XVIII. PHOSPHORYLATION BY THE LIVING BACTERIAL CELL¹

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SINCE the classical work of Harden & Young [1905] an immense amount of evidence has been brought forward favouring the intimate association of glucolysis and phosphorylation. The greater part of the investigations, however, has been carried out with unnatural systems, such as cell-free juices, acetone or toluene treated cells, or poisoned systems.

Unquestionably phosphorylation is related to the dissimilation of glucose under the unnatural conditions. The question arises, however, as to whether phosphorylation is intimately associated with glucolysis in the living cell. Macfarlane [1936] established a relationship in yeast between a decrease in orthophosphate and rapid fermentation by comparing orthophosphate in the trichloroacetic acid extracts of yeast cells fermenting glucose and not fermenting glucose. In addition to the orthophosphate, the following fractions were determined:

Labile phosphate, calculated from Lohmann's equation,

$$\text{"Labile" P} = \Delta_{7 \text{ min.} - 0 \text{ min.}} - \Delta_{30 \text{ min.} - 7 \text{ min.}};$$

total acid-soluble phosphorus, determinable after ashing with concentrated sulphuric acid and hydrogen peroxide and organic phosphorus fraction, calculated as the total acid-soluble minus the sum of the orthophosphate and the labile phosphorus. In these experiments the decrease in orthophosphate was not correlated with an increase in organic phosphate, but it has since been shown [Macfarlane, 1937] that phosphoric esters, mainly hexosediphosphate, are present in increased amount during fermentation.

In the present communication the living bacterial cell has been studied. The phosphoric esters in the trichloroacetic acid extract of the cells were classified according to ease of hydrolysis in *N* HCl at 100°. The esters from the extract of cells fermenting glucose were quantitatively compared with those from cells not fermenting glucose. Under the conditions of our experiments there exists a correlation between a decrease in readily determinable phosphorus and an increase in esterified phosphorus occurring on addition of glucose.

EXPERIMENTAL

Methods

Aerobacter aerogenes was grown in a medium of 50 g. of peptone, 50 g. of glucose, 125 ml. of 0.5 *M* potassium phosphate buffer (*pH* 7.0) and 10 l. of water. Cultures which were never over 12 hr. old were centrifuged and the cells suspended in sterile water in such a concentration that a 10 or 12 ml. sample of the

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suspension was approximately equivalent to 1 g. (wet wt.) of bacterial cells. The suspension was equally divided between two flasks with 50 ml. in each. 10 or 12 ml. samples were taken before and at various intervals after the addition of 2 ml. of a solution of glucose to one flask, and the addition of 2 ml. of water to the other. To the samples were added 5 ml. of a 25% solution of trichloroacetic acid. After storing overnight in the ice-box the samples were centrifuged and the supernatant liquid was analysed for orthophosphate which was determinable, (a) readily, (b) after hydrolysis in *N* HCl at 100° for 7, 12, 30, 120 and 300 min. respectively, and (c) after ashing with sulphuric acid and hydrogen peroxide.

Phosphorus was determined by Briggs's [1922] modification of the Bell-Doisy phosphate method in Exps. 6, 9 and 14, and by the method of Küttner & Lichtenstein [1930] in Exps. 4 and 5. The values obtained were plotted against time of treatment in *N* HCl at 100° (Fig. 1).

Fig. 1. Hydrolysis curves of phosphoric acid esters present in the trichloroacetic acid extract of bacterial cells. Broken lines are projected to total acid soluble phosphorus values obtained on ashing. (Exp. 14 *b*, Table I.)

It is seen that phosphoric acid esters are present which are completely hydrolysed in 30 min. and that the amount of these easily hydrolysable esters present may be calculated from the equation:

Easily hydrolysable P = P determinable after 30 min. hydrolysis — readily determinable P.

The difficultly hydrolysable phosphoric acid esters may be calculated from the equation:

Difficultly hydrolysable P = total acid soluble P — phosphorus determinable after 30 min. hydrolysis.

The above curves have been presented to show the types of phosphoric acid compounds present, as well as their quantitative relationship to fermentation.

In Table I are given quantities of esters present in the trichloroacetic acid extract of bacterial cells fermenting and not fermenting glucose.

Table I. *Phosphorus fractions present in the trichloroacetic acid extracts of living cells of Aerobacter aerogenes*¹

Quantities in mg. phosphorus per g. of cells (wet wt.)

Exp.	Time after addition of glucose to B min.	(A) No glucose added				(B) Glucose added			
		Readily determinable P	Easily hydrolysable P 30-0	Difficultly hydrolysable P T-30	Total acid soluble P	Readily determinable P	Easily hydrolysable P 30-0	Difficultly hydrolysable P T-30	Total acid soluble P
14 a	0	0.65	1.35	1.78	3.78	0.58	1.52	1.76	3.86
b	3	0.64	1.32	1.78	3.74	0.48	1.42	1.92	3.82
c	15	0.58	1.45	1.74	3.77	0.53	1.33	1.88	3.74
d	420	0.79	0.91	1.17	2.87	0.64	1.07	1.53	3.24
9 a	0	0.67	1.47	1.61	3.75	0.65	1.57	1.67	3.89
b	10	0.67	1.51	1.44	3.62	0.45	1.64	1.90	3.99
c	30	0.70	1.53	1.55	3.78	0.50	1.55	1.81	3.86
d	420	0.89	0.57	1.10	2.56	0.70	0.77	1.17	2.64
6 a	0	0.54	0.44	0.66	1.64	0.45	0.55	0.67	1.67
b	40	0.51	0.71	1.06	2.28	0.28	0.79	0.97	2.04
c	330	0.47	0.94	0.80	2.21	0.40	0.80	1.42	2.62
5 a	0	0.42	0.76	0.86	2.04	0.52	0.77	0.89	2.18
b	25	0.47	1.06	0.75	2.28	0.33	1.06	1.00	2.39
c	720	0.71	1.00	0.96	2.67	0.55	0.89	1.04	2.48
4 a	5	0.59	1.05	0.94	2.58	0.35	1.17	1.60	3.12
b	30	0.56	0.96	1.18	2.70	0.25	1.27	1.19	2.71
c	180	0.58	1.12	1.04	2.74	0.37	1.16	1.21	2.74

* *A. indologenes* instead of *A. aerogenes* was used in No. 4.

It is apparent that the readily determinable phosphorus is less and the esterified phosphorus is greater in the presence of glucose than in its absence. Following the addition of glucose to B (Table I), there occurs a decrease in readily determinable phosphorus, followed by a gradual rise as the latter stages of the fermentation are reached. Often it is the difficultly hydrolysable phosphorus which has increased in the presence of glucose but frequently the easily hydrolysable phosphorus is increased. The variation in the total acid-soluble phosphorus of the same suspension requires some explanation. The total phosphorus of the acid extract depends in part on the time the cells are left in contact with the trichloroacetic acid. Since the time of extraction varied with the samples taken at different times, the total phosphorus values show some variation. It should, perhaps, be noted in this connexion, that the time of extraction for any set of samples (A and B) at a given time was always the same. In Exp. 6 we were unable to correlate an increase in esterified phosphate with the decrease in orthophosphate at the 40 min. period. This result may possibly have been due to an error in determining the total phosphorus since in only one other determination (Exp. 5c) is the total acid soluble phosphorus less in the presence of glucose than in its absence. The decrease of orthophosphate with the increase of esterified phosphorus shown by Table I is a fundamental characteristic of phosphorylation.

Estimation of the number of living bacterial cells

To determine the number of living bacterial cells present, a comparison was made between microscopic and plate counts. The former was obtained by use of the Petroff-Hauser bacteria counting cell. Plate counts were determined on the following medium: infusion from 5 g. calf liver, 3 g. of peptone (Difco), 10 g. of glucose, 25 ml. 0.8M potassium phosphate buffer (pH 7.0), 20 g. of agar and 1000 ml. of water.

Table II. *Comparison of microscopic counts and plate counts of an 11 hr. culture of Aerobacter aerogenes*

Exp. 1				Exp. 2			
Theoretical count*	No. of plates	Plate count (Av.)	% viable cells	Theoretical count*	No. of plates	Plate count (Av.)	% viable cells
956	1	774	80.96	1012	1	967	95.55
478	3	476.7	99.72	506	3	459.6	90.83
239	3	217.6	91.05	253	2	249.5	98.62
119.5	3	93.3	78.08	126.5	3	109.7	86.72
47.8	1	42	87.86				
		Av.	88.67			Av.	91.71

Av. of 1 + 2 = 90.01 %.

* Calculated from the microscopic count.

Results are given in Table II. It is apparent that 90 % of the cells were capable of proliferation. Probably the percentage of living cells is higher, since some cells may be alive but not capable of proliferation.

Behaviour of cells from a 15-day culture with respect to phosphorylation

Since a small percentage of dead cells are probably present even in a 12-hr. culture of *A. aerogenes*, it is of interest to know the behaviour of a suspension of cells from an old culture with respect to phosphorylation. Exp. 7, Table III,

Table III. *The effect of the addition of glucose on phosphorus distribution of a suspension of cells from a 15-day culture of Aerobacter aerogenes*

Quantities in mg. phosphorus per g. of cells (wet wt.)									
(A) No glucose added						(B) Glucose added			
	Time after addition of glucose to B min.	Readily determinable P	Easily hydrolysable P 30-0	Difficultly hydrolysable P T-30	Total acid soluble P	Readily determinable P	Easily hydrolysable P 30-0	Difficultly hydrolysable P T-30	Total acid soluble P
Exp.		P	30-0	T-30	P	P	30-0	T-30	P
7	0	0.26	0.59	1.18	2.03	0.27	0.53	1.07	1.87
	25	0.29	0.51	1.08	1.88	0.29	0.42	1.04	1.75
	300	0.29	0.37	0.98	1.64	0.30	0.35	0.92	1.57

was carried out in the same manner as the experiments described above with the exception of the use of a 15-day in place of a 12-hr. culture. The readily determinable phosphorus does not decrease in the presence of glucose, nor does the esterified phosphorus increase.

Increase of the readily determinable phosphorus with time

It has been noted that in a suspension of bacterial cells, the readily determinable phosphorus increases with time. We have considered the possibility of this phenomenon being associated with the depletion of the carbohydrate reserve of the cell.

It seems possible that when the bacterial cell has used up the free glucose, it may then use the carbohydrate reserve of the cell as a source of energy. We may expect some phosphorus to be continuously esterified with the reserve as it is used, thus remaining in organic combination; but as the amount of reserve decreases, the quantity of unesterified phosphorus will rise, just as it does at the end of the glucose fermentation.

Such an explanation may be tested by seeing whether the reserve is phosphorylated, for, in this case, the change in the distribution of phosphorus occurring on addition of glucose should be more apparent with cells in which the reserve has been depleted, just as would be the case on adding glucose to cells with no glucose at all, as compared with cells with a small amount of glucose present. In order to test this point, 10 ml. of the bacterial suspension after 48 hr. vigorous aeration were placed in each of two centrifuge cups. 2 ml. of 12% glucose solution were added to one cup and to the other 2 ml. of water. As soon as fermentation commenced, as evidenced by the evolution of CO₂, 5 ml. of trichloroacetic acid were added to each tube. Within 48 hr. the cells were centrifuged and the supernatant liquid was analysed as in the experiments described above. Results are shown in Table IV. It is interesting to note:

Table IV. *Effect of aeration on the change of phosphorus distribution occurring on addition of glucose to Aerobacter aerogenes*

Quantities in mg. phosphorus per g. of cells (wet. wt.)

Exp.		Before aeration*				After 48 hr. aeration			
		Ortho-P	Easily hydrolysable P 30-0	Difficultly hydrolysable P T-30	Total acid soluble P	Ortho-P	Easily hydrolysable P 30-0	Difficultly hydrolysable P T-30	Total acid soluble P
4	Before addition of glucose	0.59	1.05	0.94	2.58	1.87	0.47	0.49	2.83
	After addition of glucose (30 min.)	0.25	1.27	1.19	2.71	1.38	0.76	0.76	2.90
	Difference due to addition of glucose	-0.34	+0.22	+0.25	+0.13	-0.49	+0.29	+0.27	+0.07
	Change due to aeration	—	—	—	—	-0.15	+0.07	+0.02	-0.06
5	Before addition of glucose	0.52	0.77	0.89	2.18	1.51	0.45	0.62	2.58
	After addition of glucose (25 min.)	0.33	1.06	1.0	2.39	1.19	0.47	1.34	3.00
	Difference due to addition of glucose	-0.19	+0.29	+0.11	+0.21	-0.32	+0.02	+0.72	+0.42
	Change due to aeration	—	—	—	—	+0.13	-0.27	+0.61	+0.21

* From Exps. 4 and 5, Table I; other portions of the same suspensions were used in the aeration experiments.

(1) that on aeration the esterified phosphorus is broken down with a consequent increase in orthophosphate; (2) that on addition of glucose to both suspensions of aerated and unaerated cells, significant changes in phosphorus distribution occur; and (3) that the changes occurring are greater in the case of the aerated

cells than in the non-aerated cells. The last point indicates that the reserve is phosphorylated, and hence supports the belief that a great part, at least of the increase of orthophosphate with time, is due to the depletion of reserve substances in the bacterial cell.¹

DISCUSSION

In showing that phosphorylation is associated with glucolysis in the living bacterial cells, a certain additional value is placed on the past research with non-living systems.

Considerable evidence has been brought forward favouring the importance of phosphorylation in bacterial metabolism. Virtanen & Tikka [1930] have isolated a disaccharide phosphoric ester from *Lactobacillus casei*, and Tikka [1935] has investigated the fermentation of phosphoglyceric acid, α -glycerophosphoric acid and hexosediphosphate. Neuberg & Kobel [1933] have shown that phosphoglyceric acid yields pyruvic acid and phosphoric acid in the presence of *L. delbrückii*. Werkman *et al.* [1936] were the first to isolate phosphoglyceric acid from bacteria, and Stone & Werkman [1937] have shown the rather general occurrence of this ester among members of the order Eubacteriales.

Evidence has been brought forward by Werkman *et al.* [1937] that certain bacteria which form phosphoglyceric acid can dissimilate glucose in the presence of a concentration of sodium fluoride which inhibits the dissimilation of the principal phosphoric acid esters of the Embden-Meyerhof scheme. They suggested that two mechanisms occur; one involving phosphoglyceric acid and the other not.

The objection might be raised that the phosphoric esters whose breakdown is inhibited by the concentration of sodium fluoride used constitute only stabilization products of the active forms. Evidence for this objection is lacking, however, and these experiments must be interpreted as being evidence against the Embden-Meyerhof scheme as the only route of glucose dissimilation by bacteria. Such a conclusion, of course, does not rule out the role of phosphorylation or the Embden-Meyerhof scheme.

SUMMARY

In comparing the quantities of phosphoric acid esters present in the trichloroacetic acid extracts of living bacterial cells fermenting glucose to the quantities present in the extract of cells not fermenting glucose, it has been found that:

(1) The readily determinable or unesterified orthophosphate is less in the presence of glucose than in its absence.

(2) The esterified phosphorus is greater in the presence of glucose than in its absence.

(3) These changes in the distribution of phosphorus occurring on addition of glucose are fundamental characteristics of phosphorylation, hence evidence has been given for the occurrence of phosphorylation in the living bacterial cell and its association with glucolysis.

(4) An explanation is offered for the increase of the orthophosphate with time in a suspension of cells.

¹ A certain amount of this increase may be due to autolysis, this process possibly freeing phosphorus from its various compounds.

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