LXXXV. A NEW PHOSPHORIC ESTER PRODUCED BY THE ACTION OF YEAST JUICE ON HEXOSES.

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(Received October 30th, 1922.)

THE effect of sodium phosphate in increasing the fermentative power of yeast juice was first observed by Wroblewski [1901] and again by Buchner [Buchner, E. and H. and Hahn 1903] by whom it was attributed to the alkalinity of the salt. That such was not the true explanation followed from the work of Harden and Young [1905; 1906, 1 and 2; 1908, 1 and 2; 1909; 1910, 1 and 2; 1911] whose extended investigations revealed the important rôle played by phosphates in alcoholic fermentation. It was shown by these authors that when a suitable amount of a soluble phosphate is added to a fermenting mixture of yeast juice and glucose, fructose or mannose, the rate of fermentation rapidly increases but after a short period again falls to a constant rate, which is only slightly greater than that of the original yeast juice and sugar. During this period of increased fermentative activity the total evolution of carbon dioxide is increased by an amount which is equivalent, molecule per molecule, to the phosphate added, while the latter undergoes a transformation into a form that is no longer precipitable by magnesium citrate mixture.

From such solutions Young [1907; 1909] succeeded in isolating an ester of phosphoric acid by precipitation in the form of its insoluble lead salt. He showed [1909; 1911] that this compound possessed the constitution of a hexosediphosphoric acid, $C_6H_{10}O_4(PO_4H_2)_2$, and that the same substance was formed from glucose, fructose or mannose when fermented with yeast juice or zymine in the presence of phosphate. The acid was slightly dextrorotatory ($[\alpha]_D = +3.2^\circ$) and on hydrolysis yielded phosphoric acid and a laevorotatory reducing substance from which fructose was isolated, although Young was not satisfied that the latter was the only sugar produced. A number of salts and derivatives were described, including the phenylhydrazine salt of a phenylhydrazone in which both phosphoric acid groups were retained, and an osazone in the formation of which one molecule of phosphoric acid had been split off. The evidence brought forward by Young was, however, insufficient to decide fully the constitution of the ester.

About the same time this compound was also discovered by Ivanov [1905; 1907; 1909], who attributed to it the constitution of a triosemonophosphoric ester, and at a somewhat later date was examined by Lebedev [1909; 1910] who at first supposed it to be a hexosemonophosphoric ester but afterwards [1911] came to the same conclusion as Young on this point.

During the course of some experiments carried out in 1913 by the author in conjunction with Professor A. Harden indications were noted that hexosediphosphoric acid is not the only compound of this type produced during the fermentation of sugar by yeast juice in presence of phosphate. It was observed that during the preparation of hexosediphosphoric acid from fructose and after the precipitation of its lead salt by the addition of lead acetate, the filtrate always contained very appreciable amounts of phosphorus in organic combination, and that a further precipitate containing most of this phosphorus was thrown down on the addition of basic lead acetate. On examining the acid solutions obtained by decomposing such basic lead precipitates with hydrogen sulphide it was found that the ratio of the reducing power, as determined by Bertrand's method, to the phosphorus content was much higher than that given by solutions of hexosediphosphoric acid, while polarimetric observations gave evidence of the presence of a substance much more strongly dextrorotatory than the latter.

The barium salt of this new acid was prepared, and proved to be readily soluble in both hot and cold water, differing in this respect markedly from barium hexosediphosphate. The salt was amorphous and was obviously contaminated with substances derived either from the yeast juice or formed during the fermentation of the fructose, but the results of analyses appeared to indicate that the compound was the barium salt of a hexosemonophosphoric acid, $C_6H_{11}O_6PO_4Ba$. These facts were published in a preliminary note [Harden and Robison, 1914] and the investigation of the compound was continued. Its purification, however, proved unexpectedly difficult and was not completed when the work was interrupted by the war. It was resumed in 1919 but was again held up by the difficulty experienced in obtaining yeast juice of reasonable fermentative power. The variability in the activity of juice prepared from the same type of yeast and apparently under similar conditions has been frequently observed, but no explanation could be found for the long run of inactive or feebly active juices obtained during 1919 unless it were the disheartening effect of the war beer on the yeast. That difficulty no longer exists and improved methods for the isolation and purification of the new compound have been worked out. Sufficient evidence has now been obtained to confirm the opinion already stated as to its constitution. It is, however, not identical with the hexosemonophosphoric acid prepared by Neuberg [1918], by partial hydrolysis from hexosediphosphoric acid. A specimen of barium hexosemonophosphate prepared according to the method described by Neuberg was found to be optically almost inactive but to yield a strongly laevorotatory product on further hydrolysis. The compound iso-

lated from the products of fermentation is, on the other hand, strongly dextrorotatory, and on hydrolysis yields a dextrorotatory reducing substance from which glucosazone has been obtained. The ratio of the rotatory power of this hydrolysis product to its reducing power as estimated by Bertrand's method is, however, much lower than that given by solutions of pure glucose. In this and in other respects the behaviour of the ester suggests that it may possibly be a mixture of isomeric hexosemonophosphoric acids, while the substance described by Neuberg may represent one or more other isomers. The isomerism may be due to the hexose molecule itself or to the position of the phosphoric acid radicle.

So far, only one salt of hexosemonophosphoric acid, that of brucine, has been obtained in a crystalline condition and the hope that this salt might be used to effect the purification of the acid, and its possible resolution into two or more isomers was for long disappointed owing to the difficulty with which crystallisation was effected. Within the last few weeks, however, more success has been obtained and it is hoped that the question may yet be solved in this way.

With phenylhydrazine, hexosemonophosphoric acid yields the phenylhydrazine salt of an osazone in which the phosphoric acid radicle is retained. This compound is not identical with the osazone of the same empirical formula prepared by Young, Ivanov and Lebedev from hexosediphosphoric acid.

Hexosemonophosphoric acid is only very slowly hydrolysed by boiling with mineral acids and a considerable portion of the sugar produced is destroyed during the operation. It is also hydrolysed by emulsin, yielding free phosphoric acid and a dextrorotatory reducing substance. Emulsin likewise hydrolyses hexosediphosphoric acid but, as with acid hydrolysis, the reducing substance produced is strongly laevorotatory. It is therefore improbable that hexosemonophosphoric acid is formed during the fermentation by partial hydrolysis—enzymic or otherwise—of the diphosphoric ester.

The behaviour of hexosemonophosphate towards yeast, yeast juice and zymin is being studied in conjunction with Dr Harden and is yielding interesting results that are, however, difficult to interpret. The alkali salts are readily fermented by yeast juice and by zymin and the initial rate is relatively high, even approximating to the "phosphate rate" for glucose, but rapidly falls to a lower level, which nevertheless is decidedly higher than that for hexosediphosphate.

The same compound is apparently formed during the fermentation of either fructose or glucose in presence of phosphate. It is true that some differences were observed in the specific rotations of the various preparations, but such differences were not greater between preparations made from glucose and those from fructose than between different preparations made from the same sugar. This may perhaps be taken as further evidence that all specimens were mixtures of isomeric compounds in somewhat varying proportions.

Owing to the many processes employed in the different attempts to obtain

the ester in a pure condition, and the fact that even the best of these methods involves some loss of the substance, the record of yields obtained does not at present give very trustworthy evidence as to the most favourable conditions for the production of the monophosphoric ester or for determining the relative amounts of this and the diphosphoric ester that are formed during fermentation.

Calculations based on the values of the ratio

$$\frac{\text{Reducing power (as glucose)}}{\text{Phosphorus}}$$

as determined for the acid solutions derived from both normal and basic lead precipitates would appear to indicate the presence of a much higher proportion of the monophosphoric ester than has ever been actually isolated. It is just possible that in spite of the thorough washing to which they were subjected, these precipitates still held some adsorbed sugar which would affect the above ratio. The possibility of other reducing substances being present must also be considered.

The best yields of the hexosemonophosphoric acid actually obtained from fructose and from glucose correspond with only 15 % and 7 % respectively of the sodium phosphate added during the fermentation. These figures are based on the weight of barium salt of at least 90 % purity; the total amounts formed would certainly be higher and might well correspond with double these percentages.

The question as to what rôle, if any, is played by hexosemonophosphoric acid in alcoholic fermentation must be left unanswered until more information is gained on the various points indicated above.

EXPERIMENTAL.

A typical series of values of the ratios

$$\frac{\text{Reducing power (as glucose)}}{\text{Phosphorus}} \text{ and } \frac{\text{Rotation in 4 dm. tube}}{\text{Reducing power (as glucose) in 100 cc.}}$$

as determined for the acid solutions derived from normal and basic lead precipitates, is given below. They are taken from one of the first experiments, which led to the discovery of the monophosphoric ester, and are quoted because of their bearing on the relative amounts of the two esters formed during the fermentation.

The reducing power was estimated by Bertrand's method and calculated as glucose; the phosphorus was estimated by Neumann's method.

Fermentation: 1220 cc. juice from 4 kilos of fresh pressed brewery yeast was allowed to ferment 122 g. fructose, 131 g. (0.4 Mol.) of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ being added gradually, as indicated by the rate of evolution of CO_2 , during $1\frac{1}{2}$ hours. Fermentation was stopped and protein coagulated by blowing steam through the solution. After neutralisation of the filtered solution with caustic soda and removal of free phosphate by precipitation with magnesium acetate, a slight excess of lead acetate was added to the filtrate. The precipitate was

removed by centrifuging and washed nine times with water. The filtrate and washings were treated with basic lead acetate and the precipitate again separated by centrifuging and thoroughly washed. These precipitates were decomposed by hydrogen sulphide and the excess of the latter removed by means of a current of air. The acid solutions were each neutralised to phenolphthalein by the addition of caustic soda and were again treated successively with normal and with basic lead acetate, any traces of free phosphate being first removed. These operations were repeated as shewn below (Table I).

The acid solutions derived from the various lead precipitates gave the following ratios:

Table I.

No. of sol.	Description of precipitate from which solution was derived	Reducing power as glucose P	Rotation in 4 dm. tube reducing power as glucose in 100 cc.	$[\alpha]_D$ of acid calculated on P-content
1	First precipitate by normal lead acetate (crude lead hexosediphosphate)	2.50	—	—
2	Solution 1 precipitated a second time by normal lead acetate	2.25	—	—
3	Solution 2 precipitated a third time by normal lead acetate. Precipitations carried out in two fractions: 1st fraction	1.50	—	—
4 a	Solution 3 precipitated a fourth and fifth time with normal lead acetate, the fifth time in two fractions: 1st fraction	1.21	0.647	Calculated for $C_6H_{10}O_4(PO_4H_2)_2$ + 3.56°
4 b	2nd "	1.67	—	—
5	Solution 4 a precipitated a sixth time with normal lead acetate (pure lead hexosediphosphate)	1.16	0.623	+ 3.26*
6	First precipitate by basic lead acetate after removal of first normal lead precipitate	5.50	—	—
7 a	Solution 6 precipitated a second time with normal then with basic lead acetate:			
	Normal lead precipitate	—	—	—
7 b	Basic " "	4.24	—	—
8 a	Solution 7 b precipitated a third time with normal then with basic lead acetate:			
	Normal lead precipitate	3.96	1.60	Calculated for $C_6H_{11}O_5(PO_4H_2)$ + 19.0°
8 b	Basic " "	4.36	2.40	+ 31.2°
9 a	Solution 8 b precipitated a fourth time with normal then with basic lead acetate:			
	Normal lead precipitate	4.13	2.18	+ 25.8†
9 b	Basic lead precipitate Amount too small for determination of ratios	—	—	—

* $[\alpha]_D$ of hexosediphosphoric acid = + 3.2° (Young).

† $[\alpha]_D$ of hexosemonophosphoric acid = + 25.0° (p. 817).

Solutions 9 a and 9 b were each treated with baryta until neutral to phenolphthalein and the barium salts precipitated by the addition of an equal

volume of alcohol. They were purified by redissolving in water and reprecipitating with alcohol and were then dried at 100° to constant weight and analysed:

Barium salt from solution 9 *a*. Ba = 34.33 %; P = 7.07 %.

9b. Ba = 34.86 %; P = 7.22 %.

Calculated for $C_6H_{11}O_5PO_4Ba$. Ba = 34.75 %; P = 7.85 %.

It seemed probable therefore that the chief constituent of the basic lead precipitates was the salt of a hexosemonophosphoric acid, but that this was contaminated with salts of other acids containing less phosphorus or none at all. Whether the differences in the ratios for solutions Nos. 6 to 9 are due entirely to phosphorus-free compounds, or whether they indicate the presence of yet another phosphoric ester, *e.g.* of a disaccharide, is impossible at present to say.

In the case of the solutions derived from the normal lead precipitates a steady decrease in the ratio Reducing power/P occurs as the purification proceeds. If the final ratio of 1.16¹ be accepted as correct for pure hexosediphosphoric acid, then it is obvious that solution 1 must have contained a considerable proportion of some other reducing substance. The presence of sugar would seem to be precluded by the thorough washing of the precipitate. That the precipitate contained some hexosemonophosphate is certain since, although this ester gives in concentrated neutral solutions no precipitate with normal lead acetate, a copious precipitate, probably of basic lead salt, is formed in dilute solutions.

If solution No. 1 contained no reducing substance or phosphorus compound other than the two hexosephosphoric acids, then the ratio 2.50 would indicate that nearly 50 % of the phosphorus was present in the form of the mono-ester, in addition to the amount remaining in the filtrate and precipitated by basic lead acetate. As already stated, the yields of hexosemonophosphoric acid actually isolated in the form of its barium salt have never reached this proportion and have usually been much lower.

The purification of the ester proved a problem of considerable difficulty and much time was spent in solving it. Attempts to prepare salts in a crystalline condition were unsuccessful except in the case of the brucine salt, and fractional precipitation of the metallic salts by alcohol effected only slight improvement in the analytical figures. Fractional precipitation of the syrupy acid from its alcoholic solution by ether, was also of little use. Repeated extraction of the aqueous solution of the acid with ether or ethyl acetate did remove a small proportion of crystalline substance which proved to be succinic acid, but the phosphorus content of the barium salt was thereby only slightly raised. Further fractional precipitation by lead acetate, normal and basic, gave a purer product but involved too rapid diminution in the amount of

¹ The values for this ratio calculated from Young's data lie between 2.1 and 2.3. They are, however, not comparable with those shown above as the reducing power was determined by a different method(Pavy).

substance to be practicable. The greatest improvement was effected by the use of mercuric acetate which precipitates a considerable proportion of the impurities and by combination of this with other processes, a product giving satisfactory analytical figures was at length obtained.

Preparation and purification of the hexosephosphoric esters.

The methods employed in the preparation of the esters have been considerably modified as experience suggested. Heating the fermentation mixture to destroy the enzymes and coagulate the protein was discontinued on account of the risk of causing partial hydrolysis of the diphosphoric acid. Preliminary separation of the two esters by means of their lead salts was given up owing to the large proportion of the monophosphoric acid brought down in such dilute solutions by lead acetate. The method now used is as follows:

Juice prepared from fresh pressed English mild ale yeast is mixed with 10 % of its weight of fructose or glucose and warmed to 26° in a water-bath. Fermentation having commenced a 20 % aqueous solution of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ is added from time to time in such quantity as to produce the maximum rate of evolution of carbon dioxide. The addition of phosphate solution is best regulated by means of a guide experiment carried out with 25 cc. juice in the apparatus described by Harden, Thompson and Young [1910] (v. "Alcoholic Fermentation" by A. Harden, 1923 edition, p. 28). When the total volume of phosphate solution added exceeds half the original volume of juice a further quantity of sugar should be dissolved in the fermentation mixture. When the further addition of phosphate no longer causes any considerable rise in the rate of evolution of carbon dioxide, solid barium acetate in amount equal to the total weight of crystalline sodium phosphate that has been added, is dissolved in the reaction mixture which is then rendered just alkaline to phenolphthalein with baryta, and treated with an equal volume of alcohol. The barium salts of the two hexosephosphoric acids and of any excess of free phosphoric acid are thus precipitated together with the protein of the yeast juice. The precipitate is filtered off on a large Buchner funnel, and thoroughly washed with 70 % alcohol. It is then treated with boiling absolute alcohol and allowed to remain in contact with the alcohol over night. In this way the protein present is denaturated and rendered insoluble. The crude barium salts are then dried in an evacuated desiccator over sulphuric acid or more quickly by exposure to a current of warm air. They are next thoroughly ground up with 10 parts of cold water which dissolves the barium hexosemonophosphate, but scarcely any of the hexosediphosphate. The residue is washed twice with small quantities of water (being preferably removed from the Buchner funnel and again ground up) and is then extracted with 200 parts of water which dissolves the barium hexosediphosphate, leaving behind the insoluble barium phosphate etc. After removal of any traces of mineral phosphate by precipitation with magnesium acetate, the hexosediphosphoric acid is precipitated from the filtered solution in the form of its lead salt by

the addition of lead acetate. The precipitate is filtered or centrifuged off and washed. It is then suspended in water, decomposed by a current of sulphuretted hydrogen, the clear filtrate is freed from sulphuretted hydrogen by a current of air and finally neutralised to phenolphthalein with caustic soda. The precipitation of the lead salt is repeated several times [v. Young, 1909].

The first aqueous extract of the crude barium salts contains the barium hexosemonophosphate together with a little hexosediphosphate and other barium salts of acids derived from the yeast juice or formed during the fermentation. It is treated with basic lead acetate and the insoluble basic lead salt is filtered and washed. It is then decomposed with sulphuretted hydrogen as described above and the acid solution neutralised to phenolphthalein with a hot saturated solution of baryta. The solution is filtered and the precipitation with basic lead acetate repeated, after which the barium salt is again formed and is precipitated from its solution by alcohol, filtered off, washed with absolute alcohol and dried in an evacuated desiccator over sulphuric acid. It is dissolved in 10 parts of water to which 10 % of alcohol is finally added. The filtered solution is treated with mercuric acetate so long as this causes any precipitate to form, and is allowed to stand for some hours before filtering. The basic lead salt is then reprecipitated from the clear filtrate and once more converted into the barium salt, which is finally purified by repeatedly dissolving it in 10 % alcohol, filtering and reprecipitating with an equal volume of alcohol. In presence of a small proportion of water the salt readily forms a syrup, and it should therefore be washed several times with absolute alcohol and rapidly dried in an evacuated desiccator. If the results of analysis are not satisfactory the purification by mercuric acetate etc. is repeated. The mercury precipitate and a sparingly soluble barium salt, which is obtained in very small amounts during the final stage of the purification, both contain phosphorus and are being submitted to further investigation.

Hexosemonophosphoric acid and its salts.

Barium hexosemonophosphate, prepared by the above method, is a colourless, amorphous substance very readily soluble in hot and cold water, but insoluble in organic solvents. Dried over sulphuric acid it still retains about 4.5 % moisture which is given up at 100°, the salt at the same time taking on a brownish tint. The percentage of water corresponds approximately with one molecule (calculated for $C_6H_{11}O_5PO_4BaH_2O$, $H_2O = 4.36\%$) but is not constant. The anhydrous salt takes up about the same amount of moisture with great avidity at ordinary temperatures even when kept in a closed vessel over calcium chloride.

For analysis the salt was dried to constant weight in an evacuated vessel (2 mm. pressure) at 78° over phosphorus pentoxide, only slight discoloration taking place under these conditions. Table II gives the results of analyses of specimens prepared from glucose and fructose, together with the specific

rotation of the barium salt and of the acid prepared from it by the addition of the exact amount of sulphuric acid.

The results obtained with a specimen of barium hexosemonophosphate prepared by hydrolysis of hexosediphosphoric acid (Neuberg's method) are also shown in the same table for purposes of comparison.

Table II.

No.	Sugar from which salt was prepared	Ba	P	C	H	$[\alpha]_D^{20}$ of salt	$[\alpha]_D^{20}$ of acid
1	Fructose	35.35	7.61	—	—	—	—
2	"	34.89	7.86	—	—	+12.5° (c=3.9 %)	+24.9° (c=2.1 %)
3	Glucose	35.12	7.60	—	—	—	—
4	"	35.05	7.66	—	—	+14.4° (c=14 %)	—
5	"	34.82	7.45	—	—	+12.7° (c=17 %)	—
6	"	34.83	7.73	—	—	+15.6° (c=11.2 %)	+28.8° (c=2.7 %)
7	"	34.87 35.09	7.82 7.85	18.37 18.16	2.93 2.99	+12.2° (c=3.7 %)	+25.0° (c=3 %)
Calculated for $C_6H_{11}O_5PO_4Ba$		34.75	7.85	18.20	2.81	—	—
8	Hexosemonophosphoric acid obtained by partial hydrolysis of hexosediphosphoric acid (Neuberg's method)	33.26	7.58	—	—	+0.38° (c=5.2 %)	+1.49° (c=3.3 %)
Calculated for $C_6H_{11}O_5PO_4BaH_2O$		33.22	7.51	—	—	—	—

Molecular weight determination by the cryoscopic method yielded the following results:

1. 0.0849 barium salt in 13.17 g. water gave $\Delta = 0.062^\circ$, whence apparent Mol. weight = 198.

2. 0.3037 barium salt in 13.17 g. water gave $\Delta = 0.192^\circ$, whence apparent Mol. weight = 228.

$C_6H_{11}O_5PO_4Ba$ requires Mol. weight = 395.5; if 100 % dissociated, apparent Mol. weight = 197.8.

$C_{12}H_{20}O_9(PO_4Ba)_2H_2O$ requires Mol. weight = 791; if 100 % dissociated, apparent Mol. weight = 263.7.

The experimental results are therefore consistent with the first formula but not with the second.

The brucine salt was prepared by dissolving the calculated amount of the base in an aqueous solution of hexosemonophosphoric acid prepared from the barium salt. On evaporation of the solution at a low temperature a crystalline residue was obtained. This was very soluble in water, moderately soluble in warm methyl alcohol but sparingly soluble in ethyl alcohol. Recrystallisation was not readily effected but on treating the concentrated aqueous solution with acetone and cooling to 0° , a semi-crystalline precipitate was obtained. After twice repeating this operation the salt was dried to constant weight at 78° and 2 mm. pressure over phosphorus pentoxide, no discoloration taking place.

Phosphorus estimated by Neumann's method: found P = 2.73 %.

Calculated for $C_6H_{11}O_5PO_4H_2(C_{23}H_{26}N_2O_4)_2$; P = 2.96 %.

Specific rotation of salt in water (conc. = 6.6 %); $[\alpha]_D^{19} = -23.4^\circ$.

The investigation of the brucine salt is being continued.

The normal lead and copper salts are also soluble in concentrated solutions but on diluting these, precipitates, presumably of the basic salts, are formed. Hexosemonophosphoric acid itself was obtained as a colourless, very viscous liquid by evaporating its aqueous solution *in vacuo*. It is moderately soluble in alcohol but very sparingly soluble in ether or acetone. Whether prepared from fructose or glucose it reduces Fehling's solution and gives a red coloration when heated with resorcinol and hydrochloric acid (Selivanov's reaction), though the colour is not so intense as that given by the equivalent amount of fructose. With water it yields a strongly acid solution which can be titrated sharply with alkalis (two equivalents) using phenolphthalein as indicator. All attempts to obtain the acid in crystalline condition have failed.

Osazone of hexosemonophosphoric acid.

Attempts to prepare a phenylhydrazone of hexosemonophosphoric acid were not successful but on warming the solution with excess of the base, a crystalline derivative was obtained. 10 g. barium hexosemonophosphate, prepared from fructose, were treated with the exact amount of sulphuric acid and the barium sulphate removed by filtration. The clear solution was mixed with 15 g. phenylhydrazine dissolved in acetic acid, and was heated on the water-bath during 30 minutes. It was then cooled to 0° and the yellow precipitate filtered off, washed with chloroform, and dried.

Weight of product = 8 g. M. pt. 135°–137°.

Recrystallisation was effected by dissolving the compound in boiling ethyl alcohol, adding an equal volume of hot chloroform and cooling to 0°. Short, pale yellow needles were formed. M. pt. 139° with decomposition. Moderately soluble in methyl alcohol, less soluble in ethyl alcohol, still less in chloroform and insoluble in light petroleum. Very sparingly soluble in water but readily soluble in dilute sodium hydroxide.

Analysis of substance dried at 66° *in vacuo* over phosphorus pentoxide: N = 15.33 %; P = 5.60 %.

Calculated for $C_6H_5NHNH_2H_2PO_4 \cdot C_4H_5(OH)_3C(N_2HC_6H_5)CHN_2HC_6H_5$; N = 15.38 %; P = 5.68 %.

The compound therefore appeared to be the phenylhydrazine salt of the osazone of hexosemonophosphoric acid. It is, however, not identical with the compound of the same empirical formula obtained by Young from hexosediphosphoric acid, with elimination of one molecule of phosphoric acid, since the latter melted at 151°.

The chloroform extract from the crude osazone yielded, on evaporation, a small quantity of a crystalline compound readily soluble in ethyl alcohol, which, by recrystallisation from ethyl alcohol and chloroform, was obtained in yellow plates, melting with decomposition at 190°. The substance contained phosphorus and was readily soluble in dilute sodium hydroxide, though very sparingly soluble in water. Unfortunately the quantity was insufficient for analysis.

Hydrolysis of hexosemonophosphoric acid by acids.

Hexosemonophosphoric acid is very resistant to hydrolysis by acids at ordinary temperatures.

A solution of the acid in *N* sulphuric acid, after being kept for four months at room temperature contained only a trace of free phosphoric acid. When the solution of the acid is boiled either alone or with the addition of mineral acid, free phosphoric acid is slowly liberated and a reducing substance formed. Table III gives the data of an experiment in which 5.4 g. of hexosemonophosphoric acid prepared from glucose, was heated with 250 cc. of *N*/5 sulphuric acid at 97°, the rotation and free phosphoric acid being estimated from time to time. The total phosphorus = 0.643 g.

Table III.

Time of heating, hours	Rotation in 1 dm. tube	P present as free PO ₄ , g.	P present as free PO ₄ in percentage of total P
0	+0.625°	0	0
1	+0.568°	—	—
3	+0.544°	—	—
16½	+0.507°	0.170	26.4
36	+0.427°	0.274	42.6
80	+0.327°	0.374	58.2

After 80 hours the solution had become dark brown in colour and the rotation was difficult to measure. The hydrolysis was accordingly stopped and the solution neutralised with baryta. The precipitate, which consisted entirely of barium phosphate and sulphate, was filtered off and the clear filtrate treated with an equal volume of alcohol, which precipitated the barium salt of the unchanged hexosemonophosphoric acid. The phosphorus content and specific rotation of this salt agreed with those of the original barium hexosemonophosphate. The alcoholic filtrate reduced Fehling's solution and contained only a trace of phosphorus. After distilling off the alcohol the reducing power of the solution was estimated by Bertrand's method and its rotation measured.

Reducing power calculated as glucose = 0.083 g. per 100 cc. equivalent to 0.98 for total original solution.

Rotation in 4 dm. tube = + 0.097°.

$\frac{\text{Rotation in 4 dm. tube}}{\text{Reducing power as glucose in 100 cc.}} = 1.17$. Calculated for glucose = 2.11.

The amount of sugar formed during the hydrolysis, calculated from the amount of phosphoric acid liberated is equal to 2.17 g. or more than twice the quantity actually estimated in the solution. It would seem therefore that a considerable proportion of the sugar is destroyed during the boiling. This solution yielded an osazone which, after being twice recrystallised from dilute pyridine, melted at 205°–206° and did not lower the melting point of glucosazone when intimately mixed with the latter.

In another experiment, Table IV, a solution of hexosemonophosphoric acid prepared from laevulose was boiled under a reflux condenser, the reducing power and the rotation being measured from time to time.

Table IV.

Time in hours	Rotation in 4 dm. tube	Reducing power as glucose in 100 cc.	Rotation Reducing power
0	+1.339°	0.59	2.27
2	+1.064°	0.64	1.66
4	+1.024°	0.64	1.60
10	+0.896°	0.64	1.40
16	+0.788°	0.65	1.21

At the end of 16 hours' boiling the free phosphate amounted to 0.0795 g. P per 100 cc. or 53 % of the total phosphorus (0.1499 g. per 100 cc.). After precipitating the unchanged ester by basic lead acetate the filtrate was freed from lead by sulphuretted hydrogen and the reducing power etc. determined. The value found for the ratio $\frac{\text{Rotation in 4 dm. tube}}{\text{Reducing power as glucose in 100 cc.}}$ was equal to 0.90.

Hydrolysis of hexosemonophosphoric acid by emulsin.

Hexosemonophosphoric acid is hydrolysed by emulsin yielding also in this case, free phosphoric acid and a dextrorotatory substance from which glucosazone can be obtained.

A solution of 1.876 g. anhydrous barium hexosemonophosphate in 24 cc. water was treated with 2 cc. of an extract of emulsin¹ prepared from sweet almonds, and was incubated at 37° in presence of toluene. At intervals the rotation was measured, after filtering off the precipitated barium phosphate. Table V gives the results of these determinations. On the 10th day a further 1 cc. emulsin extract was added but the rotation has been calculated for the original volume of the solution.

Table V.

Time of incubation, days	Rotation in 2 dm. tube at 20°	Percentage of salt hydrolysed as measured by free PO ₄
0	+1.680°	—
1	+1.579°	—
4	+1.376°	—
10	+1.334°	—
13	+1.339°	66

On the 13th day the remaining solution was neutralised to phenolphthalein with baryta and the total free phosphate estimated. The clear filtrate was treated with 1½ volumes of alcohol and the precipitate filtered off, dried at 78° *in vacuo*, and examined.

Weight = 0.370 g. equivalent to 0.53 g. for the total original solution.

Found Ba = 32.8 %; P = 6.87 %; $[\alpha]_D^{20} = +11.3$ %.

¹ This extract contained a slight trace of reducing substance.

The substance probably consisted chiefly of unchanged barium hexosemonophosphate. The filtrate and washings from this precipitate were made up to 100 cc. They contained a reducing substance equivalent to 0.240 g. glucose as estimated by Bertrand's method. This amount is equivalent to 0.347 g. for the original solution.

Rotation in 4 dm. tube = + 0.350°.

$$\frac{\text{Rotation in 4 dm. tube}}{\text{Reducing power as glucose in 100 cc.}} = 1.46.$$

On evaporation this solution yielded a syrup which could not be made to crystallise but with phenylhydrazine yielded an osazone. After recrystallisation from dilute pyridine this melted at 204°–205°, and did not lower the melting point of glucosazone when intimately mixed with the latter.

Hydrolysis of barium hexosediphosphate by emulsin.

The hydrolytic action of emulsin on hexosediphosphate was established by Harding [1912] who estimated the phosphoric acid set free but did not further examine the products.

In view of the possibility that the optically active substances produced under these conditions might differ from those produced by acid hydrolysis it was thought advisable to investigate this point.

1 g. of barium hexosediphosphate was incubated with 1 cc. emulsin extract and 10 cc. water at 37°, in presence of toluene. The salt only dissolved to a slight extent in the water. After 14 days the solution was filtered from the solid matter, which consisted of unchanged barium hexosediphosphate and barium phosphate, and examined polarimetrically.

$$\alpha^{18} \text{ in 1 dm. tube} = - 0.530^\circ.$$

Estimation of free and total phosphate in both precipitate and solution indicated that about 60 % of the phosphoric acid had been liberated. On neutralising the solution with baryta and adding alcohol a small amount of soluble barium salt was precipitated while the solution contained the laevo-rotatory substance.

It appears therefore that the hydrolysis of hexosediphosphoric acid by enzymes results in products similar to those obtained by acid hydrolysis. Further experiments are, however, being carried out.

The hexosemonophosphoric acid produced by partial hydrolysis of hexosediphosphoric acid.

A quantity of this ester was prepared by Neuberg's [1918] method in order to compare it with the compound obtained by the fermentation of sugar.

9.3 g. of crude barium salt was thus obtained from 19 g. barium hexosediphosphate by boiling the latter with a solution of 9.5 g. oxalic acid in 150 cc. water. After purification by repeatedly dissolving it in 10 % alcohol and precipitating the clear solution with alcohol, the product (5.3 g.) was dried at 78° *in vacuo* over phosphorus pentoxide and analysed.

Found Ba = 33.26 %; P = 7.58 %; $[\alpha]_D^{19}$ in 5.2 % aqueous solution = + 0.38°.

Calculated for $C_6H_{11}O_5PO_4BaH_2O$; Ba = 33.22 %; P = 7.51 %.

According to Neuberg the barium salt holds one molecule of water which is not given up at 105° and the above analyses are in agreement with this view.

The specific rotation of the acid, prepared from the solution of the barium salt by adding the calculated amount of oxalic acid was also determined:

$[\alpha]_D^{20}$ in 3.3 % aqueous solution = + 1.49°.

With such small polarimetric readings, however, the possible error in the above values is relatively large. The solution of the acid was boiled with such excess of oxalic acid as brought the total acidity equal to *N*.

The solution rapidly became laevorotatory, as is shown in Table VI.

Table VI.

Time of boiling (hours)	Rotation in 2 dm. tube	Percentage of salt hydrolysed as measured by free phosphate
0	+0.094	0
$\frac{1}{2}$	-0.176	—
$1\frac{1}{2}$	-0.481	—
$4\frac{1}{2}$	-0.822	68

The behaviour of fermentation hexosemonophosphoric acid on hydrolysis is thus very different from that of hexosediphosphoric acid, and of the monophosphoric acid obtained from it by partial hydrolysis, both of which yield laevorotatory products. Table VII gives the results of experiments with each of the above compounds for comparison, the figures for the diphosphoric ester (Nos. 7-9) being those of Young [1909].

Table VII.

Substance	Method of hydrolysis	Percentage of phosphate liberated	Rotation in 4 dm. tube	
			Before hydrolysis	After hydrolysis
1. Hexosemonophosphoric acid from glucose	Solution of acid in <i>N</i> /5 H_2SO_4 at 97° 80 hours	58.2	+2.50°	+1.31°
2. Do. Do.	Solution of acid boiled 27 hours	73	+0.374°	+0.234°
3. Do. from fructose	" " 16 "	53	+1.339°	+0.788°
4. Do. Do.	" " $10\frac{1}{2}$ "	42	+0.816°	+0.532°
5. Barium hexosemonophosphate from glucose	Emulsin; 13 days at 37°	66	+1.680*	+1.339°*
6. Hexosemonophosphoric acid from hexosediphosphoric acid by partial hydrolysis (Neuberg's method)	Solution of acid in <i>N</i> oxalic acid boiled $4\frac{1}{2}$ hours	68	+0.094 ^b	-0.822°
7. Hexosediphosphoric acid from glucose (Young)	Solution of acid boiled 27 hours	91	—	—
8. Do. Do.	" " 14 "	—	+0.161°	-0.658°
9. Do. from fructose (Young)	" " 10 "	—	+0.416°	-1.514°
10. Barium hexosediphosphate	Emulsin; 14 days at 37°	60	(+0.30† calc.) -0°530† (salt not in solution)	

* 2 dm. tube.

† 1 dm. tube.

It would seem improbable therefore that the hexosemonophosphoric acid found in the products of fermentation is formed from hexosediphosphoric acid by hydrolysis, enzymic or otherwise. It may conceivably be an intermediate stage in the formation of the diphosphoric ester and indeed its behaviour with yeast juice and zymine lends some weight to such a view. On the other hand it may be formed by the synthetic power of the yeast enzymes but have no part in the main cycle of reactions which constitutes alcoholic fermentation.

The analytical data so far obtained are all in agreement with the formula assigned to the compound, that of a hexosemonophosphoric ester, but it is admitted that further criteria of purity and homogeneity are desirable before this formula can be considered as established. The formation of an osazone retaining the phosphoric acid radicle indicates that the latter is not attached either to the terminal aldehydic carbon atom or that adjacent to it, but its position in the molecule cannot be definitely stated at present. The nature of the hexose is also undecided. The reducing substance produced on hydrolysis is dextrorotatory but less so than glucose. The ester itself whether produced from glucose or fructose gives Selivanov's reaction for fructose but not so strongly as an equivalent quantity of the latter sugar. These facts and the formation of glucosazone from the products of hydrolysis may perhaps indicate that the compound is a mixture of the monophosphoric esters of glucose and fructose. More cannot be said until further work has been done.

SUMMARY.

1. When fructose or glucose is fermented by yeast juice in presence of suitable amounts of a soluble phosphate, an ester having the composition of a hexosemonophosphoric acid is formed in addition to the hexosediphosphoric acid described by Young, Ivanov and Lebedev.

2. A method is described for the separation of this new phosphoric ester from the other products of the fermentation and for the purification of its barium salt.

3. Hexosemonophosphoric acid is strongly dextrorotatory; $[\alpha]_D^{20} = + 25.0^\circ$ in aqueous solution.

The metallic salts with the exception of the basic salts of the heavy metals, are all readily soluble in water and are amorphous. A crystalline brucine salt has been obtained.

4. The phenylhydrazine salt of the osazone of hexosemonophosphoric acid has been obtained. It is not identical with the compound of the same empirical formula prepared by Young and Lebedev from hexosediphosphoric acid.

5. On hydrolysis by acids or by emulsin, hexosemonophosphoric acid yields free phosphoric acid and a dextrorotatory reducing substance from which glucosazone has been obtained. The rotatory power of this product is, however, less than that of pure glucose.

6. By its specific rotation and its behaviour on hydrolysis the compound is sharply distinguished from the hexosemonophosphoric acid prepared by Neuberg by partial hydrolysis of hexosediphosphoric acid.

7. The alkali salts of hexosemonophosphoric acid are readily fermented by yeast juice and zymine.

8. It is improbable that hexosemonophosphoric acid is produced by the hydrolysis of the diphosphoric ester during fermentation. It may perhaps be an intermediate stage in the formation of the latter compound.

In conclusion I wish to thank Professor A. Harden for the interest which he has taken in this investigation.

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