosphate in living yeast was maximal in less than 5 min., the level then

remaining steady for $\frac{1}{2}$ hr., till, with the consumption of the sugar, the CO_2 rate slackened and finally ceased and the inorganic P rose towards its original value.

The initial value of orthophosphate in yeast B (Table III), i.e. after 24 hr. "resting", was decidedly higher than in the fresh sample; the final value after fermentation was the same in both samples and less than the original value in the fresh yeast. It was found that if a second addition of glucose were made to the yeast, the time taken to attain the maximum rate increased with the interval between the two fermentations; during this interval there was a slow rise in orthophosphate. The point has not been investigated in great detail, but the experiments indicated a "rejuvenating" effect of the fermentation in building up labile P compounds which are slowly hydrolysed in the "resting" cell.

Independence of orthophosphate content of the cell and that of the medium

In the experiments so far cited the fermentations were carried out with mixtures of yeast, water and sugar, so that the observed changes in P distribution were internal. The following experiment demonstrates that this change is independent of the concentration of P in the surrounding medium. Weighed amounts (0.5 g.) of yeast were placed in centrifuge pots to which either H_2O , or phosphate buffer pH 6.6, or 1 ml. 10 % glucose or glucose + phosphate was added, the volume in each case being made up to 3 ml. with water. The yeast was well mixed and kept at 20° for 20 min. After centrifuging, the supernatant was separated as quickly and completely as possible with a capillary pipette, and the residue extracted with 5 ml. 0.5 % trichloroacetic acid. Inorganic P was then estimated in the supernatant liquid (external P) and in the trichloroacetic acid extract (internal P). It will be seen (Table IV) that in the presence of glucose,

Table IV. *Internal and external P concentrations in fermenting yeast*

Additions	mg. ortho-P	
	Supernatant liquid	Trichloroacetic acid extract
(1) H_2O	0	0.46, 0.46
(2) Glucose	0	0.32, 0.32
(3) 0.36 mg. P as phosphate buffer	0.358	0.48
(4) 0.36 mg. P as phosphate buffer + glucose	0.358	0.36
(5) 0.18 mg. P as phosphate buffer	0.178	0.46
(6) 0.18 mg. P as phosphate buffer + glucose	0.178	0.33

i.e. during fermentation, a decrease in orthophosphate takes place inside the yeast cell, while the concentration in the surrounding fluid remains unchanged. It has been shown by Hevesy *et al.* [1937], by means of experiments with radioactive P compounds, that there is no transference of P between the yeast cell and the medium unless growth is taking place.

The cycle of phosphate in glycogen and glucose fermentations in the cell

The experiments which have been described illustrate the general principle that in the decomposition of a substrate to its normal end products by a living cell there is no unlimited accumulation of intermediate products but a constant and perhaps extremely low "working level" of such substances. For instance in fermentation by living yeast the concentration of phosphoric esters is constant and independent of the total amount of CO_2 produced; this is in great contrast to fermentation in yeast juice, where in presence of excess glucose and phosphate the

amount of esterified P found is in stoichiometric proportion to the CO_2 evolved [Harden & Young, 1906].

The evidence suggests that there are two cycles with many points in common in which P is concerned in the yeast cell; firstly in the slow fermentation of glycogen present in the cell (autofermentation) and secondly in the rapid fermentation of hexoses presented to the cell. The mechanism of glycogen esterification in cell-free extracts is known to differ from that of glucose; esterification takes place in the absence of cozymase [Nilsson, 1930] and is not inhibited by fluoride [Meyerhof, 1926] or by iodoacetate [Macfarlane, 1931]. Since it is not possible to remove the preformed glycogen to ascertain the true resting level of the P the only evidence that glycogen is being esterified in the living cell is the fact that in presence of NaF esterification of inorganic P takes place. No phosphoglyceric acid was isolated from the autofermentation in presence of NaF, though it was readily obtained from an equal quantity of yeast after fermentation of sugar for the same length of time (Table I, Exp. 39).

The course of events during the fermentation of hexoses in the yeast cell is consistent with the following picture, based on the relationships between P-transference and oxidoreduction reactions which have been demonstrated in cell-free extracts [see Meyerhof *et al.* 1937; Needham & Pillai, 1937]. In the resting cell there is a small amount of hexosediphosphate (Table I) and of adenylic acid, some of which is present as adenylypyrophosphate [Wagner-Jauregg, 1936]. Part of the added hexose is therefore rapidly esterified by transference of P from adenylypyrophosphate and gives rise to triosephosphate by the reactions

The coupled reaction

begins to take place, the rate of the oxidoreduction and consequently of the esterification of inorganic P rising as the concentration of triosephosphate increases autocatalytically, until the limiting concentration of phosphoric esters is reached and the fermentation rate becomes maximal (see Table III). Once this rate is established the rate of breakdown of the phosphoric esters is in fact equal to the rate of their formation, since the level of P remains constant; when the free sugar has disappeared however it is probable that the reaction

no longer predominates in the decomposition of phosphopyruvic acid but that instead the reaction

leads to the reappearance of inorganic P in the cell.

The addition of NaF to the fermenting yeast inhibits the breakdown of phosphoglyceric acid to phosphopyruvic acid and consequently both the formation of acetaldehyde and the reformation of adenylypyrophosphate are suppressed. The ultimate effect of fluoride therefore is that the fermentation cycle is immobilized so soon as all the cozymase present has been reduced by triosephosphate, with formation of phosphoglyceric acid. Since the latter reaction is stoichiometric, the amount of phosphoglyceric acid formed in the fluoride-poisoned yeast cell should be dependent on the cozymase present, but independent of the duration of the reaction. The latter was found to be the case (Table I) while the amount of "pyro"-P found in corresponding batches of

yeast was appreciably less in the presence of fluoride than in the normal fermentation, as would be expected if reformation of adenylypyrophosphate had been inhibited. Approximately 7 mg. P per 100 g. yeast were isolated as phosphoglyceric acid, equivalent to 140 mg. cozymase. Meyerhof & Ohlmeyer [1937] estimated the cozymase content of bottom yeast as 50 mg. per 100 g., a figure based on the amount in boiled extracts, which may not represent a complete extraction. The value calculated above is only in fair agreement, but no figures are available for the actual cozymase content of the yeast used. It seems probable that in a normal fermentation this figure (7 mg. P/100 g. yeast) represents the maximum sum of the P present as 3- and 2-phosphoglyceric and phosphopyruvic acids, since for the formation of further quantities of phosphoglyceric acid the decomposition of an equivalent amount of phosphopyruvic acid is necessary.

Out of the seven phosphoric esters which are known to be intermediate products in the fermentation of sugar by yeast juice only hexosediphosphate was identified with certainty in the normal fermentation of the cell. Since the increase in organic P was of the order of 30 mg. P per 100 g. yeast of which at least 10 mg. were present as hexosediphosphate, there was an accumulation of this ester relative to the other esters. It may be surmised that this ester acts as a pressure head, maintaining by an enzymic equilibrium reaction [Meyerhof & Lohmann, 1934] the optimum supply of triosephosphate.

The detection of phosphorylation in tissues

It is clear that if the initial concentration of adenylypyrophosphate present in the tissue is so high that sufficient phosphoric ester to saturate the enzymes can be produced by direct phosphorylation of the added sugar then the esterification or even the presence of inorganic P is unnecessary. When therefore the principle is recognized that glycolysis in the living cell takes place by a cycle of P transference in which the actual amount of P involved approaches catalytic dimensions, the question of practical importance for the detection of such phosphorylation is not how much but how little P need be esterified at any one time in order to provide the concentration of phosphoric esters necessary for the maximum rate.

It is known that in certain isolated tissues [Boyland & Boyland, 1935] adenylypyrophosphate is very rapidly broken down by the enzymes present. It appears probable that if a tissue is fresh and only recently removed from its natural source of carbohydrate in the body, or if the experimental procedure necessary involves little injury, the amount of inorganic P esterified is likely to be extremely small.

This point is illustrated by observations on the orthophosphate concentration during glycolysis by a young culture of *Bact. coli*. The culture was obtained by successive tenfold dilutions with 1% trypsin-broth of 0.5 ml. of a fresh subculture after 15, 7 and 6½ hr. incubation; the bacteria were separated from the broth by centrifuging 9½ hr. after the last transfer, washed twice with 0.086% NaCl and suspended in saline. The orthophosphate content during incubation in absence and presence of glucose was then estimated. It will be seen (Table V) that the initial value of the orthophosphate and the actual decrease in presence of glucose were both very small, and that here also as in living yeast the P values increased as the glucose was consumed. The lowest amount of P actually estimated in these experiments was 0.004 mg., with an accuracy of approximately ± 0.0005 mg. P. In the absence of glucose there was a rapid increase in inorganic P, presumably by autolytic changes in an unfavourable medium; by comparison with these values the decrease during glycolysis was marked, and quite outside the experimental error.

Table V. *Inorganic P during glycolysis by Bact. coli*

2 ml. bacterial suspension (15.6 mg. dry wt. in 0.05 *M* NaHCO₃, 0.015 *M* NaCl) + 1 ml. glucose or water incubated at 37°; + 3 ml. 5% trichloroacetic acid. 4 ml. samples for P estimations.

Time min.	No sugar mg. P	Sugar added	
		mg. P	mg. glucose
0	0.009	0.009	9.9
30	0.023	0.006	6.5
60	0.027	0.006	3.9
120	0.033	0.014	0.7

In experiments on the glycolysis of retina Bumm & Fehrenbach [1931] found "in glukose-freier Ringer-Lösung immer etwas mehr Phosphat abgespalten als im Ansatz mit Glukose", though the glycolysis could take place even if no detectable inorganic P were present in the tissue. The differences observed were of the order 0.002–0.004 mg. P per mg. dry wt. of retina. The glycolytic rates of yeast, *Bact. coli* and retina are high, while the observed differences of P concentration between fermenting and non-fermenting cells are of the order 1–2 mg. P/g. dry wt. If the amount of P esterified is a function of the glycolytic rate the detection of phosphorylation in tissues of low glycolytic rate will require an extremely delicate technique; especially if the tissue examined is not readily available in large amounts.

Generally speaking the rapid formation of inorganic P in an isolated tissue indicates a physiological lesion. The retina, which is often classed as having an "abnormally high" glycolytic rate, is one of the few tissues which can be detached with little injury and used entire in a manometric experiment; it is interesting to note that in the experiments of Bumm & Fehrenbach the glycolytic rate (mg. lactic acid/mg. dry wt./hr.) averaged 0.234 in 9 expts. where no inorganic P was detectable and only 0.14 in 8 expts. in which the inorganic P had increased to 0.02 mg./mg.

The formation of glycogen in living yeast. The occurrence of an induction period in the fermentation of sugar by living yeast has been pointed out by Willstätter & Rohdewald [1937] who found a marked increase in glycogen during this period and therefore advanced the view that glucose is not directly fermented but is first transformed into glycogen. The rapid formation of glycogen by fresh yeast has been confirmed by Goda [1938] who however does not support Willstätter & Rohdewald's hypothesis, since he found that in old yeast there was a rapid fermentation of glucose but no parallel formation of glycogen. It seems clear that glucose is directly fermented by yeast juice since the sugar is fermented more rapidly than glycogen either present in or added to the juice; it is difficult to imagine how the juice obtains this property if it is not also one of the living cell, or why glycogen newly formed from added glucose should be more rapidly fermented than glycogen preformed in the cell. Though no glycogen estimations were made in the present experiments, the experimental conditions were very similar to those of the previous workers; it seems probable therefore that the esterification of inorganic P and the formation of glycogen are contemporaneous. It was suggested by Robison [1932] that esterification of sugar might take place by a preliminary enolization and phosphorylation on carbon atom 1 of the enol sugar, followed by migration of the P group to carbon atom 6. The discovery however that the fermentation of mannose by yeast preparations may result in the almost exclusive accumulation of mannosephosphate [Jephcott & Robison, 1934] and that muscle and yeast extracts contain an enzyme, phosphohexokinase [Lohmann, 1931; Tankó & Robison, 1935] which can transform the individual

hexose-6-phosphates into an equilibrium mixture of aldose and ketose esters, indicates that enolization takes place in the phosphorylated rather than in the free sugar. Glucose-1-phosphate, which is an intermediate product in glycogen breakdown, can also be transformed enzymically into the equilibrium mixture of 6-phosphoesters [Cori *et al.* 1937]. It seems possible that the fermentable sugars are primarily phosphorylated on the reducing group, and that the observed formation of glycogen in living yeast takes place by condensation of glucose-1-phosphate with elimination of phosphate. It would be of interest to know the behaviour of fructose under similar circumstances.

Non-phosphorylating glycolysis

The demonstrated changes in P distribution during fermentation in the living yeast cell are so consistent with the theory of alcoholic fermentation developed from experiments on cell-free extracts [see Meyerhof *et al.* 1937] that there can be little doubt that the processes are analogous. Yet there are a number of differences between the cell and the cell-free extract which may be summarized as follows: fresh yeast does not ferment added glycogen or hexosediphosphate; the rate of fermentation of sugar is not increased by inorganic P, cozymase or arsenate; there is no stoichiometric relationship between the CO₂ evolved and the actual decrease in inorganic P. These apparent inconsistencies may be ascribed to the fact that the cell is a well-concerted enzyme system protected by a selectively permeable membrane, so that the possession of these negative attributes by other tissues cannot furnish any evidence either for or against the existence of a phosphorylating glycolysis in these tissues. It is, however, these attributes which have been advanced as the primary experimental basis of the hypothesis of non-phosphorylating glycolysis [see Ashford & Holmes, 1929; Ashford, 1934; Needham & Nowinski, 1937; Needham *et al.* 1937]. The conclusions which have been drawn in such cases are frequently due to the expectation, either implicit or avowed, that an intact tissue or tissue slice will behave like a cell-free extract of muscle or yeast, whereas they may be expected to differ from such extracts in the same way as living yeast. For instance, the fact that the glycolysis of retina is not accelerated by phosphate or arsenate [Bumm & Fehrenbach, 1931] is more reasonably interpreted as a similarity between this tissue and living yeast than as a dissimilarity from glycolysis in yeast juice.

The demonstration of esterification during glycolysis is experimentally more difficult in tissues such as brain, kidney, tumour and chick embryo which show a rapid autolytic formation of inorganic P than in yeast where the formation of inorganic P in the resting cell is negligible in short experimental periods. In the former cases a small and limited decrease in orthophosphate due to esterification in glycolysis may be opposed to an increase due to hydrolysis by phosphatases of cell substrates which may perhaps have no connexion with the formation of lactic acid such as aminoethylphosphate or diphosphoglycerate.

Nevertheless the esterification may still be detectable; thus in the experiments of Ashford [1934] with brain tissue, the inorganic P per 100 g. tissue was 10–25 mg. less after incubation in the presence of glucose than in its absence, though both values were much greater (30–40 mg. P) than the zero value. This is indirect evidence of phosphorylation during glycolysis; the fact that the esterification was independent of the total glycolysis is consistent with the theory of a phosphorylating cycle.

The experiments of Needham *et al.* [1937] with chick embryo do not provide any evidence on this point, since no values are given for the orthophosphate in embryos incubated without glucose, a necessary control where a rapid hydrolysis

of phosphoric esters is shown to be taking place. Their values for the P distribution during glycolysis in absence and presence of fluoride show a relative increase of esterification in the latter case, particularly in the phosphoglyceric acid and hexosediphosphate fractions.

Thus in one experiment (Table XIV, *loc. cit.*) the distribution of P (in % of the acid-soluble P) was: inorganic P 48.5 % and 34.7 %, insoluble "resistant" ester P 0.7 % and 3.6 %, in absence and presence of NaF respectively; in another experiment (Table XV) inorganic P 39.3 and 27 %, hexosediphosphate-P 0 and 19.8 % in absence and presence of NaF. The authors' comment on these figures is that it is "at once noticeable that no accumulation of the 'hexosediphosphate' fractions has taken place...but a piling up of inorganic phosphate" at the expense of phosphagen, adenylypyrophosphate, hexosediphosphate and insoluble "resistant esters". It is true that the accumulation was only relative, but if a relative accumulation amounting to 10 or even 20 % of the total acid-soluble P was insignificant, the technique was clearly inadequate for the problem.

In the interpretation of other experiments which have been adduced as evidence of non-phosphorylating glycolysis—the effect of dialysis or NaF on the glycolysis of tissue slices or brei, or the substrate-selectivity displayed by these enzyme systems—the limitations imposed by the presence of the cell membrane or by the location of the enzyme in the residual cell debris have sometimes been given insufficient weight. It is well known that the ease with which a diffusible constituent may be removed from a tissue brei or even from a cell-free extract is very variable. Thus, by dialysis against water, cozymase may be readily removed from yeast preparations but less readily from muscle extract, while for the removal of cocarboxylase alkaline washing is necessary. It cannot be assumed that because, for example, glutathione is removed by dialysis under arbitrary conditions other coenzymes which are ordinarily diffusible are also removed. The lack of response to added coenzymes in such cases may be due simply to the fact that the tissues still contain the optimum supply. On the other hand if the coenzymes have all been removed by dialysis it is necessary to restore them all to obtain reactivation; in the experiments of Needham & Lehmann [1937, p. 1230] on reactivation of dialysed chick embryo brei, for instance, in no case were adenylypyrophosphate, cozymase and Mg^{++} added simultaneously.

The difficulty in removing even inorganic P from tissue brei is illustrated in the following experiment. Weighed samples of about 1.0 g. rabbit brain were ground in a mortar with 0.5 g. silver sand and transferred quantitatively to centrifuge pots with 6 ml. borate buffer, two samples at pH 6.0 and two at pH 8.4; 2 ml. *M* $CaCl_2$ were added with stirring to each pot and after 20 min. at room temperature the residue was spun down and washed with the same buffer as before. All the residues were then separately extracted three times with borate buffer at pH 6.0 and finally twice with 5 ml. 5 % trichloroacetic acid; the inorganic P in all the extracts was estimated. The results in Table VI show that only 80–85 % of the total inorganic P present in the tissue could be extracted by buffer at pH 6.0, whether or not it had been previously rendered insoluble. It is not improbable that a residual cell structure which can prevent the outward diffusion of P can also prevent the inward diffusion of Ca. For this reason previous experiments on the immobilization of inorganic P by Ca salts in alkaline solution [e.g. Ashford & Holmes, 1929] do not give very satisfactory evidence on the participation of inorganic P in glycolysis, and moreover do not exclude the esterification of sugar by transference of P from adenylypyrophosphate.

There is other reason to suppose that there is an intracellular structure (perhaps for instance a lipoprotein complex) regulating the diffusion of sub-

Table VI. *Extraction of inorganic P from brain tissue*

g. tissue	A	B	C	D
	0.87	0.93	0.92	0.84
	mg. P/g. tissue			
Extracts 1 + 2, pH 8.4	0.024	0.014	—	—
Extracts 1 + 2, pH 6.0	—	—	0.198	0.198
Extract 3, pH 6.0	0.168	0.192	0.025	0.027
Extract 4, pH 6.0	0.058	0.054	<0.005	<0.005
Extract 5, pH 6.0	<0.005	<0.005	<0.005	<0.005
Extract 6, trichloroacetic acid	0.049	0.046	0.052	0.055
Extract 7, trichloroacetic acid	<0.005	<0.005	<0.005	<0.005
Total inorganic P extracted	0.299	0.306	0.275	0.280
% extracted in buffer	84	85	81	81

stances throughout the cytoplasm, which may persist in cell fragments even if the outer membrane is disrupted. It is known for instance that if toluene is added to fresh yeast, the autofermentation rate is greatly increased [Harden, 1911]; it is difficult to account for this fact without postulating an internal change in the accessibility of the enzyme to the glycogen. It is clear that in cases where a glycolytic enzyme has not been obtained in a cell-free extract the existence of an intracellular structure affecting the accessibility of substrates has not in fact been excluded. For these reasons the view of Lehmann & Needham [1938] that "substrate-preference experiments cannot be interpreted as due to selective penetration of cell-membranes" is hardly justified till they have obtained from chick embryo a cell-free glycolysing extract which shows the same substrate-selectivity as the enzyme in the cell or cell-brei. It has been shown by Euler *et al.* [1936] and Boyland & Boyland [1938] that in brain and tumour, the selectivity exhibited by intact tissue or tissue slices to fructose and glycogen is not a property of the cell-free extract. The latter authors have pointed out that the differential inhibition of the reaction phosphoglyceric $\rightarrow$ phosphopyruvic acid observed in brei at concentrations of NaF which do not inhibit glycolysis is probably due to the accessibility of the enzyme exposed on the injured surface both to added phosphoglyceric acid and to fluoride. The behaviour of chopped brain tissue towards glycogen, glucose and hexosediphosphate [Ashford, 1934; Ashford & Holmes, 1929] is also explicable on this basis, glucose being broken down rapidly within the cell fragment, glycogen and hexosediphosphate by enzymes exposed on the surface and partly inactivated by loss of coenzymes.

Apart from this type of experiment the most important evidence put forward in support of the hypothesis of a non-phosphorylating glycolysis is the specific inhibition of glycolysis in tumour, chick embryo etc. by *l*-glyceraldehyde. It has been shown that the phosphorylating glycolysis of dialysed muscle extracts and of washed dried yeast is also inhibited by glyceraldehyde [Adler *et al.* 1937; Boyland & Boyland, 1938]. These findings have been criticized by Lehmann & Needham [1938] on the ground that the concentration necessary for inhibition was so large that the effect is probably not the specific effect observed in tumour etc. They find it also difficult to see how the normal glycolysis of the embryo can involve the formation of hexosediphosphate, since the latter should give rise to dihydroxyacetonephosphate, and the inhibiting glyceraldehyde should in that case be removed by condensation to ketosemonophosphate. The following hypothesis explains this discrepancy and is based on the assumption that the glycolytic cycle in chick embryo is essentially similar to that of living yeast. It is known that the enzymic condensation of *d*- or *l*-glyceraldehyde with dihydroxy-acetonephosphate leads to the irreversible formation of 1-fructose- and 1-sorbose-

phosphates respectively; these esters do not give rise to the equilibrium mixture of hexose-6-phosphates [Meyerhof *et al.* 1936] and take no further part in the glycolysis. Since glycolysis is inhibited by *l*- and not by *d*-glyceraldehyde, it seems probable that the fixation of triosephosphate, which can take place with either isomeric form, is not the point of primary inhibition; the actual point is immaterial to the argument. The concentration of glyceraldehyde necessary for inhibition in a particular case will however be determined by the amount of triosephosphate—practically speaking by the amount of hexosediphosphate—available for fixation of the inhibitor. In the intact chick embryo the amount of hexosediphosphate present at one time will be very small; if the added glyceraldehyde can trap all the triosephosphate formed, then the glycolytic cycle will be immobilized since the further formation of adenylypyrophosphate and consequently of hexosediphosphate will be suppressed, the primary inhibition being reinforced by a secondary blockage. If however some proportion of the triosephosphate escapes fixation, the glycolytic cycle can proceed, even if diminished in extent or slowed in rate, until eventually the glyceraldehyde is decreased, by condensation, below the inhibitory concentration. The inhibition by glyceraldehyde in low concentrations is in fact transitory; with $M/800$ in chick embryo the duration was about 1 hr.

On the other hand, both dried yeast and yeast juice normally contain hexosediphosphate; if none is present, and in the absence of adenylypyrophosphate, it is necessary to add hexosediphosphate or triosephosphate to initiate glycolysis. Moreover, yeast and muscle preparations have a relatively high esterifying power (2 g. dried yeast for instance can esterify 31 mg. P in about 10 min.) and the esterified P, mainly hexosediphosphate, remains in this type of fermentation as a by-product; it can be utilized for the removal of glyceraldehyde without affecting the subsequent fermentation. The concentration of glyceraldehyde necessary for inhibition will therefore be much higher than in a tissue of low glycolytic activity, in which a limited amount of P is taking part in a cycle.

It is premature to stress too much the lack of positive evidence of a non-phosphorylating glycolysis, for instance the extraction of an enzyme which does not require the presence of cozymase etc., since the history of the preparation of similar extracts is one of frequent trials and many failures. Nevertheless the evidence is at present negative and is based chiefly on the failure to demonstrate phenomena which are characteristic of the glycolysis of cell-free extracts rather than of cells. A critical examination of the evidence shows that in at least two of the tissues discussed, brain and retina, esterification of P during glycolysis can be inferred from the experimental data; in other cases, e.g. the inhibition by glyceraldehyde and the behaviour of tissue brei, the facts can be as consistently explained on the basis of a phosphorylating glycolysis as on any other basis. It appears that the existence of a glycolytic enzyme of a non-phosphorylating type has not yet been experimentally established.

SUMMARY

1. In the normal "resting" yeast cell hexosediphosphate is present in small amounts, approximately 1 mg. P/100 g. yeast.

2. During the fermentation of hexoses by living yeast the organic P present in the yeast is increased by about 30 mg. P/100 g. yeast, of which at least 10 mg. P are hexosediphosphate. This ester was identified and estimated by the formation of the crystalline phenylhydrazine salt of 6-phosphogluconic acid.

3. When the fermentation of sugars was inhibited by the addition of NaF, phosphoglyceric acid in amount equal to 7 mg. P/100 g. was isolated from the fresh yeast.

4. The observed changes in the distribution of P during fermentation in the living cell are shown to be in accord with the Embden-Meyerhof theory of alcoholic fermentation in cell-free extracts.

5. The evidence which has been put forward in favour of a theory of non-phosphorylating glycolysis in certain tissues is discussed. It is concluded that this theory is not yet experimentally justified.

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