The Action of Enzymes on Hexosephosphate.

By Victor J. Harding.

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(Biochemical Department of the Lister Institute.)

According to the theory advanced by Harden and Young,* the presence of phosphate is essential for the alcoholic fermentation of sugar by yeast-juice. In the presence of the fermenting complex, the phosphate and sugar react together, with the simultaneous production of equivalent quantities of carbon dioxide, alcohol, and hexosephosphate. The hexosephosphate is then hydrolysed by an enzyme present in yeast-juice, with formation of a hexose and free phosphate, and the latter then again undergoes the first reaction with more sugar. The phosphate thus repeatedly passes through a cycle which may be represented by the following equations :—

(1) $2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O$,

(2) $C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4$.

The normal rate of fermentation of excess of sugar by active yeast-juice is therefore dependent upon the rate at which phosphate is set free from the hexosephosphate, and any acceleration of this reaction would increase the rate of fermentation of the sugar. Thus the addition to yeast-juice and sugar of a hexosephosphatase, that is, an enzyme capable of hydrolysing hexosephosphate, would be expected to bring about this result, and the following experiments on the action of various enzyme preparations on sodium hexosephosphate have been carried out primarily with the object of finding such an enzyme.

As a result, it was found that enzymes of very different origin (Ricinus lipase and almond emulsin) were capable of hydrolysing hexosephosphate, but the effect of adding these to fermenting yeast-juice has not yet been ascertained.

Preparation of Hexosephosphoric Acid.—The solution of hexosephosphoric acid used in these experiments was prepared as described by Young,† by decomposing the lead salt with hydrogen sulphide. The solution of free hexosephosphoric acid was made neutral to litmus by a known volume of sodium hydrate solution, and such a neutral solution was used in all the experiments. The amount of hexosephosphoric acid was previously deter-

* 'Roy. Soc. Proc.,' 1908, B, vol. 80, p. 299.

† 'Roy. Soc. Proc.,' 1909, B, vol. 81, p. 531.

mined by titration of 1 c.c. with N/10 KOH, phenolphthalein as indicator. In all experiments the term hexosephosphate is used to designate the solution neutral to litmus. The extent of hydrolysis was in each case ascertained by precipitating the liberated phosphate with magnesium citrate mixture, igniting and weighing as magnesium pyrophosphate. All results, as well as the concentration of the original hexosephosphate solutions, are expressed in terms of magnesium pyrophosphate.

The following enzyme preparations were employed :—

(*a*) lipase from castor oil seeds; (*b*) autolysed ox pancreas; (*c*) emulsin; (*d*) zymin extract; (*e*) autolysed yeast-juice.

(*a*) *Lipase from Castor Oil Seeds* (Ricinus communis).—This was prepared by extracting the freshly ground seeds of *Ricinus communis* with ether until fat free, and using the residue as the lipase preparation. It was found to be highly active.

The results of three experiments are given in the accompanying table, which refers in each case to the following set of mixtures:—

A. 0·3 grm. lipase + 3 c.c. N/5 acetic acid + 15 c.c. water.
B. „ „ „ + 15 c.c. hexosephosphate.
C. Same as B, but boiled before incubation.

The experiments were carried out at 25° in presence of toluene (0·5 c.c.), and different hexosephosphate solutions were employed in each set of experiments.

No.	Original hexosephosphate as $Mg_2P_2O_7$.	Time of incubation in hours.	Free phosphate, grm. $Mg_2P_2O_7$.			Phosphate liberated, B−C.
			A.	B.	C.	
1	0·5835	24	0	0·1259	0·0428	0·0831
		48	0	0·1836	0·0320	0·1516
2	0·5665	120	0	0·2746	0·0416	0·2330
3	0·6274	240	0	0·3655	0·0359	0·3296

It is seen that the preparation had a considerable hydrolytic action on the hexosephosphate.

(*b*) *Ox Pancreas.*—The fresh ox pancreas, freed as far as possible from connective tissue, was allowed to undergo autolysis for four days under toluene at 37°. The liquid was then filtered through a coarse muslin cloth to remove any undigested pancreas, and the filtrate used in the experiments.

The following mixtures were incubated for 48 hours at 37° in presence of toluene, and the free phosphate estimated as before:—

A. 5 c.c. autolysed ox pancreas + 15 c.c. water.
B. „ „ + 15 c.c. hexosephosphate.
C. The same as B, but the pancreas mixture employed was boiled.

Original hexosephosphate = 0·5351 grm. $Mg_2P_2O_7$.
Free phosphate in A ..... = 0·0 „
„ B ..... = 0·0915 „
„ C ..... = 0·0814 „
Phosphate liberated ..... = 0·0101 „

Practically no action had taken place.

(*c*) *Emulsin.*—The emulsin was prepared as described by H. E. and E. F. Armstrong and Horton.*

The experiments were carried out at 37° in presence of toluene, the following mixtures being employed:—

A. 10 c.c. emulsin solution + 15 c.c. water.
B. „ „ + 15 c.c. hexosephosphate.
C. Same as B, but boiled emulsin employed.

Original hexosephosphate = 0·6274 grm. $Mg_2P_2O_7$.

	After	
	48 hours.	144 hours.
Free phosphate in A	0·0	0·0
„ „ B	0·1750	0·3064
„ „ C	0·0342	0·0563
Phosphate set free by enzyme ...	0·1408	0·2501

Emulsin has, therefore, a considerable hydrolytic action on hexosephosphate.

(*d*) *Zymin Extract.*—10 grm. of "zymin" preparation were digested four days with 30 c.c. water at 25°. The resulting mixture was filtered and used in the experiment. It was found to contain considerable amounts of phosphate.

An aqueous extract of zymin (yeast treated with acetone, was found only to have a slight action on hexosephosphate. This is seen from the following experiment carried out at 25° in presence of toluene, the mixture being incubated for three days:—

* 'Roy. Soc. Proc.,' 1908, B, vol. 80, p. 324.

A. 5 c.c. zymin extract + 15 c.c. water.
B. „ „ + 15 c.c. hexosephosphate.
C. Same as B, but zymin extract boiled.

Original hexosephosphate..... = 0·6274 grm. $Mg_2P_2O_7$.
After incubation, free phosphate in—
A = 0·0625 „
B = 0·1618 „
C = 0·0910 „
Phosphate set free by enzyme..... = 0·0708 „

(*e*) *Autolysed Yeast-juice.*—A sample of yeast-juice which had been allowed to remain at 0° for a fortnight was used. It was found to be incapable of fermenting glucose.

The following mixtures were incubated at 25° for 24 hours in presence of toluene, and the free phosphate estimated:—

A. Autolysed yeast-juice 25 c.c. + 10 c.c. water.
B. „ „ + 10 c.c. hexosephosphate.
C. As B, but boiled yeast-juice employed.

Original hexosephosphate ..... = 0·3776 grm. $Mg_2P_2O_7$.
After incubation, free phosphate in—
A = 0·1389 „
B = 0·2232 „
C = 0·1409 „
Phosphate set free by enzyme..... = 0·0823 „

Hexosephosphate is thus hydrolysed to some extent by autolysed yeast-juice. This is in agreement with with the experiments of Harden and Young, who showed that in actively fermenting yeast-juice most of the phosphate was present in the combined form, but was gradually set free by an enzyme hexosephosphatase, after the juice had lost its fermentative activity.*

An attempt was made to ascertain if hexosephosphatase could be isolated from the autolysed yeast-juice: 50 c.c. of autolysed yeast-juice were added with constant stirring to a mixture of 400 c.c. alcohol and 400 c.c. ether. The white precipitate was filtered immediately at the pump, washed once with ether, and air dried. A very small amount of a brownish sticky precipitate remained. This was dissolved in 50 c.c. of water and allowed to stand overnight with a little toluene. It was then filtered through muslin, and the solution thus obtained examined as to its hydrolytic properties towards hexosphosphate.

* 'Roy. Soc. Proc.,' 1908, B, vol. 80, p. 299.

The mixtures A, B, C were incubated with toluene at 25° for 24 hours :—

A. 10 c.c. above solution + 15 c.c. water.
B. „ „ + 15 c.c. hexosephosphate.
C. As B, but enzyme solution previously boiled.

Original hexosephosphate ..... = 0·5665 grm. $Mg_2P_2O_7$.
After incubation, free phosphate in—
A = 0·0494 „
B = 0·1268 „
C = 0·0755 „
Phosphate set free by enzyme ..... = 0·0513 „

The hexosephosphatase, therefore, can be precipitated by means of alcohol and ether.

Summary.

(*a*) Ricinus lipase and emulsin possess a slow hydrolytic action on hexosephosphate.

(*b*) Autolysed ox pancreas is almost without action upon hexosephosphate.

(*c*) "Zymin" extract hydrolyses hexosephosphate slowly.

(*d*) Autolysed yeast-juice possesses a marked action on hexosephosphate.

(*e*) An alcohol-ether precipitation of autolysed yeast-juice possesses considerable activity towards hexosephosphate.