CLXXXVIII. THE OCCURRENCE OF PHOSPHO- GLYCERIC ACID IN THE BACTERIAL DISSIMILATION OF GLUCOSE¹

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SINCE Harden & Young [1906] first obtained a hexose ester from yeast juice fermentation, phosphorylation and phosphorylated compounds have been of increasing importance in the reactions proposed to explain glycolysis. The work of Nilsson [1929], Embden *et al.* [1933] and particularly that of Meyerhof and associates [cf. Meyerhof, 1935] has produced convincing evidence that triosephosphoric acid and phosphoglyceric acid are intermediates in both muscle and yeast glycolysis. Furthermore, these workers do not believe that methylglyoxal normally functions as an intermediary, and Lohmann [1932] has demonstrated that lactic acid formation in muscle can be independent of methylglyoxal.

As most schemes of bacterial dissimilation are based on methylglyoxal as an intermediate, it is desirable to determine whether the reactions of Embden and Meyerhof may not be applicable to bacterial dissimilation. The literature contains little information. Antoniani [1933, 1, 2] showed that *Escherichia coli* can dissimilate both glyceric and phosphoglyceric acids. Either fresh or dried bacteria formed pyruvic acid from the phosphate ester in the presence of toluene. Neuberg & Kobel [1933, 1] showed that *Lactobacillus delbrückii* also formed pyruvic acid under these conditions. Tikka [1935] showed that *Escherichia coli* dissimilates hexosediphosphoric acid, α -glycerophosphoric acid, phosphoglyceric acid and pyruvic acid to normal end products. Although he was unable to isolate phosphoglyceric acid, the quantitative analysis was fitted to a scheme of glycolysis based on the work of Meyerhof & Kiessling [1934]. None of the proposed intermediate phosphoric acid esters was isolated. The first isolation of phosphoglyceric acid from a bacterial fermentation was reported by Werkman *et al.* [1936]. With *Citrobacter freundii* a small quantity of barium phosphoglycerate was obtained in the presence of toluene, glucose and phosphate buffer. The salt was identified by optical rotation and analysis of carbon, hydrogen, barium and phosphorus. Stone & Werkman have since reported the isolation of this phosphate ester from toluene-treated fermentations of propionic acid bacteria [1936, 1] and *Escherichia coli* [1936, 2]. The phosphoglyceric acid is believed to arise according to reaction D proposed by Meyerhof & Kiessling [1935] for alcoholic fermentation. Catalysed by hexosediphosphate:

Sodium fluoride is added to prevent further dissimilation of phosphoglyceric acid while toluene apparently aids in its production. Wood *et al.* [1937] showed that neither sodium fluoride, toluene nor added hydrogen acceptor was necessary for the formation of the ester by the propionic acid bacteria; however, these substances or similar compounds were required to produce significant quantities.

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Because of the ease of formation of phosphoglyceric acid by yeast, the propionic acid bacteria and *Esch. coli*, it was considered desirable to determine whether the ability to form this ester is a property of bacteria in general. The propionic acid and colon bacteria have been chosen for further study of some of the conditions affecting formation of the ester.

EXPERIMENTAL

The procedure followed in the isolation of phosphoglyceric acid was based on the method of Vercellone & Neuberg [1935] for the production of the ester by yeast. This method requires the addition of hexosediphosphate. However, it has been found that when phosphoglyceric acid is obtained with the addition of the hexose ester, it can be obtained also from glucose alone by preceding the regular incubation period with a phosphorylation period as described by Neuberg & Kobel [1933, 2]. The organisms were cultured in large flasks of broth (usually 10 l.), removed by passing the liquid through a supercentrifuge and used immediately. The experiments were based on the following quantities of reactants: 7 g. moist cell paste, 7 ml. 0.67 *M* phosphate buffer, 6 ml. 20 % glucose, 5 ml. 2.5 % sodium hexosediphosphate, 0.1 ml. 1 % MgCl_2 and 1 ml. toluene. The quantities of 0.2 *M* NaF and 2 % acetaldehyde, or other hydrogen acceptor, were varied according to the experiment. The final volume of the mixture was adjusted to approximately 35 ml. with distilled H_2O .

The buffer was prepared by mixing 500 ml. of 0.67 *M* Na_2HPO_4 and 150 ml. of 0.67 *M* KH_2PO_4 , resulting in a pH of 6.85.

The hexosediphosphate solution was prepared from the commercial calcium salt by precipitating the calcium with $\text{Na}_2\text{C}_2\text{O}_4$. About 15 % of the phosphorus in the resulting solution was not hydrolysed in 2 hr. at 100° in *N* HCl—this was presumably present as hexosemonophosphate.

The reactants were well mixed and warmed and the cotton-stoppered flasks were incubated for a specified time at 37° with frequent shaking. At the termination of the incubation period, the flasks were chilled and placed at 5°. The mixtures were treated within 24 hr. to obtain any phosphoglyceric acid present. The inorganic phosphate was precipitated by addition of 4 ml. of 20 % Mg acetate and a few drops of 20 % NH_3 solution. The mixture was centrifuged and the supernatant liquid treated with 3.5 ml. glacial acetic acid and 1.7 ml. 50 % barium acetate. The precipitate sometimes formed at this point was removed immediately by centrifuging. The supernatant liquid was placed at 5° and if a considerable quantity of phosphoglyceric acid was present, the barium salt crystallized out in 24–48 hr. If no crystals formed after 2 days, alcohol was added equivalent to about 10 % of the volume of the liquid. If still no precipitate resulted in 2 days, a like volume of alcohol was added.

The crystalline barium salt of phosphoglyceric acid was filtered, dried and weighed. In case of the first isolation from an organism or where any doubt existed concerning the nature of the precipitate, the ester was purified by solution in 70 times its weight of 0.05 *N* HCl and reprecipitation by addition of twice this volume of alcohol. The identification was based on the optical rotation of the ester $[\alpha]_D^{25} = -13 \pm 0.3^\circ$, phosphate analysis and microscopical examination of crystals. The Sr and Ca salts can also be used to identify phosphoglyceric acid by microscopic examination.

A rough measure of the phosphate uptake was made available by centrifuging out the MgNH_4PO_4 precipitate in graduated centrifuge tubes and comparing the amount with that obtained from a control at 5°.

(1) *The formation of phosphoglyceric acid by the propionic acid bacteria*

Two species of propionic acid bacteria were used, *Propionibacterium arabinosum* and *P. pentosaceum* (described by Werkman & Brown [1933]). The bacteria were grown in a medium consisting of 0.5% glucose, 0.3% Difco yeast extract and 0.2% K_2HPO_4 and incubated for 3 days at 30°. From 3 to 4 g. of moist cell paste per litre of culture medium were obtained on centrifuging.

In Table I are given the results of several experiments showing the yields of salt and approximate phosphate uptake (based on the total 35 ml. mixture) under varying conditions of time, fluoride and acetaldehyde concentrations etc.

Table I. *Formation of phosphoglyceric acid by Propionibacterium arabinosum and P. pentosaceum**

Exp.	Time hr.	0.2 M NaF ml.	2% acetalde- hyde ml.	Special treatment	Phosphate uptake mg.	Yield of salt mg.
41	4	1.0	4.2	—	.65	73
	4	1.5	4.2	—	75	80
	6	1.5	8.4	—	85	116
	6	2.0	8.4	—	90	119
65	4	1.5	8.0	—	75	112
	6	1.5	8.0	—	95	143
	4	1.5	8.0	Aerated	75	139
	6	1.5	8.0	Aerated	90	174
	4	1.5	8.0	Nitrogen	80	104
	6	1.5	8.0	Nitrogen	55	114
69	6	2.0	8.0	—	60	101
	6	2.0	8.0	Aged 6 days	30	4
74	5.5	2.0	8.0	Worked up at once	75	24
	5.5	2.0	8.0	Worked up in 2 days	55	66
	5.5	2.0	8.0	Worked up in 17 days	80	74
86	6	2.0	8.0	—	30	127
	6	2.0	8.0	Aged 1 day	30	93
	6	2.0	8.0	Aged 3 days	35	52
	6	2.0	8.0	Aged 7 days	20	7

* *P. pentosaceum* was used in Exp. 86 only.

Exp. 41 indicates that a change in time, in acetaldehyde or fluoride may influence the formation of the ester. In Exp. 65 aeration is shown to increase the yield of phosphoglyceric acid, whereas if nitrogen is passed through the reaction flask instead of air there is less product formed than in the cotton-stoppered controls. The action of the air may be due to oxygen acting as a hydrogen acceptor to supplement acetaldehyde. Exps. 69 and 86 show that to obtain phosphoglyceric acid it is advisable to use the organisms immediately after removal from the original medium. The "ageing" was carried out by storing the moist paste at 5° in a sterile Petri dish. There was very little loss of moisture, but the activity of the phosphoglyceric acid enzyme system fell off rapidly with time of storing. It is noteworthy that there is some correlation between the phosphate uptake and quantity of phosphoglyceric acid obtained. It is also apparent that there is much more phosphate esterified than can be accounted for by the yields of ester even if it is assumed that the methods are only 50% efficient for the smaller yields. It is probable that the phosphate may be held as an intermediate ester, perhaps a precursor of phosphoglyceric acid. Exp. 74 lends support to this suggestion. Several fermentations were run under the same conditions, and the yield of phosphoglyceric acid determined after varying

times of storage at 5°. As the time of storage lengthened, the yield of ester increased, with no corresponding increase in phosphate uptake; an indication that some intermediate ester may be undergoing a slow conversion into phosphoglyceric acid.

(2) *The formation of phosphoglyceric acid by the colon-aerogenes bacteria*

Two typical members of the colon-aerogenes group were chosen for study, *Aerobacter indologenes* and *Escherichia coli*. The isolation of phosphoglyceric acid from the glucose fermentation (without hexosediphosphate) of these organisms has been reported by Stone & Werkman [1936, 2]. The results given in Tables II and III show the effects of various conditions on yields of the

Table II. *Formation of phosphoglyceric acid by Aerobacter indologenes*

2 ml. 0.2 <i>M</i> NaF unless otherwise noted					
Exp.	Time hr.	H acceptor ml.	Special treatment	Phosphate uptake mg.	Yield of salt mg.
62	6	Acetaldehyde 8	—	60	152
	6	Acetaldehyde 8	No toluene	30	1
	3	None	1 ml. NaF	25	42
	3	None	1 ml. NaF, no toluene	25	0.4
66	6	Acetaldehyde 8	—	20	48
	6	Acetaldehyde 8	Xylene, no toluene	50	68
	6	Acetaldehyde 8	CHCl ₃ , no toluene	20	60
	6	Acetaldehyde 8	No toluene	20	6
83	6	Acetaldehyde 8	—	20	99
	6	None	—	20	27
	6	Pyruvate 8	—	20	123
	6	Amc. 8	—	30	169
90	6	Propaldehyde 8	—	35	58
	6	Acetaldehyde 8	—	35	102
	6	Acetaldehyde 8	Aged 1 day	30	79
	6	Acetaldehyde 8	Aged 3 days	30	90
92	6	Acetaldehyde 8	Aged 5 days	10	30
	6	Acetaldehyde 8	Aged 7 days	5	7
	6	Amc. 8	—	25	90
	6	Amc. 8	Aerated	30	140
95	6	Amc. 8	Nitrogen	35	102
	4.5	Acetaldehyde 8	—	55	34
	4.5	Acetaldehyde 8	Aerated	50	64
	4.5	Acetaldehyde 8	Nitrogen	50	38

Table III. *Formation of phosphoglyceric acid by Escherichia coli*

Time 6 hr.; 1.5 ml. 0.2 M NaF				
Exp.	H acceptor ml.	Special treatment	Phosphate uptake mg.	Yield of salt mg.
91	None	—	40	88
	Acetaldehyde 8	—	35	165
	Amc. 8	—	65	234
	Pyruvate 8	—	50	79
	Propaldehyde 8	—	25	93
93	Amc. 8	—	75	345
	Amc. 8	Aged 1 day	60	310
	Amc. 8	Aged 3 days	35	178
	Amc. 8	Aged 7 days	35	260
94	Amc. 8	—	80	245
	Amc. 8	Aerated	75	452
	Amc. 8	Nitrogen	50	319

barium salt from toluene-treated mixtures of glucose, hexosediphosphate, etc., as outlined above. The organisms were grown in a medium consisting of 0.5 % glucose, 0.3 % Difco peptone and 0.2 % K_2HPO_4 , and incubated at 30°. Cultures of *Aerobacter indologenes* were aerated and the organisms removed by centrifuging after 36 hr. The cultures of *Escherichia coli* were neutralized two or three times with NaOH using bromothymol blue as an indicator and centrifuged after 48 to 60 hr.

Exp. 62, Table II, shows that acetaldehyde is unnecessary for the isolation of phosphoglyceric acid with *Aerobacter indologenes*. Toluene may also be omitted and a small quantity of ester isolated. Exp. 66 indicates that toluene is not specific in its action as xylene and chloroform may be substituted with good results. Similar findings were reported for the propionic acid bacteria by Wood *et al.* [1937]. A comparison of different hydrogen acceptors, each equivalent to 2 % acetaldehyde in dehydrogenating capacity, is presented in Exp. 83. Pyruvic acid proves to be a good hydrogen acceptor, propaldehyde is fair and acetylmethylcarbinol (recorded as Amc.) is excellent. Other experiments with this organism indicate that acetaldehyde functions as well as pyruvic acid. For *Escherichia coli*, Exp. 91, Table III, only acetylmethylcarbinol surpasses acetaldehyde. This organism readily yields phosphoglyceric acid with no added hydrogen acceptor present. It is of interest to note that for the propionic acid bacteria [Wood *et al.* 1937] pyruvic acid is a better acceptor than acetaldehyde and acetylmethylcarbinol is much poorer; while with the colon-aerogenes organisms the situation is quite different.

The effect of ageing is shown in Exps. 90 and 93, Tables II and III. Apparently ageing does not diminish yields by *Esch. coli* as in the case of *Aerobacter indologenes* or the propionic acid bacteria. The formation of phosphoglyceric acid under aeration and in the presence of nitrogen is shown in Exps. 92, 94 and 95. Aeration measurably increases the yield whilst agitation with nitrogen gives results comparable with the control. The constant agitation of the mixture may be the cause of slightly higher yields under anaerobic conditions.

As in the results with the propionic acid bacteria there is a correlation between the quantity of phosphate taken up and the ester formed. Three values in Table II are negative, showing that some of the original hexosediphosphate must have been dissimilated to give additional inorganic phosphate.

(3) *Formation of phosphoglyceric acid by various bacteria*

In order to determine whether the occurrence of phosphoglyceric acid is general in bacterial glycolysis, several representative species were used. The choice was determined by the organisms readily available that gave reasonably good growth in appropriate broth cultures. The cells were centrifuged from the medium near the end of the log-growth phase and used immediately. The isolations were carried out by the previously outlined method of Vercellone and Neuberg, based on 7 g. of moist cells. Results are shown in Table IV. In general the formation of the ester was accompanied by an uptake of inorganic phosphate, but owing primarily to the small yields obtained, the correlation was not significant. It is probable that had further experiments on the most favourable conditions for the formation of phosphoglyceric acid been carried out, the yields could have been materially increased. The results show that phosphoglyceric acid can be isolated from the dissimilation of glucose by *Serratia marcescens*, *Lactobacillus pentoaceticus*, *L. plantarum*, *Bacillus subtilis*, *B. mycoides*, *Azotobacter vinelandii*, *Streptococcus paracitrovorus*, and *Staphylococcus albus*.

Table IV. *Formation of phosphoglyceric acid by various bacteria*

Exp.	Organism	Time hr.	0.2 M NaF ml.	2% acetalde- hyde ml.	Yield of salt mg.
44	<i>Serratia marcescens</i>	4.5	1.5	8.4	22
	"	6	1	8.4	35
	"	6	2	8.4	20
47	<i>Lactobacillus pentoaceticus</i>	3	1	6	0
	"	4	1	8.4	13
	"	8	2	8.4	25
52	<i>Bacillus subtilis</i>	4	1	8.4	83
	"	6	1.5	8.4	95
57	<i>Lactobacillus plantarum</i>	6	1.5	8.4	35
	"	9	2.5	8.4	42
62	<i>Azotobacter vinelandii</i>	6	2	8	0.5
78	<i>Streptococcus paracitrovorus</i>	4	1	8	13
	"	6	2	8	10
80	<i>Streptococcus paracitrovorus</i>	3	1	8	14
	"	6	2	8	Trace
104	<i>Bacillus mycoides</i>	4	1	6	Trace
	"	6	2	8	14
107	<i>Staphylococcus albus</i>	4	1	6	9
	"	8	2	8	13

Table V. *Summary of attempts to isolate phosphoglyceric acid with Clostridium butylicum*

Exp.	Time hr.	0.2 M NaF ml.	2% acetalde- hyde ml.	Special treatment	Phosphate uptake mg.	Pre- cipitate B ml.
30	3.5	1.4	8.4	—	—	—
31	3.5	1.5	8.4	2 ml. 0.2 M Na oxalate	—	—
42	3	1.0	4.2	—	30	8.5
	3	2.0	8.4	—	35	6.5
	6	1.0	4.2	—	40	7.0
	6	2.0	8.4	—	75	8.0
45	7.5	2.0	8.4	—	45	8.0
	4	1.0	4.2	2 ml. 0.2 M Na oxalate	30	8.0
	7.5	2.0	8.4	—	50	5.0
50	8	3.0	12.0	—	25	2.0
	8	3.0	8.0	2 ml. 0.2 M Na oxalate	25	3.5
	6	2.0	2.0	8 ml. 4% amc.	25	3.5
55	8	2.0	8.4	Anaerobic	45	9.5
	4	1.0	6.0	Anaerobic	35	8.0
	6	1.0	8.4	—	15	4.5
	6	2.0	8.4	—	60	8.5
	6	2.0	8.4	2 ml. 0.2 M cysteine	65	7.5
	4	3.0	8.4	No toluene	85	11.5
105*	4	1.0	6.0	—	—	4.5
	8	2.0	8.0	—	—	5.5
115†	3	1.0	6.0	—	—	5.5
	6	2.0	9.0	—	—	6.0

* *Cl. sporogenes*.† *Cl. histolyticum*.

The only organisms failing to yield phosphoglyceric acid after several experiments were members of the genus *Clostridium*. Three species were tested, *Cl. butylicum*, *Cl. sporogenes* and *Cl. histolyticum*, and of these a number of trials

were carried out with *Cl. butylicum* using the same procedure as before with variations listed in Table V. The organisms were inoculated in large flasks of glucose-yeast extract broth under semi-anaerobic conditions and incubated at 40° for 18–20 hr. *Cl. sporogenes* and *Cl. histolyticum* were incubated at 37° in a medium containing 2% Difco tryptophan broth and 0.5% glucose. Crystallized liver extract (0.5%) was added to the medium for *Cl. histolyticum*. At the point in the procedure where acetic acid is added, a considerable amount of a yellow-white precipitate, termed precipitate B, came out of solution. The amount obtained from each mixture is recorded in Table V in ml. of moist precipitate. In experiments with yeast [Stone, 1936] there is a similar precipitation at this point and the quantity of the precipitate correlates inversely with the yield of phosphoglyceric acid. With most of the bacteria studied, this precipitate was altogether lacking or present in negligible quantities. However, with members of the genus *Clostridium* not only were there relatively large quantities of the precipitate, but they correlated directly with the phosphate uptake. The precipitate in question is converted into a dark brown glue-like mass on drying and is evidently not a pure substance, since it contains varying small amounts of phosphorus and nitrogen.

DISCUSSION OF RESULTS

In order to compare the results of experiments on the isolation of phosphoglyceric acid, the significance of the varying yields of phosphoglyceric acid should be considered. It seems reasonable to assume that a large yield of ester indicates favourable conditions for the corresponding enzymes to function, while continued small yields show the presence of the necessary enzyme either in very small quantity or functioning under unfavourable conditions. The yields are subject to a large variation even within one experiment (i.e. from one culture of organisms). Between different experiments the variation is even larger, and results must be compared with great caution.

The isolation of phosphoglyceric acid from every organism studied excepting the strict anaerobes (i.e. genus *Clostridium*) indicates that the ability to form this intermediate is widespread among the bacteria. The fact that none of the three species of *Clostridium* formed the ester under the conditions studied, and showed a distinctly different chemical behaviour during the experiment, suggests that these organisms as a genus do not possess an enzyme mechanism capable of producing phosphoglyceric acid. Until more is known of the nature of precipitate B, it is difficult to attach any significance to its appearance. However, it is quite similar (if not identical) to the substance found in this procedure with yeast. Small amounts were formed by several of the bacteria, particularly the cocci. As the results on the isolation of phosphoglyceric acid appear to set apart the anaerobic organisms, it is possible that the formation of this ester may be related to an aerobic dissimilative mechanism. The beneficial effect of aeration compared to that of agitation with nitrogen adds support to this idea. However, if one attempts to correlate the degree of aerobiosis with ease of formation of the ester, it is apparent that the facultative colon organisms or micro-aerophilic propionic acid bacteria produce phosphoglyceric acid much more readily than the aerobic *Bacillus mycoides* or *Azotobacter vinelandii*. The increased yields due to aeration may be explained by the action of oxygen as a hydrogen acceptor. In this connexion, it is apparent that for best results in experiments of this type it is advisable to choose a hydrogen acceptor appropriate to the organism. Acetaldehyde is quite satisfactory for yeast and works well with most bacteria. However, acetylmethylcarbinol functions much better as an oxidizing agent for

the colon-aerogenes group, whereas pyruvic acid is superior to acetaldehyde or propaldehyde for the propionic acid bacteria.

The isolation of phosphoglyceric acid from dissimilations by a number of bacteria does not prove that this substance is an intermediate in bacterial glycolysis. The fact that it is present, however, indicates that many bacteria possess an enzyme system capable of producing the ester. From the standpoint of its formation phosphoglyceric acid is more readily obtained than methylglyoxal in the case of the propionic acid bacteria and the colon-aerogenes organisms. However, it is necessary to conduct further investigations on the dissimilation of phosphoglyceric acid and other phosphate esters before it can be accepted that the Embden-Meyerhof reactions play a primary role in bacterial dissimilation. It is possible that there may be two or more mechanisms of glycolysis present in the bacterial cell, one of which gives rise to phosphoglyceric acid, and another that may cause breakdown of glucose without involving the formation of phosphorylated intermediates.

SUMMARY

Phosphoglyceric acid has been isolated from fermentation mixtures using *Propionibacterium arabinosum*, *P. pentosaceum*, *Aerobacter indologenes*, *Escherichia coli*, *Lactobacillus pentoaceticus*, *L. plantarum*, *Serratia marcescens*, *Bacillus subtilis*, *B. mycoides*, *Azotobacter vinelandii*, *Streptococcus paracitrovorus* and *Staphylococcus albus*. It was not found with *Clostridium butylicum*, *Cl. histolyticum* and *Cl. sporogenes*.

Aeration of the fermentation mixtures increases, whilst storing the cells at 5° decreases, the formation of the ester. Acetylmethylcarbinol is a better hydrogen acceptor for use with the colon-aerogenes bacteria than either pyruvic acid or acetaldehyde. The possible role of phosphoglyceric acid in bacterial glycolysis is discussed.

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