CCXXV. STUDIES ON BACTERIAL PHOSPHATASES.

I. THE DECOMPOSITION OF PHOSPHORIC ACID ESTERS BY *CLOSTRIDIUM ACETOBUTYLICUM* WEIZMANN.

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THE conception of the phosphoric esterification of sugars as a necessary step in their metabolism in bacterial cells follows logically from considerations of carbohydrate breakdown in muscle and in yeast. But the experimental evidence in support of this conception in the case of bacteria is not very extensive, and information regarding the bacterial phosphatase systems which are responsible for synthesis and hydrolysis of phosphoric acid esters is very limited indeed. Virtanen and Tikka [1930] succeeded in isolating from a mixture containing, initially, glucose, inorganic phosphate and dried *Bacterium casei* € two hexose-phosphoric acid esters. One of these resembled the Robison ester, while the barium salt of the other had the formula $C_{12}H_{19}O_{16}P\text{Ba}_2$. This appears to be the only reported case where the product of the phosphoric esterification of a sugar, as effected by bacteria, has been isolated. Virtanen had previously obtained some evidence of such a transformation with *Bacterium casei* € [1924], *Streptococcus lactis* [1925, 1] and *Bacterium acidi propionici* b [1925, 2], but the ester was not isolated in any of these experiments. However it has been shown that several species of bacteria possess an enzyme system which is able to form methylglyoxal from hexosephosphates. Such species are *Bacillus delbrücki* [Neuberg and Kobel, 1929], *Bact. coli* [Fromageot, 1929], *Bact. lactis aerogene* [Neuberg and Scheuer, 1930], tubercle and timothy bacilli [Kuroya, 1931], *Cl. acetobutylicum* [Pett and Wynne, 1932, 1] and *Propionibacterium jensenii* [Pett and Wynne, 1932, 2]. These findings suggest that hexosephosphates play a significant part in the carbohydrate metabolism of these organisms.

Very little information is available in the literature with respect to the hydrolytic action of bacteria upon phosphoric esters. Manning [1927] has shown that *Bact. coli commune* ferments the sodium salts of both hexosediphosphoric acid and "hexosemonophosphoric" acid synthesised by yeast. Under either aerobic or anaerobic conditions decomposition proceeded with the formation of products normally found in glucose fermentations. The concentration of the diphosphate used was about 2.5 %. The fact that the ester is fermented by *Bact. coli* is not, in itself, evidence that a phosphatase is present, since it is conceivable that the glycolytic mechanism of the organism is able to ferment the glucose portion of the ester without preliminary hydrolysis. Recent observations of Boivin and Mesrobian [1933], however, have led to the conclusion

that a phosphatase is actually present in *Bact. coli*. In the present work it is shown that *Cl. acetobutylicum* possesses an active phosphatase, although sodium hexosediphosphate even in low concentration is not fermented by the living cells when no other carbohydrate is present.

EXPERIMENTAL.

In order to test the ability of the living organisms to ferment directly sodium hexosediphosphate, sterile solutions containing 1.2 % of the ester, 0.6 % of peptone, traces of NaCl and MgSO₄, and shredded filter-paper were inoculated with portions of suspensions of washed and of unwashed organisms removed from an active glucose-peptone culture at the age of 20 hours. The test media, contained in tubes, were incubated both aerobically and anaerobically at 37° and any gases formed in non-evacuated tubes were collected over dilute H₂SO₄. Similar tubes containing 3 % glucose-0.6 % peptone were treated in the same manner. The glucose tubes all yielded normal volumes of mixed gases, but not even after 2 weeks' incubation did any tube containing hexosediphosphate evolve a trace of gas. In all cases, however, growth occurred, due to the presence of peptone. The results indicate that sodium hexosediphosphate in the relatively low concentration of 1.2 % is not fermented by the living organism in the manner in which glucose is fermented. No attempt was made to determine whether any hydrolysis of the ester took place. In view of Manning's results with *Bact. coli*, the fermentation of the same hexosediphosphate preparation by the latter organism was tested. Media similar to those described above were inoculated with *Bact. coli* prepared according to the method described by Manning. Small portions of a suspension of organisms added to sterile glucose-peptone and hexosediphosphate-peptone solutions caused vigorous fermentation in 24 hours. The sodium hexosediphosphate used in these experiments and in those which follow was prepared synthetically with the aid of zymin made from brewer's top yeast.

The hydrolysis of sodium hexosediphosphate by Cl. acetobutylicum in active glucose fermentations.

Into each of nine tubes containing shredded filter-paper were introduced 15 cc. of 5 % glucose-1.0 % peptone solution; the tubes were sterilised, and varying amounts of sterile sodium hexosediphosphate solution of known strength in addition to sufficient sterile water to make the volume 25 cc. were added. Each tube was inoculated with 0.5 cc. of an active maize culture of the organism and incubated for 5 days at 37.5°. The liberated gases were collected over dilute H₂SO₄ and the volumes, corrected to 20° and constant pressure, were measured at intervals during the fermentation and at the end. The final inorganic phosphate content of all tubes was estimated by the Briggs method, final reducing power by the method of Shaffer and Hartmann. Sodium hexosediphosphate allowed the fermentation to proceed only when present in low concentrations. When the degree of hydrolysis of the added ester was plotted against concentration of the ester as in Fig. 1 it was observed that greatest hydrolysis occurred when the ester was present in a concentration of approximately 0.05 %. But it is also apparent from Fig. 1 that with initial concentrations of the hexosediphosphate higher than about 0.2 % practically no additional inorganic phosphate was liberated. This may possibly be explained on the basis that the available enzyme was practically completely saturated by 0.2 % hexosediphosphate or it may be due to an inhibitory effect of the accumulated inorganic

phosphate. The added hexosediphosphate did not affect the rate of fermentation except in concentrations higher than 0.35 % where there was definite retardation.

Fig. 1. Hydrolysis of sodium hexosediphosphate in active cultures of *Cl. acetobutylicum*.

Since it was shown in the last experiment that added sodium hexosediphosphate was partially hydrolysed in active glucose fermentations, the rate of hydrolysis in relation to the general progress of the fermentation as indicated by the development of acid was next studied. For this purpose four flasks were prepared, containing the solutions specified.

(1) Experimental flask: 300 cc. of a sterile solution containing 3 % glucose, 0.6 % peptone and 0.04 % sodium hexosediphosphate; (2) three control flasks: (a) 100 cc. of a sterile 0.04 % solution of sodium hexosediphosphate; (b) 100 cc. of a sterile 0.04 % solution of the diphosphate in which were dissolved quantities of acetic and butyric acids such that their concentrations approximated to the amounts present in a normal 3 % glucose culture at maximum acidity; (c) 300 cc. of sterile 3 % glucose-0.6 % peptone solution.

Flasks 1 and 2 c were inoculated with 5 cc. of an active maize culture and all flasks were incubated at 37°. The changes in the total acid and inorganic phosphate contents of flask 1 are illustrated in Fig. 2. No significant change in

Fig. 2. Rate of hydrolysis of sodium hexosediphosphate in an active culture of *Cl. acetobutylicum*. a, titratable acidity; b, inorganic phosphorus.

inorganic phosphate occurred in any of the control flasks in 170 hours, but there was quite extensive hydrolysis of the hexosediphosphate in flask 1. The enzyme responsible for this hydrolysis did not come into effective action until the second phase of the fermentation. In this phase there is little or no further increase in the number of cells but frequently a decrease; during this period part of the accumulated acetic and butyric acids is converted into acetone and butyl alcohol at a rate which is comparable with the rate of accumulation of the acids in the first phase. The marked increase in the rate of hydrolysis in the second period suggests either that some of the cells have become more permeable to the enzyme or that the enzyme may have been liberated from cells which have actually undergone disintegration. It is not unlikely that a small portion of the liberated inorganic phosphate had its origin in the complex esters of the cell. The slight decrease in free phosphate in the early part of the fermentation suggests the formation of such esters during active growth.

Hydrolysis of glycerophosphate.

In the previous experiments the presence of a hexosephosphatase in active cultures was demonstrated: it was of interest to learn whether glycerophosphate, under similar conditions, would undergo hydrolysis. A series of flasks was prepared containing 3 % glucose-0.6 % peptone solution in which were dissolved varying amounts of sodium β -glycerophosphate (Boots); water was added to provide equal volumes in all flasks. The sterile media were inoculated with equal portions of an active maize culture of the organism and incubated at 37.5° for 5 days. From the amount of inorganic phosphate finally present in each flask the degree of hydrolysis of the glycerophosphate was calculated. The results are summarised in Table I. The amount of liberated phosphate increased with the concentration of glycerophosphate but the degree of hydrolysis calculated as % of added ester was low in all flasks, reaching a maximum when the concentration of the ester was approximately 0.02 %. Since glycerol is not fermented by this organism there can be no possibility that the accumulation of free phosphate is due to the operation of a glycerolytic mechanism which might conceivably release free phosphate by fermenting away the glycerol portion of the ester. It can be concluded, therefore, that a true phosphatase is present in the actively fermenting culture of the organism.

Table I. *Hydrolysis of sodium β -glycerophosphate in active cultures of Cl. acetobutylicum.*

Flask	Added glycerophosphate %	Total inorganic P after 5 days mg. per 10 cc.	Inorganic P derived from ester mg. per 10 cc.	Ester hydrolysed %
1	0.00	0.086	—	—
2	0.00	0.087	—	—
3	0.015	0.130	0.044	20.45
4	0.030	0.172	0.086	20.00
5	0.075	0.177	0.091	8.46
6	0.300	0.343	0.257	5.97
7	0.350	0.407	0.321	6.40
8	0.500	0.330	0.244	3.40
9	0.750	No fermentation		—

Attempts to demonstrate synthesis of phosphoric esters.

Several attempts were made to demonstrate phosphoric esterification of glucose and fructose, in the presence of inorganic phosphate, by various preparations of the organisms. These included air-dried organisms, alcohol-ether-dried

organisms, dried organisms suspended in a small quantity of water and ground for several hours in an agate mortar with powdered glass and autolysed organisms. The results were all negative: in every case a definite increase rather than a decrease in inorganic phosphate was observed and, simultaneously, the reducing value of the mixture increased. Detailed consideration of these experiments is reserved until further studies have been made.

SUMMARY.

1. Sodium hexosediphosphate in 1.2 % concentration was not fermented by living *Clostridium acetobutylicum*. When present in 0.75 % concentration the diphosphate completely inhibited the fermentation of glucose in an otherwise favourable medium. A similar concentration of sodium β -glycerophosphate inhibited the fermentation of glucose.

2. In active glucose-peptone cultures of the organism both sodium hexosediphosphate and sodium β -glycerophosphate were appreciably hydrolysed. The presence of a true phosphatase in active cultures has been established.

3. The hydrolysis of sodium hexosediphosphate proceeded most rapidly during the second phase of the fermentation following the time at which maximum acidity was attained.

4. Attempts to demonstrate the synthetic activity of the phosphatase failed.

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