34. *The Nature of the Phosphorus Compounds Present in Cells of* Bact. lactis aerogenes.

By P. C. Caldwell.

The rate of hydrolysis by N-sulphuric acid at 100° of the phosphate ester linkages in cells of *Bact. lactis aerogenes* has been investigated. Three distinct stages in the reaction can be detected and it has proved possible to analyse the curves obtained into three separate first-order components. The first-order constants obtained are compared with those found for the breakdown of relevant model substances. The results suggest that the first two stages represent the hydrolysis of different linkages present in the nucleic acids and that the third represents the breakdown of phospholipoid. There is an indication that the nucleic acid in cells which have been subjected to phosphorus starvation may contain phosphate linkages not present in the nucleic acids derived from yeast and thymus.

Various results are available to give an overall picture of the distribution of phosphorus in the different forms in which it occurs in the bacterial cell (preceding paper; Taylor, *J. Biol. Chem.*, 1946, **165**, 271). About 80—90% of the phosphorus appears to be in the form of the nucleic acids and their simple components, while the remainder probably consists of phospholipoid. Furthermore, of the nucleic acid fraction, about 20% can be accounted for as deoxyribose nucleic acid and about 60% as ribose nucleic acid and ribose nucleotides.

To attempt any further subdivision of the cell phosphorus is difficult. Large quantities of culture must be grown to make identification of the phosphorus compounds by normal chemical methods practicable. It was shown in the previous paper, however, that a crude distinction could be detected in the cell phosphorus in so far as it was possible to distinguish two components which differed in the rate of hydrolysis of their phosphate ester linkages under the action of N-sulphuric acid at 100°. In the present paper the rate of hydrolysis of the different phosphate ester linkages in the cells has been examined in greater detail, and an attempt made to carry out a kinetic analysis. The rates observed with the cell components have been compared with those of deoxyribose and ribose nucleic acids and with those of simple ribose nucleotides.

The Rate of Hydrolysis of the Phosphate Ester Linkages in the Cells.—In these experiments a complete survey was made of the organic phosphates present in both the cells and the medium in which they were growing at the time of sampling. Only traces of organic phosphorus could be detected in the latter, and this appeared to consist chiefly of substances which were decomposed by N-sulphuric acid after 60 minutes at 100°. Of the phosphorus in the cells

themselves, about 10% was found to be in the form of adsorbed inorganic phosphate which could be removed by the action of acid at room temperature, whereas the remainder was in organic form.

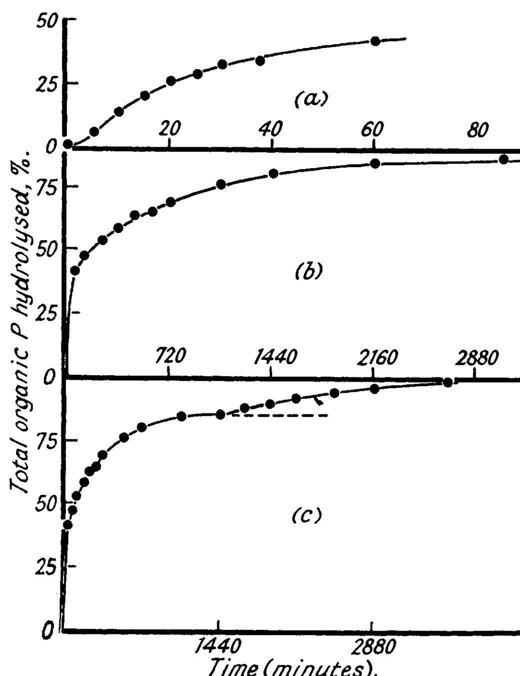
Fig. 1 shows the percentage of this organic phosphorus which had undergone decomposition after varying times in N-sulphuric acid at 100°, for cells which were sampled during the logarithmic-growth phase. Fig. 2 shows the same for cells which were sampled after a period of phosphorus starvation.

These two curves show that the organic phosphorus in the cells can be divided into three well-defined groups :

(1) a fraction which is completely decomposed after 60 minutes,
(2) a fraction which is decomposed after about 24—36 hours, and
(3) a fraction which is still not completely decomposed after 5 days.

FIG. 1.

Hydrolysis of organic phosphates present in actively growing cells (by N-*sulphuric acid at* 100°).



In the third of these groups the curves reveal a delay before decomposition sets in at all This effect has been studied closely and appears to be reproducible. The possible reasons for it will be discussed later.

The decomposition of phosphate ester in the presence of hydrogen ions usually shows first-order kinetics provided that the phosphoric acid formed is not sufficient materially to alter the pH. An attempt has been made, therefore, to analyse the three stages, in which the cell phosphorus decomposes, into three first-order reactions. Though this analysis is complicated by the fact that the third component shows the delayed hydrolysis, a visual inspection of the curves in Figures 1 and 2 will show that the second stage is almost completed before the third stage has begun. The end-point for the second stage can thus be obtained by inspection.

In a first-order reaction log (residual reactant), *i.e.*, log $(a - x)$, is a linear function of time. Fig. 3*a* shows the first-order plot for the third stage of the curve shown in Fig. 1. The end-point was known from the total phosphorus of the cell. Fig. 3*b* shows a similar plot for the second stage of the curve shown in Fig. 1, the end-point being obtained by inspection. This plot shows an abrupt deviation from linearity between 60 minutes and zero time, since during this period the first stage of the hydrolysis is also taking place. The second stage can be separated from the first by the following procedure. The linear portion of the curve (B to C),

which relates solely to the second stage, is extrapolated to A to give the complete first-order plot for the second stage.

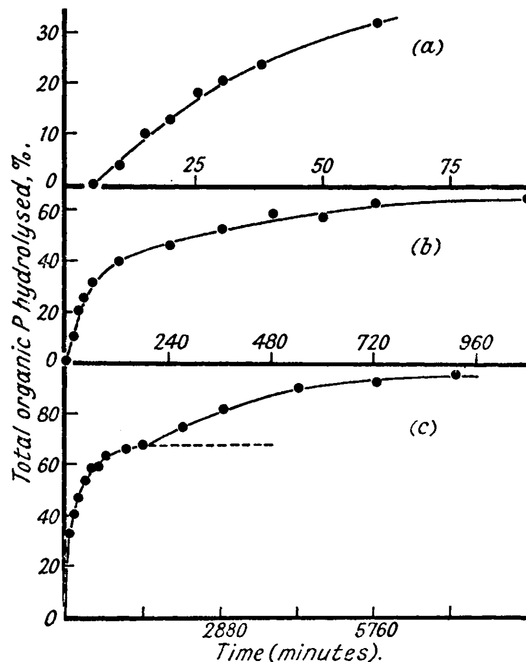
$(a_2 - x)$ for the second group in the range $t = 0$ to $t = 60$ minutes can be obtained from points on AB. If these values are subtracted from the total organic phosphorus, the values of $(a_1 - x)$, *i.e.*, organic phosphorus of the first group which remains undecomposed at these times, can be obtained. A plot of log $(a_1 - x)$ against t may now be made for the first stage and this is shown in Fig. 3*c*.

Figs. 4*a*, 4*b*, and 4*c* show the various components of the curve in Fig. 2 after a kinetic analysis carried out in a similar manner.

The velocity constants for the decomposition of the various components can be evaluated from the slope of the log $(a - x)/t$ curves, and the results are summarised in Table I.

Fig. 2.

Hydrolysis of organic phosphates present in cells which have been starved of phosphorus.



The numbers show that the rate of hydrolysis of the components in the first group remains unchanged on transition from the phase of active growth to conditions of phosphorus starvation. The velocity constants for the third stage also remain sensibly constant. In the second group, however, a remarkable change is observed, the velocity constant increasing by 100%, a change which is outside the limits of experimental error.

Before the velocity constants obtained for the breakdown of the phosphates present in the cells are compared with those obtained with model compounds, the approximate nature of such a comparison must be emphasised. Differentiation of two phosphorylated substances in a composite hydrolysis curve is only possible if their velocity constants are reasonably widely separated (numerical trials suggest by about 100%). It would be erroneous, therefore, to regard each stage in the hydrolysis curve found with the cells as arising necessarily from one single type of phosphate linkage. Within each of the three groups there are probably a number of different substances, with rates of hydrolysis close enough to give the impression of an approximately first-order reaction, the velocity constant for which represents some sort of average of those for the various components. (These considerations apply to the curves obtained with the nucleic acids, since these break down to give a variety of phosphorylated products.)

Comparison of the Rates of Hydrolysis of the Components of the Cell Phosphorus with those of the Nucleic Acids.—With a view to a possible identification of the components which contribute

TABLE I.

Velocity constants for the first-order hydrolysis of the phosphate ester linkages in cell suspensions in the nucleic acids and in various nucleotides.

	Cell suspensions		Nucleic acids	
	(1) cells actively growing.	(2) cells starved of phosphorus.	(1) yeast ribose nucleic acid.	(2) thymus deoxyribose nucleic acid.
The first stage :				
10^2k (min.$^{-1}$) …………	5·4	5·3	6·1	7·0
% of the total organic phosphorus …………	38	25; 32	52	30
The second stage :				
10^3k (min.$^{-1}$) …………	1·4	3·1; 2·9	0·81	1·7
% of the total organic phosphorus …………	47	42; 34	48	70
The third stage :				
10^4k (min.$^{-1}$) …………	4·0	3·3; 4·7		
% of the total organic phosphorus …………	15	33; 34		

Nucleotides, etc.

	(1) Guanosine-3 phosphate.	(2) Uridine-3 phosphate.	(3) Adenosine-5 phosphate.	(4) Lipoid extract of the cells.
k (min.$^{-1}$) ……………	$7·0 \times 10^{-2}$	$0·47 \times 10^{-3}$	$2·7 \times 10^{-3}$	$2·4 \times 10^{-4}$

FIG. 3.

First-order plots for the various stages of the hydrolysis of phosphates present in actively growing cells.

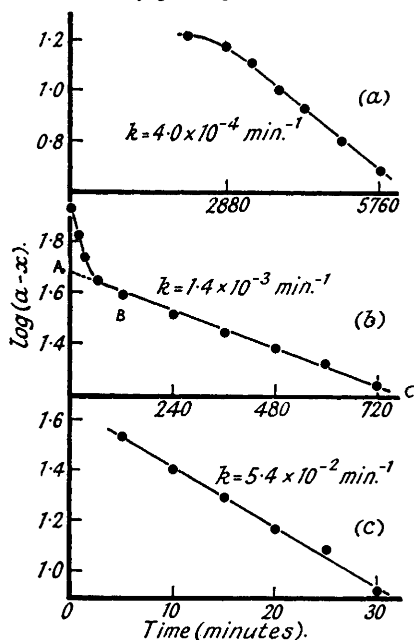
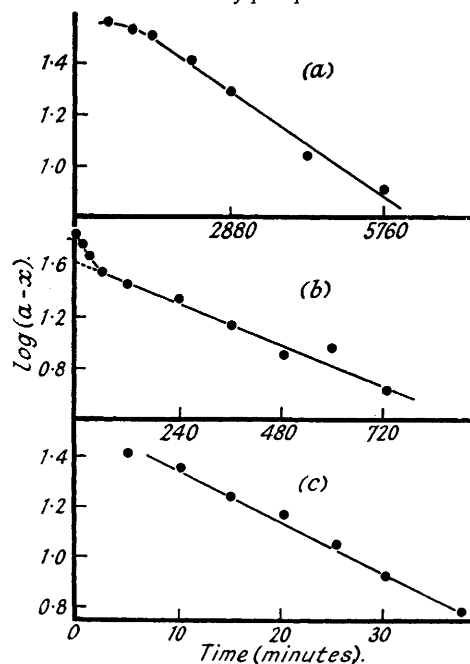


FIG. 4.

First-order plots for the various stages of the hydrolysis of phosphates present in cells which have been starved of phosphorus.



to the hydrolysis curves obtained with the cells, the hydrolysis of the phosphate ester linkages in ribose and deoxyribose nucleic acids have been studied. The results for ribose nucleic acid are shown in Fig. 5. This shows two distinct stages which are similar to the first two stages observed with the cells. A kinetic analysis, as described, into two first-order reactions has been carried out.

Fig. 6 shows the hydrolysis curve for deoxyribose nucleic acid, which also decomposes in two stages. This curve also has been analysed into two components. The similarity between these curves and those for the first two stages in the breakdown of the cell phosphates strongly suggests that these stages represent the breakdown of nucleic acid. This identification would not be inconsistent with the analytical figures given earlier, since the first two stages account for 66—85% of the cell phosphorus.

FIG. 5.

Hydrolysis of phosphate ester linkages in ribose nucleic acid from yeast.

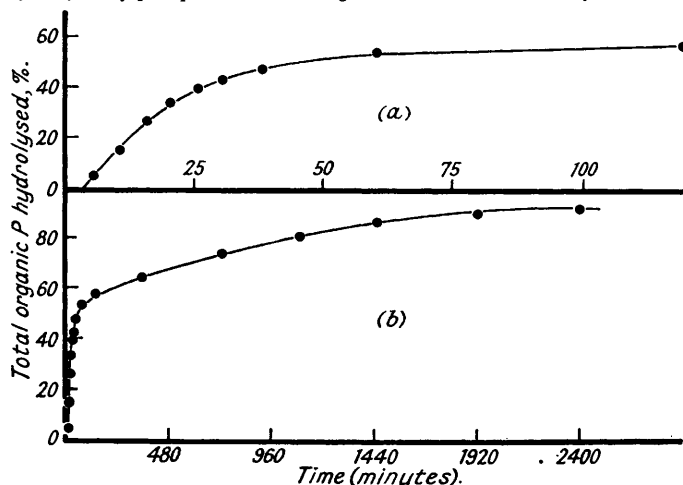
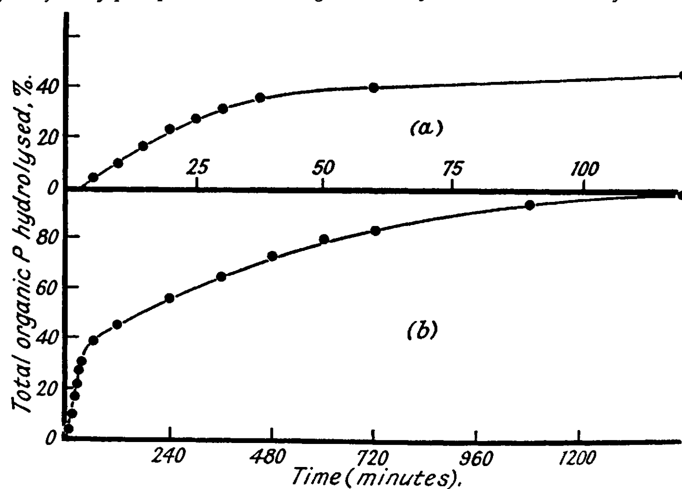


FIG. 6.

Hydrolysis of phosphate ester linkages in deoxyribose nucleic acid from thymus.



The velocity constants for the decomposition of the various components of the two nucleic acids are recorded in Table I. The values obtained for the first stage coincide within 10% and are in good agreement with those obtained for the first stage hydrolysis of the cell material. The values for the second stage differ by about 100%. The value for the second stage of the cells in logarithmic growth lies between those found for the two nucleic acids, and may arise from the combined hydrolysis of both types. On the other hand, the value for the second stage with cells which have been deprived of phosphorus is considerably higher than the values obtained with the two nucleic acids. This would suggest that in cells grown under these conditions the nucleic acid contains a phosphate ester linkage which is not found in the nucleic acids prepared from yeast and thymus.

The velocity constant for the third stage in the breakdown of the cell phosphates is considerably lower than that for any component of the nucleic acids, which suggests that this third group consists of something other than nucleic acid.

Comparison of the Rates of Hydrolysis of the Components of the Cell Phosphorus and of the Nucleic Acids with those of Certain Simple Nucleotides.—The hydrolysis of the following simple nucleotides has been studied : guanosine-3 phosphate, uridine-3 phosphate, and adenosine-5 phosphate. In N-sulphuric acid at 100° these all hydrolyse with first-order kinetics. The velocity constants obtained are summarised in Table I. This shows that the values for guanosine-3 phosphate (which is typical of ribose-3 phosphate and of purine nucleotides containing a ribose-3 phosphate linkage) and those for the first stage in the hydrolysis of both the cell phosphorus and the nucleic acids all fall within about 20% of one another. Thus the first stage appears to be due to the breakdown of purine nucleotides containing a 3-phosphate linkage. (This assumes that the kinetics of the hydrolysis of the deoxyribose nucleotides is similar to that of the ribose nucleotides.) The second stage in the breakdown of the cell phosphates and the nucleic acids may contain contributions from both pyrimidine nucleotide-3 phosphates and nucleotides (either purine or pyrimidine) containing other phosphate linkages. It is not possible, however, to make a satisfactory identification of the components which contribute to this stage of the hydrolysis in either the cells or the nucleic acid since certain important model compounds (in particular, ribonucleotides containing a 2-phosphate linkage) are not available.

The value obtained with uridine-3 phosphate indicates that substances of this type may contribute to the second stage of the hydrolysis of yeast ribose nucleic acid. That obtained with adenosine-5 phosphate suggests that the nucleic acid present in cells which have been subjected to phosphorus starvation may possibly contain a 5-phosphate linkage.

The Nature of the Phosphorus Compounds which Contribute to the Third Stage of the Hydrolysis of the Cell Phosphates.—The proportion of the cell phosphorus present as phospholipoid was found to be about 8—9% of the total. Since the first two stages in the hydrolysis of the bacterial phosphorus, which account for between 70 and 85% of the total, can be attibuted to the breakdown of nucleic acid, it is likely that the third stage arises from the decomposition of phospholipoids. To test the correctness of this point of view the rate of hydrolysis of the organic phosphates from a chloroform extract of the cells was investigated. The reaction is of the first order, and the velocity constant is given in Table I. The value is somewhat lower than that found with the whole cells, but lies within the same range, and the assumption that the third stage arises from the breakdown of the phospholipoids seems probably correct.

No definite explanation can at present be offered for the fact that the third stage shows a delayed hydrolysis. None of the model compounds tried (including the lipoid extract) shows an induction period of this type. It may perhaps be assumed that the phospholipoid is dissolved in small globules of fat formed during the disintegration of the cells and that these must be destroyed by the acid before it can have any action on the phospholipoids themselves.

EXPERIMENTAL.

Experiments on the Decomposition of the Cell Phosphates.—The cells were grown in glucose–veronal media having the general composition described in the preceding paper. That used for the cells which were to be removed in the logarithmic phase contained 15 mg./l. of phosphate and that in which the cells were to be subjected to conditions of phosphorus starvation was buffered at an initial pH of 7·8 and contained 10 mg./l. of phosphate.

When the culture had reached the required phase of the growth cycle, 500 ml. of it were centrifuged. After removal of the supernatant liquid, which was kept for further examination, the cells were suspended in distilled water and diluted to a volume of 500 ml.

The rate of liberation of phosphoric acid from the phosphate esters in the cells was measured in the following way : 10-ml. samples of the cell suspension were pipetted into glass tubes and treated with 1 ml. of 11N-sulphuric acid. The tubes were then sealed and heated for varying times at 100°. The cells disintegrated after about 5 minutes and the various phosphate esters were dispersed into the solution.

After the heating, the tubes were opened and the inorganic phosphate formed was measured by the Berenblum–Chain method. In samples which had been heated for less than 5—10 minutes, sufficient cell debris remained to interfere with the separation of the *iso*butanol layer from the aqueous layer during the estimation, and this was therefore removed by centrifugation beforehand.

Determination of the total phosphorus in the suspensions was carried out after oxidation of the cell material with sulphuric acid.

The organic phosphates present in the supernatant liquids were investigated by the methods described above for the cell suspension.

Experiments on the Rates of Hydrolysis of the Phosphate Esters in the Nucleic Acids and their Derivatives.—The two nucleic acid samples and the guanosine-3 phosphate were purchased. The deöxyribose nucleic acid and the guanosine-3 phosphate were not subjected to any further purification. The ribose nucleic acid was purified by precipitation in mildly acid solution, after which it was centrifuged and washed with distilled water. It was slowly treated with sufficient alkali to bring the pH just to 7·0 and then reprecipitated as the sodium salt by the addition of alcohol. The samples of adenosine-5 phosphate and cytosine-3 phosphate had been prepared by synthetic methods.

Solutions which contained 40 mg./l. of each of these compounds were made in distilled water. The decomposition of the ester linkages by N-sulphuric acid at 100° was investigated by the method used for the examination of the cell suspensions.

Experiments on the Rate of Hydrolysis of the Phosphate Esters in a Lipoid Extract of the Cells.—A culture was grown in the normal glucose medium, and a quantity of this was centrifuged. The cells were subsequently suspended in isotonic saline and re-centrifuged. They were then dispersed in acetone and set aside for 24 hours. Extraction of the lipoid material was completed by addition to the suspension in acetone of an equal volume of chloroform and the heating of the mixture. The acetone and chloroform were then evaporated off, and the extracted fatty material was removed from the cell debris by partition between chloroform and water. The chloroform extract was emulsified in distilled water, and the chloroform itself removed by aeration to leave an aqueous emulsion of the lipoids.

10-ml. portions of this emulsion were treated with 1 ml. of 11N-sulphuric acid and heated for varying periods in sealed tubes. After the heating phosphorus estimations were carried out. A determination of the total phosphorus content of the emulsion was also made.

The author expresses thanks to Professor A. R. Todd, F.R.S., and to Dr. E. Bergel for the gift of specimens of synthetic nucleotides.

PHYSICAL CHEMISTRY LABORATORY, OXFORD UNIVERSITY. [*Received, September 19th*, 1950.]