CCXXVI. STUDIES ON BACTERIAL PHOSPHATASES.

II. THE PHOSPHATASES OF *CLOSTRIDIUM ACETOBUTYLICUM* WEIZMANN AND *PROPIONIBACTERIUM JENSENII* VAN NIEL.

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INTRODUCTION.

In a previous paper [Heard and Wynne, 1933] the presence of a phosphatase in active glucose-peptone cultures of *Clostridium acetobutylicum* Weizmann was reported. It is the purpose of the present communication to describe some of the properties of this enzyme and of a similar enzyme present in *Propionibacterium jensenii* van Niel. Each of these organisms ferments various carbohydrates and certain related compounds. The first produces, as the chief end-products of glucose or starch breakdown, carbon dioxide, hydrogen, acetone, ethyl and butyl alcohols; the acetone and alcohols are derived from acetic and butyric acids formed as intermediary compounds. The second organism may be regarded as being representative of a rather large group of bacteria which ferment sugars and some related compounds with the formation of acetic and propionic acids [van Niel, 1928].

Phosphatases exhibit marked differences in their p_H -activity relationships. Those of mammalian tissues are, in most cases, much more active under alkaline than acid conditions, irrespective of the nature of the substrate. Usually the speed of hydrolysis reaches a maximum at about p_H 9, although, as Kay [1932] points out, this is probably only an apparent optimum since inactivation of the enzyme in more alkaline media may affect the exact identification of the optimum reaction. Roche [1931] observed that the phosphatase of certain mammalian erythrocytes appeared to differ from other mammalian phosphatases since it had a p_H optimum of 5.8 to 6.8 varying with the substrate. Among fungi and higher plants the few phosphatase systems which have been investigated are all most active under acid conditions. For example, the enzymes of *Aspergillus oryzae* and *A. niger* have p_H optima varying from 3.1 to 5.5 according to the method of preparation and the nature of the substrate [Asakawa, 1929]. Yeast phosphatase, whether acting hydrolytically [Kertesz, 1930] or synthetically [Euler and Nordlund, 1921], is most active at p_H 6.4, soya bean phosphatase at about p_H 5.3 [Kay and Lee, 1931] and rice phosphatase at p_H 4.3 [Horiuchi, 1931]. Regarding bacterial phosphatases very little is known. Virtanen [1927; Virtanen and Tikka, 1930] specified p_H 6.2 as the optimum for phosphoric esterification by *Bact. casei* ϵ . The fact that the phosphatase of *Cl. acetobutylicum* was found by Heard and Wynne [1933] to be active, hydrolytically, in growing cultures of the organism suggests that the enzyme prefers an acid medium. Quite recently it has been stated by Boivin and Mesrobian [1933] that both *Bacterium coli*

and *Staphylococcus aureus* possess two phosphatase systems which exhibit optimum activity at p_H 7 and 10. This conclusion was based on studies involving the autolytic liberation of free phosphate from the complex esters of the cell. No systematic study of the phosphatase of any bacterial species has come to the attention of the authors.

Regarding the specific behaviour of the various phosphatases no conclusion can yet be reached. Strong evidence was advanced by Kay [1928; 1932] in favour of the identity of the phosphatases of mammalian bone, intestinal mucosa, kidney and blood-plasma. Recent observations, however [Roche, 1931; Jacobsen, 1931; Edlbacher and Kutscher, 1932] indicate that certain other mammalian phosphatases exhibit marked differences of behaviour. Moreover, the fact that phosphatases of higher plants and fungi have p_H optima which are widely divergent from the values characteristic of the mammalian enzymes may possibly be regarded as evidence of certain fundamental differences in the enzyme systems. The phosphatases of the two bacterial species investigated in the present work are shown to differ not only from mammalian and plant phosphatases but also from each other in several respects.

EXPERIMENTAL.

(A) *Materials and methods.*

Substrates. The glycerophosphates used were "sodium glycerophosphate 644" prepared by the Eastman Kodak Company and shown by Kay and Lee [1931] to be almost entirely the β -ester, and sodium α -glycerophosphate (Boots) which contained only small amounts of the β -ester. Unless otherwise stated, the term glycerophosphate, wherever employed in this paper, refers to the β -ester. Sodium hexosediphosphate was prepared from commercial calcium hexosephosphate somewhat after the manner described by Neuberg and Sabetay [1925]. The preparation, as finally used, still contained a small quantity of a monophosphoric ester and a trace of inorganic phosphate. The sodium pyrophosphate employed was Baker's C.P. grade and was heated to remove traces of orthophosphate. All these compounds give alkaline solutions; they were therefore adjusted to the desired p_H with HCl before using.

General experimental conditions. The reaction mixtures were usually buffered with 0.025 *M* potassium hydrogen phthalate, this concentration being an effective buffer but having no appreciable inhibitory effect on the enzyme activity. Toluene was added, and the mixtures were rocked in L-shaped tubes in a water-bath at 37.5°. At suitable intervals 2 cc. samples were removed and added to 10 cc. portions of 5 % trichloroacetic acid solution in centrifuge-tubes. After centrifuging, 10 cc. of the clear liquid were removed with a pipette, and the inorganic phosphorus was determined by the Briggs method. Control tubes containing substrate + buffer and enzyme + buffer were included in all experiments. Phosphorus values are expressed as mg. of phosphorus present as inorganic phosphate in 10 cc. of reaction mixture.

Preparation of the enzyme. Attempts were made in the case of each organism to determine whether phosphatase was present in the culture medium in which the bacteria had been grown. When the number of cells had reached a maximum they were removed in the Sharples centrifuge, the clear liquid was concentrated at low temperature to a twentieth of the original volume and was tested for enzyme activity. No phosphatase could be detected in any such preparation: the treatment was probably not destructive since amylase was always present in the concentrates of the liquid obtained from the *Clostridium* cultures.

It was found, however, that in the case of both organisms phosphatase can be shown to be present in the dried cells as well as in suspensions of living cells to which toluene is added. Dried preparations were found to be more stable and more convenient and therefore they were employed in preference to preparations of toluene-treated organisms.

Cl. acetobutylicum was grown at 37° in a medium containing 4 % glucose, 1 % peptone and shredded filter-paper. After about 20 hours' incubation the culture was filtered through cheese cloth and centrifuged. The organisms were washed twice with distilled water, spread on a glass plate, dried at room temperature in a current of air, ground to a powder and preserved in a desiccator. This dry material loses about one-third of its phosphatase activity in a month at 4° and becomes practically inactive after 5 months. Some variations were observed in the activity of the dried organisms depending on the age of the culture at the time of the removal of the cells. Greatest activity per g. of dried cells usually occurs at the age of 18 to 20 hours: this period in the fermentation of carbohydrate by *Cl. acetobutylicum* is a critical one, for it is at this time that the cell count reaches its maximum, the acidity reaches its peak, and the second phase of the fermentation begins. Just after the period of maximum acidity the phosphatase activity per g. of dried cells usually drops appreciably, but it then rises again to a value approaching the maximum, and, after several hours, it finally falls to a value which may be only a small fraction of the maximum activity. This final drop is frequently very rapid in cultures more than 60 hours old. The physiological significance of these variations is not clear. It is possible partially to remove the enzyme from the dried cells by simple aqueous extraction. From such extracts and others, more concentrated and considerably purified preparations of the enzyme can be made. In the present work, however, only the dried cells were employed.

Propionibacterium jensenii was grown at 30° for 7 to 10 days in a medium containing 3 % glucose, or 2 % glycerol, and 0.6 % peptone. Sterilised extract of fresh starch-free yeast constituted 10 % of the liquid of the medium; the remainder was distilled water. The medium was adjusted to p_H 7.1 before final sterilisation. After incubation the organisms were removed by centrifuging and were treated in the manner described above. The phosphatase of the dried powder is quite stable: in 6 months in the desiccator at room temperature about one-half of the activity disappears.

In the experiments reported the dried organisms were used in the proportion of 0.10 g. to 10 cc. of reaction mixture, unless otherwise stated.

(B) *Some properties of the enzymes.*

(1) p_H -activity. Each tube of a series received 5 cc. of phthalate buffer having approximately the desired p_H except in the alkaline range where no buffer was used, 0.10 g. dried bacteria and 5 cc. of 0.3 % substrate. The p_H of each mixture was determined electrometrically at the beginning and end of each digestion and if it changed by more than 0.2 unit the experiment was repeated. Under these conditions the rate of hydrolysis is constant for the first 90 minutes, but during the next half-hour the velocity begins to fall off quite appreciably. It is usually preferable in such experiments to use initial velocities as criteria of activity rather than the changes brought about during an arbitrary time interval or at equilibrium. In the present work, however, the same optimum p_H is obtained whether initial velocities or equilibrium values are plotted.

Results with Cl. acetobutylicum. With sodium glycerophosphate, either α - or β -, the optimum is at approximately p_H 5.1, the β -ester being hydrolysed more rapidly than the α -form. The optimum for sodium hexosediphosphate is at p_H 6.0. Fig. 1 illustrates the result of a typical experiment in which glycerophosphate

Fig. 1.

Fig. 2.

Fig. 1. The p_H -activity curves of the phosphatases of *Cl. acetobutylicum* acting on glycerophosphate (broken line), and on hexosediphosphate.

Fig. 2. The p_H -activity curve of the pyrophosphatase of *Cl. acetobutylicum*.

phosphate and hexosediphosphate were hydrolysed. The organism also possesses a very active pyrophosphatase whose p_H -activity relationships are illustrated in Fig. 2. The optimum is at p_H 7.2.

Results with P. jensenii. With sodium glycerophosphate, either α - or β -, the optimum is at about p_H 7.0, the α -ester in this case being hydrolysed faster than the β -form. With sodium hexosediphosphate the optimum is at about p_H 6.0 and with sodium pyrophosphate at p_H 7.0. The pyrophosphatase is here

Fig. 3. The p_H -activity curves of the phosphatases of *Jensenii* acting on glycerophosphate (broken line), on hexosediphosphate and on pyrophosphate (with crosses).

very much weaker than the other two phosphatases whereas in the *Clostridium* it is much more active. Fig. 3 shows the results of a typical experiment in which the propionic acid organism was used.

(2) *Relation of reaction velocity to enzyme concentration.* In establishing a correlation between enzyme concentration and speed of reaction it is necessary to choose a concentration of substrate such that the largest amount of enzyme present in any digest is completely saturated. Furthermore, since progressive inactivation of the enzyme frequently occurs in all but the very early stages of the reaction, consideration must be limited to the linear portions of the progress curves such as those illustrated in Fig. 4. By making use of such constant initial

Fig. 4. Progress curves of the phosphatases of *Cl. acetobutylicum* acting on 0.15% glycerophosphate (broken line), on 0.15% hexosediphosphate, and on 0.5% pyrophosphate (with crosses).

velocities it was found that, with the bacterial phosphatases, strict proportionality between speed of reaction and amount of enzyme was readily demonstrated. For this purpose a series of digests was prepared, each tube containing 5 cc. of buffer at the optimum p_H , 5 cc. of 1% substrate, either glycerophosphate or hexosediphosphate, and increasing amounts of enzyme. In Fig. 5 is

Fig. 5. The relation between enzyme concentration and rate of hydrolysis.

shown the result with the glycerophosphatase of the *Clostridium*, the speed of the reaction being expressed as mg. of phosphorus liberated as inorganic phosphate in 1 hour. The linearity is similar to that observed with other substrates and with the other enzyme preparation.

(3) *Effect of free phosphate.* Three solutions of NaH_2PO_4 adjusted to p_H 5.1 were prepared, of such strength that when 1 cc. portions of the solutions were diluted to 10 cc. the final concentrations of inorganic phosphate were those

listed in Table I. The inhibitory effects of these concentrations on phosphatase activity were determined in digests containing 5 cc. buffer, 0.1 g. dried organisms (*Clostridium*), 4 cc. of 0.4 % sodium glycerophosphate and 1 cc. of the phosphate solution. Control digests without added phosphate were run concurrently. The inhibitory effects, summarised in Table I, are of the same order of magnitude as those reported for mammalian phosphatase [Kay, 1928].

Table I. *The inhibition of phosphatase by inorganic phosphate.*

Initial concentration of phosphate	Degree of inhibition %
<i>M</i> /500	8
<i>M</i> /200	20
<i>M</i> /100	80

(4) *Effect of toluene.* Toluene was added in increasing amounts, up to 1 cc., to a series of tubes containing 0.1 g. dried organisms, 0.15 % substrate and suitable buffer in a total volume of 10 cc. Both organisms were used and both glycerophosphate and hexosediphosphate were employed as substrates but in none of the four experiments did the added toluene influence the rate or degree of the digestion. This result is at variance with the finding of Myrbäck and Euler [1929] who reported an inhibitory effect of toluene on the hexosephosphatase of dried yeast.

(5) *Effect of magnesium ions.* For the purpose of investigating the possible activating effect of magnesium ions the increase in inorganic phosphate in 1 hour was measured in a series of tubes each containing 0.1 g. dried organisms, 5 cc. buffer of the proper p_H , 4 cc. of substrate solution and 1 cc. of $MgCl_2$ solution of such strength as to give the desired final concentration. The concentrations of the three substrates, sodium glycerophosphate, sodium hexosediphosphate and sodium pyrophosphate, initially present in the digests, were 0.50, 0.18 and 0.50 % respectively. Suitable controls were run in all experi-

Fig. 6. The activity at different magnesium concentrations of the phosphatases of *Cl. acetobutylicum* acting on glycerophosphate (broken line), on hexosediphosphate and on pyrophosphate (with crosses).

ments. No difference could be detected between the action of MgCl_2 and MgSO_4 . No significant change in p_{H} was observed in any digest during the period of the experiment. The range of magnesium concentrations investigated extended from 0.3 to 0.000003 M . Since such a wide range of concentrations is difficult to plot conveniently, the practice of Jenner and Kay [1931] has been adopted: the symbol q_{Mg} has been used to represent the negative logarithm of the molar concentration of magnesium. This symbol has a meaning similar to that of p_{H} but avoids the question as to whether the magnesium is present entirely in the ionic form.

In Fig. 6 the effects of different concentrations of magnesium on the phosphatase activity of dried *Clostridium* are illustrated. For the hydrolysis of glycerophosphate and hexosediphosphate the optimum concentration is q_{Mg} 2.5 and for that of pyrophosphate q_{Mg} 4.5. In each case it is observed that a sufficiently high concentration of magnesium retards the action of the enzyme.

With the propionic acid organism much greater activating effects of magnesium were observed. Typical results are illustrated in Fig. 7 from which it is seen that the optimum concentration is q_{Mg} 2.5 for the hydrolysis of all three substrates.

Fig. 7. The activity at different magnesium concentrations of the phosphatases of *P. jensenii* acting on glycerophosphate (broken line), on hexosediphosphate and on pyrophosphate (with crosses).

(6) *Effects of other ions.* In Table II are summarised the results of a study of the effects of several ions on the activity of the phosphatase systems of the two organisms. For purposes of comparison the value 100 is assigned to the activity in the absence of added ion and the relative activities as influenced by the various concentrations of ions are those listed in Table II. In the case of each substrate the same concentration was used throughout the series, namely 0.5 % sodium glycerophosphate, 0.18 % sodium hexosediphosphate and 0.5 % sodium pyrophosphate. The procedure adopted was the same as that described above for the study of the effect of magnesium, 1 hour's hydrolysis being chosen as a measure of activity except in the case of the hydrolysis of pyrophosphate by the propionic acid organism where 0.2 g. dried organisms was used and the

Table II. *Effect of various ions on phosphatase activity.*

Figures give the percentage hydrolysis under the influence of ions shown, as compared with the hydrolysis without added ions.

Ion Conc. of ion ...	<i>Clostridium acetobutylicum</i>										<i>Propionibacterium jensenii</i>									
	Glycerophosphate			Hexosediphosphate			Pyrophosphate				Glycerophosphate			Hexosediphosphate			Pyrophosphate			
	M/10	M/333	M/2000	M/10	M/333	M/2000	M/10	M/333	M/2000		M/10	M/333	M/2000	M/10	M/333	M/2000	M/10	M/333	M/2000	
Magnesium	88	175	150	51	148	125	82	150	200		500	1500	900	150	515	315	0	400	100	
Beryllium	0	50	92	0	49	87	0	89	100		0	23	40	0	10	50	—	—	—	
Calcium	77	89	95	98	99	100	50	100	99		30	55	90	25	60	90	20	70	94	
Zinc	80	86	96	60	74	94	0	100	140		30	35	69	22	48	68	0	100	160	
Strontium	46	56	100	34	81	94	0	90	103		20	30	90	18	29	80	—	—	—	
Cadmium	50	62	100	42	82	99	0	85	100		30	60	97	22	48	85	—	—	—	
Barium	40	90	95	60	79	89	0	66	95		98	99	96	90	98	101	—	—	—	
Mercuric	0	35	40	0	26	60	0	30	39		0	45	50	0	0	22	—	—	—	
Silver	0	50	80	0	10	50	1	59	95		0	0	35	0	0	50	—	—	—	
Potassium	99	98	100	102	100	100	97	98	100		94	96	98	105	100	100	—	—	—	
Sodium	100	100	101	100	99	99	95	97	100		99	99	100	98	99	100	99	98	100	
Lead	0	90	100	0	100	100	52	72	96		0	40	80	0	30	60	—	—	—	
Acetate	101	100	100	101	100	99	98	100	101		96	99	100	98	99	100	—	—	—	
Nitrate	100	102	100	102	100	100	85	90	100		100	102	100	92	100	100	—	—	—	
Sulphate	96	98	99	97	98	99	90	99	100		90	95	98	93	95	96	—	—	—	
Chloride	99	100	100	100	100	101	95	99	100		101	100	99	100	99	100	97	99	99	
	M/100	M/500	M/2000	M/100	M/500	M/2000	M/100	M/500	M/2000		M/100	M/500	M/2000	M/100	M/500	M/2000	M/100	M/500	M/2000	
Cyanide	95	95	100	96	98	100	95	100	98		100	100	100	100	100	100	97	98	99	
Fluoride	70	96	98	100	100	100	73	80	97		72	84	100	100	100	100	60	86	100	

digestion was allowed to proceed for 4 hours. This reaction is much slower than the hydrolysis of the other two substrates by this organism. The figures in Table II are comparable for each substrate and are reproducible to within about 5 % with a given enzyme preparation. Portions of the same enzyme preparation of each organism were used throughout.

An examination of Table II shows that magnesium was the only ion which accelerated the rate of hydrolysis of all substrates by the phosphatases of both organisms. Zinc, in low concentrations, caused definite activation of the pyrophosphatase of both organisms. Fluoride, in certain concentrations, retarded the hydrolysis of glycerophosphate and pyrophosphate but not that of hexose-diphosphate.

In conducting these experiments careful consideration was given to several important factors. The various solutions added to the digests were first adjusted to the p_H ultimately desired; the final p_H at the end of the experiment was determined in every case and, if a change greater than 0.3 unit occurred, the experiment was repeated. Secondly it was observed that in the presence of certain concentrations of some of the ions, particularly beryllium, barium, mercury, silver and lead, precipitation of the substrates occurred, a factor which may have led indirectly to retardation of the rate of hydrolysis. It is undoubtedly true also that the heavy metals have a direct inhibitory effect in certain concentrations. Finally it was necessary to consider the effect of each ion on the determination of inorganic phosphate from the points of view of the precipitation of the reagents and the development of the blue colour. Thus lead, silver and barium give such precipitates and were therefore removed with the proteins previous to the Briggs determination. Nitrate, cyanide and fluoride ions in 0.1 *M* concentration intensify the blue colour; a correction can be applied in the case of nitrates but not so accurately in the other cases. However, with cyanide and fluoride, no such disturbing influence was observed when the concentration was 0.01 *M* or lower.

DISCUSSION.

Points of interest arising from the present work are those concerned with (1) the question as to the specificity of magnesium activation and the possible rôle of magnesium as a coenzyme of phosphatase systems, (2) the question as to the specificity of the phosphatases themselves.

Of all the ions investigated, zinc is the only one other than magnesium which exhibited any activating influence in the concentrations employed¹. Zinc, it is true, appears to have a very limited, though quite definite, effect exerted only on the pyrophosphatase systems of both organisms; the degree of activation is of the same order of magnitude in the two cases and is confined to low concentrations of zinc. The limitation of the zinc effect to the pyrophosphatase in each case suggests that this enzyme differs in some manner from the hexosephosphatase and glycerophosphatase of the same organism. On the other hand the activation by zinc may be conditioned by the nature of the substrate rather than by that of the enzyme. The final interpretation of differences of this sort in relation to enzyme specificity must await further experimentation.

Magnesium, however, appears to stand apart from all other ions as an activator of all the phosphatase systems investigated in the present work. This result is in general accord with the findings of previous investigators who used

¹ Since these experiments were completed the arsenate ion has been found to cause a definite acceleration of the rate of hydrolysis of certain substrates by dried propionic acid organisms. An account of these observations will appear in a later paper.

mammalian phosphatases [Jenner and Kay, 1931]. The apparently specific response to magnesium of phosphatases in cells so different as those of mammalian tissues and bacteria, acting on various substrates, suggests that magnesium must play a fundamental rôle in the normal functional activity of these enzymes. It has been suggested that magnesium is a true coenzyme of the phosphatase system of mammalian tissues. In the case of the bacterial phosphatases studied in this work, this is rather unlikely: dried cells of *Cl. acetobutylicum* free from detectable magnesium were found to possess very appreciable phosphatase activity. For the purpose of testing this point the organisms were grown in a medium containing no magnesium other than that in the small amount of peptone present. 1 g. of the dried organisms was carefully ashed and the residue was examined for magnesium by a spot test, using *p*-nitrobenzoylazonaphthol [Feigl, 1931]. This test, sensitive to 0.0002 mg. of magnesium, gave negative results; the amount of magnesium present in 0.1 g. of the dried cells must indeed be negligible. In spite of this, when acting on glycerophosphate, 0.15 % in a total volume of 10 cc., the phosphatase present in 0.1 g. of the dried cells liberated from the ester 0.194 mg. of inorganic phosphorus in 2 hours. Obviously the enzyme did not require for its activity the presence of more than an infinitesimal amount of magnesium; it would therefore be incorrect to designate the magnesium ion as a coenzyme in the system under investigation.

Table III. *Comparative rates of hydrolysis of different substrates by bacterial phosphatases.*

Organism	Sodium glycerophosphate	Sodium hexosediphosphate	Sodium pyrophosphate
<i>Clostridium acetobutylicum</i>	1.0	2.0 $\pm$ 0.4	6.0 $\pm$ 0.6
<i>Propionibacterium jensenii</i>	4.5 $\pm$ 0.5	8.0 $\pm$ 0.2	0.4 $\pm$ 0.3

The experiments have also a bearing on the subject of the specificity of the phosphatases, though it is realised that a study of the enzymes of only two bacterial species does not justify any wide generalisation. Points of difference and of similarity in the respective systems of the two organisms are apparent. For example the propionic acid organism has a pyrophosphatase which is very much weaker than the other phosphatases, whereas with the other organism the reverse is true. This difference and some other relationships are illustrated in Table III. In this table are summarised data based upon experiments in all of which the concentrations of substrate employed were such as to make possible the complete saturation of the enzyme, the amount of the latter being constant throughout. It is apparent that the ratio of the rates of hydrolysis of the different substrates is not the same for the two enzyme preparations. There are other differences between the phosphatases of the two organisms. The p_H optima with sodium glycerophosphate as substrate are rather widely separated, p_H 7.0 for the enzyme of one organism and p_H 5.1 for that of the other. The propionic acid organism hydrolyses α -glycerophosphate more rapidly than the β -ester, whereas with the other organism the reverse is true. The phosphatases of the propionic acid organism are apparently much more extensively activated by magnesium than are those of the other organism, though this difference may be related to differences in the physical conditions of the crude enzyme-containing preparations which were used. In general however it can be said that the effects of the several ions on the activities of the enzymes are similar for the two organisms. The behaviour of the enzymes of the two species reveals other

similarities. The p_H optima for the hydrolysis of either hexosediphosphate or pyrophosphate are the same for the two organisms. Finally the peculiar effects of zinc and of fluoride ions are essentially the same for both bacteria.

From the point of view of the substrates themselves differences have been observed. Sodium fluoride in concentrations up to $0.01M$ had no retarding effect on the hydrolysis of hexosediphosphate, whereas in considerably lower concentrations it retarded the rate of hydrolysis of glycerophosphate and of pyrophosphate. The differences in p_H optima were found to be sufficiently great in certain instances to suggest some form of specificity; it is possible that the use of purified enzyme preparations would reduce the differences, but it is unlikely that such optima as p_H 5.1 and 7.0 for the hydrolysis of different substrates could be shifted to the same level by methods of enzyme purification. Finally it may be noted that the optimum concentration of magnesium, namely q_{Mg} 4.5, for the hydrolysis of pyrophosphate by the propionic acid organism differs rather sharply from the optimum value, q_{Mg} 2.5, which characterises all other combinations of enzyme and substrate studied in the present work. This latter value is also optimum for the activity of mammalian phosphatases [Jenner and Kay, 1931].

Since the results reported reveal both similarities and differences a final decision with respect to the possible specific behaviour of the hydrolytic systems investigated cannot, as yet, be reached.

SUMMARY.

1. The phosphatase systems of *Clostridium acetobutylicum* Weizmann and *Propionibacterium jensenii* van Niel have been investigated in a series of experiments in which sodium glycerophosphate, sodium hexosediphosphate and sodium pyrophosphate were employed as substrates.

2. Various properties and relationships of the enzymes have been established, including p_H optima, correlation between enzyme concentration and reaction velocity, the effects of free phosphate, of toluene and of magnesium and several other ions on the rate or extent of hydrolysis.

3. The significance of the observations has been discussed in relation to (a) the questions of the specificity of magnesium activation and of the coenzymic nature of magnesium ions and (b) the problem of the specificity of the enzymes.

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REFERENCES.

- Asakawa (1929). *J. Biochem. (Japan)*, **11**, 143.
Boivin and Mesrobian (1933). *Compt. Rend. Soc. Biol.* **112**, 611.
Edlbacher and Kutscher (1932). *Z. physiol. Chem.* **207**, 1.
Euler and Nordlund (1921). *Z. physiol. Chem.* **116**, 229.
Feigl (1931). *Qualitative Analyse mit Hilfe von Tüpfelreaktionen* (Leipzig).
Heard and Wynne (1933). *Biochem. J.* **27**, 1655.
Horiuchi (1931). *J. Biochem. (Japan)*, **14**, 163.

- Jacobsen (1931). *Biochem. Z.* **242**, 292.
Jenner and Kay (1931). *J. Biol. Chem.* **93**, 733.
Kay (1928). *Biochem. J.* **22**, 855.
—— (1932). *Physiol. Rev.* **12**, 384.
—— and Lee (1931). *J. Biol. Chem.* **91**, 135.
Kertesz (1930). *J. Amer. Chem. Soc.* **52**, 4117.
Myrbäck and Euler (1929). *Z. physiol. Chem.* **183**, 226.
Neuberg and Sabetay (1925). *Biochem. Z.* **161**, 240.
Roche (1931). *Biochem. J.* **25**, 1724.
van Niel (1928). The propionic acid bacteria (Haarlem).
Virtanen (1927). *Z. physiol. Chem.* **166**, 21.
—— and Tikka (1930). *Biochem. Z.* **228**, 407.