CXIX. THE ACTION OF ARSENATE ON HEXOSEPHOSPHATASE.

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(*Received July 4th, 1930.*)

According to the view of Harden and Young [1910] the rate of fermentation of sugar by yeast preparations is dependent, after initial esterification of the free phosphate, upon the rate at which phosphate is liberated by decomposition of the hexosephosphates formed; this takes place by means of an enzyme, hexosephosphatase, yielding a fermentable sugar and phosphate. They showed [1911] that, where hexosediphosphate is the only source of carbohydrate, the rate both of fermentation and of liberation of phosphate is greatly increased in the presence of arsenate; the accelerating effect of arsenate on the fermentation of sugar solutions by these preparations was therefore ascribed to a direct influence on the hexosephosphatase.

Neuberg and Leibowitz [1927] showed that the rate of hydrolysis of hexosephosphate by yeast in the presence of chloroform and toluene was increased five times by the addition of arsenate; they claimed that no fermentation of sugar took place, but gave no proof that in these conditions hexosephosphate was not fermented. Meyerhof [1927] has put forward the theory that hexosephosphate may also be directly fermented without previous hydrolysis, which might involve a different explanation of the action of arsenate.

Raymond [1928] showed that a decrease in the rate of hydrolysis by zymin occurs after washing, more marked in presence of arsenate; he suggested that there was a residual hydrolysis by the washed preparation, increased by the addition of coenzyme, or alternatively that the coenzyme could not be wholly removed by washing and was necessary for any reaction to take place. His experiments are open to criticism on several grounds, notably that the only reliable index of the absence of coenzyme is failure to ferment hexosephosphate, not merely sugar as in his experiments; moreover, he did not show that the washed zymin would ferment on addition of coenzyme. It has been found in this laboratory that it is often difficult, especially with top yeasts, to obtain a potentially active zymin free from coenzyme. It was accordingly decided to investigate the matter further.

The hexosephosphatase of washed zymin.

Dried baker's (Dutch) yeast was washed four times with eight parts of water, and dried with acetone and ether (DY_1). The washed material was tested for its fermenting capacity in a microfermentation apparatus at 30°.

Flask (*a*) 0·2 g. DY_1 + 0·25 cc. 40 % fructose + 0·5 cc. *M*/10 K hexosediphosphate + 0·05 cc. 2 *M* K_2HPO_4 + 1·5 cc. H_2O; (*b*) similar to above, substituting 1 cc. coenzyme solution for 1 cc. H_2O.

The coenzyme used was a 10 % boiled extract of yeast freed from phosphate by precipitation with lead acetate at p_H 6·0. No fermentation was observed in flask (*a*), while in (*b*) 4·65 cc. CO_2 were evolved in 2 hours. This method of testing for coenzyme was used throughout.

The hydrolysing power of the preparation (DY_1) was tested on various substrates and under different conditions. Series of flasks containing a weighed amount of DY_1 and the requisite solutions were placed in a thermostat at 30°. From each series a flask was removed at intervals, the action stopped by the addition of trichloroacetic acid and the free phosphate estimated by the Briggs method. Table I shows typical results.

Table I. *Hydrolysis by washed dried yeast.*

0·2 g. DY_1 in 2·0 cc. total volume, incubated at 30°.

	Substrate	Conc. Na_2HAsO_4 molar	Time (hours)	mg. P liberated per cc.
(*a*)	2 cc. H_2O	0	2	0·07
(*b*)	1 cc. 0·1 *M* K hexosediphosphate + 1 cc. H_2O	0	2	1·39
(*c*)	,, ,, ,,	0·001	2	1·25
(*d*)	,, ,, ,,	0	2	1·16
(*e*)	,, ,, ,,	0·005	2	0·91
(*f*)	1 cc. 0·1 *M* K hexosediphosphate + 0·02 cc. toluene	0	2	1·28
(*g*)	1 cc. K hexosemonophosphate (Robison) + 1 cc. H_2O	0	1½	1·32
(*h*)	,, ,, ,,	0·001	1½	1·07
(*i*)	,, ,, ,,	0·005	1½	1·11

Similar results to the above were also obtained with a washed preparation of bottom yeast (Scotch). It seems certain, therefore, that hydrolysis of hexosemono- and di-phosphate can take place in the absence of coenzyme.

The interesting fact emerges from these experiments that under these conditions the hydrolysis is not accelerated by the addition of arsenate and is, indeed, slightly inhibited by its presence in concentrations which accelerate the fermentation of unwashed zymin. This indicates that the part played by the coenzyme, or by some other factor removed by washing, is intimately connected with the accelerating action of arsenate and not with the normal hydrolysis.

Starting from the premiss that the decomposition of hexosephosphate takes place only by a hydrolytic enzyme, it was obviously desirable to test the effect of coenzyme on preparations containing hexosephosphatase but no zymase, in order that the hydrolysis should not be masked by re-esterification. 100 g. of dried baker's yeast were incubated at 26° with 250 cc. water and 5 cc. toluene for 18 hours. After filtration on a Büchner funnel, the clear brown filtrate was evaporated to dryness in a vacuum desiccator; 120 cc. liquid yielded 14 g. of brown powder, readily soluble in water (DH_1). The dry preparation kept without change for several weeks; fresh solutions were made for each experiment.

A measured quantity of the enzyme was placed in each of a series of flasks together with the desired substrates and made up with water to a definite volume. The p_H was adjusted to 6·8 after saturation with CO_2. 1 cc. samples from each flask were transferred by an Ostwald pipette into a series of test-tubes placed in a thermostat at 30°. Tubes from each series were removed at intervals, the enzyme action stopped by the addition of trichloroacetic acid, and the contents transferred quantitatively to a 20 cc. flask and filtered. The inorganic P was then measured by the Briggs method. Identical samples were placed in microfermentation flasks and the CO_2 evolved was measured. A typical case is Exp. 29.

Exp. 29. The following solutions were used.
Enzyme. 10 % solution of dried autolysed yeast. DH_1.
Yeast extract. 10 % boiled extract of zymin.
Arsenate. 0·1 *M* Na_2HAsO_4.
Hexosephosphate. 0·1 *M* potassium hexosediphosphate.

	Substrate	mg. P liberated per cc.	cc. CO_2 per cc.
(*a*)	1 cc. enzyme + 2 cc. hex. phosphate + 3 cc. H_2O	0·17	0
(*b*)	" " " + 1 cc. yeast ext.	0·16	0·06
(*c*)	" " " + 0·3 cc. arsenate	0·10	0·01
(*d*)	" " " + 1 cc. yeast ext. + 0·3 cc. arsenate	1·19	0·52

This experiment was repeated many times with different preparations of the enzyme, giving substantially the same results, although the extent of the acceleration varied somewhat. The preparation therefore contained a hexosephosphatase but no coenzyme; with added coenzyme the hydrolysis was unaffected and a slight fermentation took place, though frequently there was a lag of 2–3 hours in CO_2 production. This lag proved misleading, as the earlier experiments were made over a period of only 1–2 hours and it appeared as if there were no fermentation, but it can only be concluded that in all cases there was a small amount of "zymase" present. The addition of arsenate in a concentration of 0·005 *M* caused a slight inhibition of hydrolysis, but by addition of both arsenate and coenzyme a striking acceleration of the rate of hydrolysis was produced, up to five-fold, together with an increased evolution of CO_2. The excess volume of CO_2 was equivalent to the excess of P liberated in the presence of arsenate over that in the normal hydrolysis, as shown in the following experiment:

Exp. 35. (*a*) 40 cc. 0·1 *M* K hexosediphosphate + 10 cc. enzyme + 10 cc. yeast ext. + 3 cc. H_2O.
(*b*) 40 cc. 0·1 *M* K hexosediphosphate + 10 cc. enzyme + 10 cc. yeast ext. + 3 cc. arsenate.
60 cc. of each were incubated at 30° for 4 hours.

	cc. CO_2	mg. P liberated
(*a*)	7·29	44·2
(*b*)	64·35	121·6
Excess (*b* − *a*)	57·06	77·4
Equiv. to 79·0 mg. P		

Purification of enzyme.

Various attempts were made to fractionate the preparation DH_1, and obtain a hexosephosphatase capable of acceleration with arsenate, but not of fermentation; none of these was successful. The enzyme was incubated for 16 hours at 26° at p_H 6·8; after this treatment it was found that the hydrolysis was only slightly decreased, while both the fermentation and the power to accelerate in the presence of arsenate had disappeared.

Exp. 31. 2 cc. 0·1 *M* K hexosediphosphate in total volume of 6 cc. Incubated 1 hour at 30°.

Substrate	mg. P liberated per cc.	cc. CO_2 per cc.
(*a*) 1 cc. enzyme + 1 cc. boiled yeast ext.	0·159	0·07
(*b*) 1 cc. enzyme + 1 cc. boiled yeast ext. + 0·3 cc. 0·1 *M* arsenate	1·052	0·52
(*c*) 1 cc. incubated enzyme + 1 cc. boiled yeast ext.	0·121	—
(*d*) 1 cc. incubated enzyme + 1 cc. boiled yeast ext. + 0·3 cc. 0·1 *M* arsenate	0·100	—

Alcohol precipitation. 70 cc. of crude hexosephosphatase solution, prepared from dried baker's yeast by autolysis, were treated with an equal volume of absolute alcohol. The white curdy precipitate was filtered on a Büchner funnel, washed with alcohol and dried in a vacuum desiccator over H_2SO_4, yielding 3·8 g. solid (*DHa*). 2 g. of this precipitate were ground with 40 cc. of water, dissolving almost completely, 1 cc. being equivalent to 0·92 cc. of the original extract. The hydrolysing capacity was then tested.

Exp. 65. 5 cc. 0·1 *M* K hexosediphosphate + 5 cc. enzyme (*DHa*) in total volume 15 cc.

Additions	mg. P liberated by 1 cc. enzyme in 80 mins. at 30°
(*a*) 5 cc. H_2O	0·546
(*b*) 0·5 cc. 0·1 *M* arsenate + 4·5 cc. H_2O	0·087
(*c*) 3·0 cc. coenzyme + 2 cc. H_2O	0·570
(*d*) 3·0 cc. coenzyme + 0·5 cc. 0·1 *M* arsenate + 1·5 cc. H_2O	0·066

0·92 cc. of the original extract liberated 1·09 mg. P in 80 minutes, so that only 50 % of the activity was retained in the precipitate. The inhibition of the enzyme by arsenate is in striking contrast to the acceleration obtained in the crude extract; addition of the alcoholic filtrate, after removal of the alcohol *in vacuo* at 30°, did not restore the power to respond to arsenate.

Purification by dialysis and by ultrafiltration in a Bechhold filter through collodion membranes had the same effect as alcohol precipitation; the residues, though retaining the same order of activity as the original and thus confirming the view that a dialysable coenzyme is not necessary for hydrolysis, were inhibited by arsenate even in the presence of added coenzyme and of the dialysate or ultrafiltrate.

The effect of fluoride.

Lipmann [1928], investigating the effect of fluoride on muscle extract prepared by incubation at 37°, stated that it inhibited the hydrolysis of hexosephosphate only slightly less than the lactic acid production from the

ester. It has been found here that the esterification of sugar by yeast preparations is inhibited by much smaller concentrations of fluoride than are required to affect the hydrolysis of hexosephosphate. The action of fluoride on the hexosephosphatase described above is very interesting, as the following experiment shows:

Exp. 33. 2 cc. 0·1 *M* K hexosediphosphate + 1 cc. enzyme (DH_1) in total volume of 6 cc. Incubated at 30° for 2 hours.

	Control		0·005 *M* NaF		0·01 *M* NaF		
Additions	mg. P liberated per cc.	CO_2 cc.	mg. P liberated per cc.	CO_2 cc.	mg. P liberated per cc.	% inhibition	CO_2 cc.
1 cc. yeast extract ...	0·336	0·0	0·344	0·0	0·225	24·5	0·0
1 cc. yeast extract + 0·3 cc. 0·1 *M* arsenate	1·502	0·51	0·853	0·25	0·440	70·5	0·1
mg. P excess in presence of arsenate	1·166	—	0·599	—	0·185	84·5	—

While a concentration of 0·01 *M* NaF causes a 24 % inhibition of the normal hydrolysis, 84 % of the increased liberation of phosphate due to the presence of arsenate is inhibited, together with approximately the same proportion of the CO_2 evolved.

Phosphatases from other sources.

Takadiastase contains an enzyme which hydrolyses hexosediphosphate very readily. It was found that this enzyme could not be accelerated under any condition by arsenate.

Exp. 34. 2 g. takadiastase suspended in 5 cc. H_2O.
1·0 cc. 0·1 *M* K hexosediphosphate + 1 cc. takadiastase, in total volume 3 cc.
Incubated for 3 hours at 30° at p_H 7·0.

Additions	mg. P liberated per cc.
(*a*) 0·5 cc. boiled yeast ext.	0·709
(*b*) ,, ,, + 0·3 cc. 0·01 *M* arsenate	0·180
(*c*) ,, ,, + 0·1 cc. ,,	0·306
(*d*) ,, ,, + 0·03 cc. ,,	0·406

A specimen of bone phosphatase was also examined and behaved in the same way as takadiastase.

Fractionation of coenzyme solution.

In the earlier experiments the coenzyme solutions used, 10 % boiled extract of zymin, were purified by precipitation with lead acetate at p_H 6·0, in order to remove the free phosphate which somewhat inhibits the hydrolysis. Later it was found that this process affected the accelerating mechanism to some degree, and the matter was therefore investigated more closely.

A 10 % boiled extract of zymin was treated with lead acetate at p_H 6·0 until no further precipitate was formed. The excess lead was removed with H_2SO_4 and the p_H adjusted to the original. 1 cc. of the filtrate = 0·5 cc. original. The lead precipitate was decomposed with H_2S and aerated, 1 cc. = 1 cc. original extract. The effect of the two fractions on the accelerating mechanism was then tested.

Exp. 29. 2 cc. 0·1 *M* K hexosediphosphate + 1 cc. enzyme (DH_1) in total volume 6 cc. Incubated for 1 hour at 30°.

Additions	mg. P per cc. hydrolysed	mg. excess P over control
(*a*) 3 cc. H_2O	0·17	—
(*b*) 0·3 cc. 0·1 *M* arsenate + 2·7 cc. H_2O	0·114	− 0·06
(*c*) 0·5 cc. yeast ext. + 2·5 cc. H_2O	0·19	+ 0·02
(*d*) „ + 0·3 cc. 0·1 *M* arsenate ...	0·38	+ 0·21
(*e*) 1 cc. Pb filtrate	0·14	—
(*f*) „ + 0·3 cc. 0·1 *M* arsenate ...	0·26	+ 0·12
(*g*) 0·5 cc. Pb precip.	0·17	—
(*h*) „ + 0·3 cc. 0·1 *M* arsenate ...	0·11	− 0·06
(*i*) „ + 1 cc. Pb filt. + 0·3 cc. 0·1 *M* arsenate	0·39	+ 0·22

Both fractions were tested for coenzyme which was found only in the lead acetate filtrate. Some acceleration was produced on the addition of this fraction in the presence of arsenate, while the lead precipitate did not relieve the inhibition produced by arsenate. On adding both fractions together with arsenate, the acceleration was of the same order as that found with an equivalent quantity of the original extract. This points to some other factor besides coenzyme being concerned in the mechanism, and further experiments on these lines will be carried out.

All the results quoted above indicate that the action of arsenate in accelerating the rate of production of free phosphate from hexosediphosphate is not due, as was previously thought, to a direct effect upon the hydrolysis, but is part of a more complex system. The facts that the accelerated production of phosphate from hexosediphosphate was never obtained except with a parallel evolution of CO_2, and that the acceleration and the fermentation disappeared together on further treatment of the enzyme, suggest very strongly that the accelerating power of arsenate is related to the fermentation of hexosediphosphate rather than to its hydrolysis. A direct fermentation of hexosediphosphate was suggested by Meyerhof [1930] as an explanation of the fermentation of this substance by muscle and maceration extracts incapable of esterifying sugar. The possibility that the sugar formed by hydrolysis of hexosediphosphate is a labile form more readily fermentable cannot be completely ignored in this connection.

If the decomposition of hexosediphosphate takes place always by a hydrolysis to sugar and phosphate, it might be expected that fluoride would prevent the subsequent esterification, and so the fermentation of the sugar, without greatly affecting the rate of the preliminary hydrolysis; in presence of both arsenate and fluoride one might expect an increased liberation of phosphate and an accumulation of sugar. Actually, as shown, fluoride inhibits the increased phosphate production due to arsenate to the extent of 84 %, while the normal hydrolysis is only decreased 20 % by the same concentration of the salt.

While no definite conclusion that the accelerating action of arsenate on the liberation of phosphate is invariably accompanied by the fermentation of hexosediphosphate can yet be reached, the evidence here offered indicates

that the phenomenon is certainly more complex than has hitherto been realised.

I wish to record here my gratitude to Prof. Harden for his help and advice throughout this work.

SUMMARY.

1. The hydrolysis of hexosemono- and di-phosphate can be effected by washed dried yeast in the absence of coenzyme.

2. The accelerating action of arsenate on the rate of production of phosphate from hexosediphosphate takes place only in the presence of yeast extract and has not been obtained without an accompanying fermentation. Fluoride inhibits this acceleration.

3. Fractionation of the boiled yeast extracts used indicates that some other factor besides the coenzyme is necessary for the reaction.

REFERENCES.

Harden and Young (1910). *Proc. Roy. Soc. Lond.* B **82**, 321.
—— —— (1911). *Proc. Roy. Soc. Lond.* B **83**, 451.
Lipmann (1928). *Biochem. Z.* **196**, 3.
Meyerhof (1927). *Biochem. Z.* **183**, 176.
—— (1930). Die chemische Vorgänge im Muskel, 163 (Julius Springer, Berlin).
Neuberg and Leibowitz (1927). *Biochem. Z.* **191**, 460.
Raymond (1928). *J. Biol. Chem.* **79**, 637.