XLIX. THE INFLUENCE OF ARSENATE AND ARSENITE ON THE ENZYMIC BREAKDOWN OF PHOSPHORIC ACID ESTERS.

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THE experiments of Harden and Young [1906; 1911] showing that arsenate and arsenite ions accelerate the rate of liberation of inorganic phosphate from hexosediphosphate by the enzymes of yeast juice have led to much speculation as to the mechanism involved. Recent investigations of the effects of arsenate on the activity of the phosphatases and the glycolytic enzyme systems present in different kinds of biological material have yielded results which have been variously interpreted. All but the most recent of this work has been summarised by Harden [1932].

The view, originally expressed by Harden and Young, that the stimulating effect is exerted on the hexosephosphatase of yeast juice is open to criticism as the result of investigations in several laboratories. The experiments of Meyerhof [1927] with yeast and muscle, of Macfarlane [1930] and of Harden [1933] with yeast, and of Braunstein [1931] with rabbit erythrocytes support the conception that the effect is exercised not on the phosphatase but on the glycolytic system. This hypothesis is not considered by Morgulis and Pinto [1932] to be generally applicable in view of their results with rabbit and dog blood. Recently Braunstein and Lewitow [1932] have observed progressive diminution of the inorganic arsenate in mixtures of yeast, sugar, arsenate, toluene and water and they suggest that the accelerating effect of arsenate on fermentation and glycolysis depends on the formation of a labile hexosearsenate.

The present paper represents a study of the effects of arsenate and arsenite ions on the enzymic breakdown of phosphoric acid esters. The work was undertaken with the purpose of determining whether stimulation of phosphatase activity by these ions can be regarded as a general phenomenon or whether their effects on the rate of breakdown of phosphoric acid esters concern rather the mechanisms by which the organic portions of the esters are degraded by the accompanying glycolytic or similar enzymes. The materials chosen as the source of enzyme included four bacterial species, *Clostridium acetobutylicum* Weizmann, *Propionibacterium jensenii* van Niel, *Bacillus lactis aerogenes* and *Bacillus subtilis*, in addition to takadiastase, dried yeast, pig kidney and ox erythrocytes. Both sodium hexosediphosphate and sodium glycerophosphate were employed as substrates since the restricted use of the former in the investigation of mixtures containing both phosphatase and glycolytic systems might conceivably lead to erroneous conclusions. Furthermore, since the early work of Buchner [1903] and of Harden and Young [1911] showed that arsenite also accelerates the rate of fermentation by yeast juice, examination of the influence of this ion has been included in the present study. The method described by Pett [1933] for the

determination of inorganic phosphate in the presence of arsenate permits greater accuracy in the investigation of the effects of this ion in a wide range of concentrations than was hitherto possible.

EXPERIMENTAL.

Enzyme preparations.

Bacteria. *Cl. acetobutylicum* and *P. jensenii*, in the dried form, were prepared in the manner previously described [Pett and Wynne, 1933, 1]. *B. lactis aerogenes* was grown for 4 days in a sterilised medium containing 3 % glucose and 0.6 % peptone in diluted yeast extract. The organisms were centrifuged, washed once, dried and ground. *B. subtilis*, grown for 6 days in a similar medium, was treated in the same manner. The bacteria were used in the proportion of 0.1 g. dried organisms per 10 ml. enzyme digest.

Yeast. *S. cerevisiae*, isolated from baker's yeast, was grown in a medium containing 5 % glucose, 1 % peptone and suitable inorganic salts dissolved in diluted yeast extract. After 5 days the organisms were centrifuged, washed, dried and ground as usual; 0.05 g. dried cells was used in 10 ml. of digestion mixture. An extract of dried yeast was prepared by shaking 5 g. of the powder with 100 ml. of chloroform-saturated water for 24 hours. The mixture was then centrifuged and filtered; 1 ml. of the clear filtrate was used in 10 ml. digest.

Takaphosphatase. 20 g. of commercial takadiastase were suspended in 50 ml. of water, chloroform was added and the mixture was shaken for 15 hours. A small insoluble residue was removed; the solution contained a large amount of free phosphate which was eliminated by adding the equivalent amount of $MgCl_2$ and dilute NH_4OH until precipitation was just complete. Of the filtrate from this precipitate 1 ml. was used in 10 ml. of enzyme digest.

*Mammalian kidney*¹. Fresh pig kidney was ground with twice its weight of water and allowed to stand for several hours. The mixture was filtered, and the filtrate was adjusted to p_H 6.0; after standing for 15 hours the solution was again filtered. The filtrate was adjusted to neutrality and stored in a refrigerator; 0.5 ml. of this solution was used in 10 ml. of digestion mixture.

Ox erythrocytes. The oxalated blood was treated in the manner described by Roche [1931]. Two ml. of the haemolysed solution were added to 0.5 ml. of buffer, 0.5 ml. of solution of arsenate or arsenite and 2 ml. of 2.5 % sodium glycerophosphate or of 1.25 % sodium hexosediphosphate.

Substrates.

The sodium glycerophosphate employed was obtained from The Eastman Kodak Company and consisted very largely of the β -ester. Sodium hexosediphosphate was prepared from the commercial calcium salt; the final preparation contained a small amount of monophosphate and a trace of inorganic phosphate.

General conditions.

The p_H optima of the phosphatases investigated are shown in Table I. The optimum for the yeast enzyme acting on hexosephosphate was reported by Kertesz [1930], for takaphosphatase on glycerophosphate by Kay and Lee [1931] and for kidney-phosphatase by Kay [1926]. The optima for the enzymes of the first two species of bacteria listed in the table have been reported by the present

¹ The authors are grateful to Mr G. A. McVicar for a supply of kidney-phosphatase prepared in this manner.

authors [1933, 1]. All the other recorded optima were determined as a part of the present investigation.

Table I. Showing the p_H optima of various phosphatases.

Enzyme source	Substrate	
	Hexosephosphate	Glycerophosphate
<i>Cl. acetobutylicum</i>	6.0	5.1
<i>P. jensenii</i>	6.0	7.0
<i>B. lactis aerogenes</i>	5.8	5.5
<i>B. subtilis</i>	6.8	10.8
<i>Sacc. cerevisiae</i>	6.3	4.0
Takadiastase	4.2	4.0
Mammalian kidney	8.9	8.9
Ox blood cells	6.5	6.1

Potassium hydrogen phthalate buffers were used in the acid range and glycine buffers in the alkaline range; at p_H 7.0 no buffer was used. All solutions were adjusted to the desired p_H before mixing and the p_H of each mixture was determined after hydrolysis. No change greater than 0.3 p_H was observed.

Solutions of Na_3AsO_4 and of Na_2HAsO_3 having the following concentrations were prepared: M , $M/4$, $M/10$, $M/25$, $M/50$, $M/75$, $M/100$ and $M/200$. Of these solutions 1 ml. portions were added to 9 ml. of digestion mixture.

The total volume in each case was 10 ml., made up of 4 ml. of substrate (2.5 % glycerophosphate or 1.2 % hexosediphosphate), 4 ml. of buffer, 1 ml. of enzyme if liquid or 1 ml. of water together with the previously indicated weight of dried organisms, and 1 ml. of arsenate or arsenite solution. When other solutions, such as NaF, were added simultaneously simple adjustments of the volume of buffer were made, the concentration being kept constant. The mixtures were rocked in L-shaped tubes in a water-bath at 37.5°. At zero time and after 1 hour 2 ml. samples were added to 10 ml. portions of 5 % trichloroacetic acid solution. The precipitates were centrifuged and 10 ml. portions of the clear liquids were placed in 25 ml. volumetric flasks. If arsenate were present reduction was then effected in the manner described by Pett [1933]. Inorganic phosphate was determined by Fiske and Subbarow's method, using 3*N* acid; in several experiments duplicate results were obtained with Briggs's modification of the Bell and Doisy method.

Control tubes containing (a) enzyme, added ions and buffer, (b) substrate, added ions and buffer were incubated with the experimental tubes. The amount of inorganic P in the latter at zero time was subtracted from the 1-hour values, and from the figures so obtained was subtracted the sum of the increases in inorganic P in the two control tubes. Other tubes containing enzyme, buffer and substrate but no arsenic were included in all experiments. To the net amount of inorganic P (after allowing for the P released in suitable controls) liberated in such a digest containing a given enzyme preparation was assigned a value of 100. All the other results obtained with the same enzyme were compared with this standard value. The results are expressed in Table II.

Effect of fluoride.

In every instance of acceleration by arsenate or arsenite the acceleration could be lessened or removed by NaF in suitable concentration. This observation is complicated by the fact that fluoride retards the phosphatase activity of certain cells, such as yeast [Lipmann, 1928] and muscle [Lipmann, 1929]. However, fluoride up to $M/100$ concentration does not affect the hexosephosphatase

Table II. Effects of arsenate and arsenite on the activity of various phosphatase preparations on glycerophosphate and hexosephosphate.

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

[illegible]

activity of *P. jensenii* [Pett and Wynne, 1933, 1]. This system is therefore well adapted for the investigation of the relation of fluoride to arsenate acceleration. Table III shows the results of an experiment of this kind.

Table III. *Diminution of the accelerating effect of arsenate by fluoride.*

Arsenate	Fluoride	mg. P released in 10 ml.
none	none	0.542
<i>M</i> /100	none	1.381
none	<i>M</i> /40	0.530
<i>M</i> /100	<i>M</i> /1000	1.368
<i>M</i> /100	<i>M</i> /400	1.287
<i>M</i> /100	<i>M</i> /200	1.207
<i>M</i> /100	<i>M</i> /100	1.123
<i>M</i> /100	<i>M</i> /40	0.873

The series can be carried no farther than *M*/40 NaF because higher concentrations affect the phosphate determination. The process used in reducing the arsenate has no effect on the recovery of phosphate in the presence of fluoride. It is evident that fluoride definitely reduces the arsenate acceleration.

A different type of experiment is of interest in this connection. The phosphorus reagents were added to a flask containing 2 ml. *M*/500 arsenate and to another containing 2 ml. *M*/500 arsenate and 2 ml. *M*/100 fluoride. After standing for 1 hour at 50° the flask containing fluoride developed only about 75 % of the intensity of colour in the other flask. Na₃AsO₄ does not yield a precipitate with NaF, even in a solution saturated with respect to both, so that the method by which arsenate is removed from action is not one of precipitation.

It was considered possible that the Na ions in the NaF solution appreciably depressed the dissociation of the Na₃AsO₄, but experiments with NaCl and Na₂SO₄ indicated that up to *M*/40 concentration these salts could not depress the arsenate acceleration to an extent equivalent to more than one-quarter of the fluoride effect.

While these facts do not fully explain the fluoride action they suggest that the fluoride ion forms a "complex ion" with the arsenate ion. Arsenate is thus converted into an ineffective form.

DISCUSSION.

The results recorded in Table II lead to several observations. (a) The liberation of inorganic phosphate by the various enzyme preparations was not uniformly affected by arsenate and arsenite ions. The release of phosphate was accelerated in some instances, unaffected in others and definitely retarded in the remainder. If it be assumed that the phosphatases of the various preparations are identical in their mode of action on both substrates, an assumption which is not yet free from difficulties, the enzymes might be expected to respond similarly to the influence of arsenate and arsenite. Since they do not, it can be concluded that the influence of these ions is exercised not on the phosphatase but on some other mechanism which by its operation liberates inorganic phosphate. (b) In cases in which arsenate accelerated the reaction, arsenite had a similar though usually less marked effect; the optimum concentrations of the two ions were not always identical. (c) No stimulation occurred with an aqueous extract of dried yeast though the activity of the dried yeast preparation itself was stimulated by both arsenite and arsenate. (d) Acceleration with glycerophosphate as substrate was observed only with the *P. jensenii* preparation.

It is probable that all the enzyme preparations possessed in addition to phosphatase a glycolytic mechanism capable of breaking down, at least partially, the hexose portion of hexosediphosphate. However, in only four of the nine cases in which the influence of arsenate on the degradation of this ester was studied was there any evidence of acceleration. It is apparent therefore that, in addition to having no consistent effect on the hydrolytic activity of the various phosphatases, the arsenate ion has no universally demonstrable accelerating influence on the activity of the glycolytic enzyme systems. This assumes that, under the conditions of the experiments, accelerated breakdown of the organic component of the ester would lead incidentally to accelerated accumulation of free phosphate.

The increase in inorganic phosphate as the result of the action of yeast, kidney, takadiastase, erythrocyte and *Cl. acetobutylicum* preparations on glycerophosphate can be regarded as proof of the presence of a true phosphatase because it is probable that none of these preparations is able to attack the glycerol portion of the ester. But no stimulation by arsenate or arsenite occurred with any of these glycerophosphatases. On the other hand all the other bacteria used can metabolise glycerol; it has been shown, furthermore, that *P. jensenii* [Pett and Wynne, 1933, 2] and *B. lactis aerogenes* (unpublished work of the authors) can, after drying, partially break down the glycerol residue of the ester. However, in the case of only one organism, *P. jensenii*, did arsenate or arsenite exert any accelerating influence on the rate of glycerophosphate breakdown.

A satisfactory interpretation of these results is not yet possible. Although it can be concluded that arsenates and arsenites do not accelerate the activity of phosphatases in general, the lack of uniformity of the effects of these ions on the activity of the various preparations, as measured by phosphate liberation, is somewhat confusing. The evidence supports the hypothesis that any observed accelerating effect is due to a stimulation of the enzyme system responsible for the degradation of the organic part of the ester. Furthermore the specificity observed suggests that the influence is exercised at some particular stage of this breakdown, common to some but not all of the preparations used. Further speculation must be supported by more complete experimental evidence.

In this connection it is of interest to observe that, although the aqueous extract of dried yeast possessed an active phosphatase which was not stimulated by arsenate or arsenite, the dried yeast itself responded to the influence of both ions. When tested in a 1 % solution of sodium hexosediphosphate in micro-fermentation tubes the yeast extract failed to cause the liberation of gas. It is evident that the complete zymase system was not present in the extract: possibly this deficiency was reflected in the failure of arsenate and arsenite to cause stimulation.

The experiments using cytolysed ox erythrocytes are of interest in view of the recent findings of Morgulis and Pinto [1932] and of Braunstein [1931]. The latter was unable to demonstrate arsenate stimulation of the phosphatase activity of rabbit serum and erythrocytes on hexosediphosphate, but in experiments in which the esters of the cells constituted the substrates for the accompanying enzymes he obtained results which were interpreted as indicating activation of the glycolytic mechanism by arsenate. On the other hand Morgulis and Pinto [1932] do not regard such stimulation of glycolytic systems as a general phenomenon because concentrations of arsenate which definitely accelerated the rate of glycolysis in mixtures containing rabbit blood preparations caused retardation of glycolysis using dog blood; in all cases the liberation of inorganic phosphates was accelerated. They therefore concluded that the increased rate of phosphate liberation in blood-glycolysing systems under the

influence of arsenate is due to a stimulation of the blood-phosphatases. In the case of ox erythrocytes, in the present work, arsenate acceleration of the rate of liberation of inorganic phosphate was observed with hexosediphosphate as substrate but not with glycerophosphate. If the stimulation were exercised on the phosphatase mechanism one would have expected acceleration of the hydrolysis of both substrates to occur. The evidence strongly suggests that in the case of ox erythrocytes the arsenate effect is exercised on the glycolytic system rather than on the phosphatase.

SUMMARY.

The rate of liberation of inorganic phosphate from glycerophosphate and hexosediphosphate through the action of several enzyme preparations was studied in relation to the influence of varying concentrations of arsenate and arsenite ions. Preparations of the following were employed: *Cl. acetobutylicum*, *P. jensenii*, *B. subtilis*, *B. lactis aerogenes*, yeast, takadiastase, pig kidney and ox erythrocytes.

No evidence was obtained to show that the activity of phosphatase systems in general is accelerated by arsenate or arsenite. The observations support the hypothesis that the accelerated enzymic liberation of phosphate under the influence of these ions is due to an effect exercised at some stage of the breakdown of the organic portion of the phosphoric acid ester.

The diminution of the stimulating effect of arsenate by fluoride was investigated and the suggestion is advanced that this may be due to the formation of a complex ion, thus rendering a portion of the arsenate ineffective.

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