XL. HEXOSEMONOPHOSPHORIC ESTERS.

By ROBERT ROBISON and EARL JUDSON KING[1].

From the Biochemical Department, Lister Institute, London.

(*Received January 16th, 1931.*)

The hexosemonophosphoric ester isolated from the products of the fermentation of glucose and fructose by yeast juice [Harden and Robison, 1914; Robison, 1922] was considered, from its properties and behaviour on hydrolysis, to be a mixture of isomers, probably the monophosphoric esters of glucose and fructose. The differences observed in the specific rotations of specimens prepared from different fermentation experiments suggested that these isomers occurred in varying proportions.

The presence of aldose and ketose derivatives in this ester was confirmed by Meyerhof and Lohmann [1927], who compared its reducing power as estimated by Bertrand's method with the value found by the iodimetric method of Willstätter and Schudel [1918] and, from the ratio of these values, calculated that a sample of the ester contained 17 % of the fructose derivative. A higher percentage of the ketose component can, however, be deduced from their data if the calculation is based on the Willstätter number, *i.e.* the ratio of total hexose to the iodimetric value. While the reducing power of these esters towards Bertrand's copper solution is always lower than that of the unsubstituted hexose, the reducing power of an aldosephosphate towards hypoiodite should be the same as that of the free aldose, a conclusion supported by Sobotka's [1926] experiments with methylated glucoses and by our present results. On this basis Meyerhof and Lohmann's figures indicate the presence of only 66 % and 68 % aldosemonophosphate in two of their preparations and 57 % in a preparation obtained from this laboratory. The last figure is in agreement with our own value (55 %) for the same preparation (see Table I, No. 1). Lohmann [1928] provided further evidence for the dual nature of the ester by investigating the kinetics of its hydrolysis in *N* HCl. Having shown that the rate of hydrolysis falls considerably after a certain period he made use of this method to remove as much as possible of the more easily hydrolysable ketose ester, thereby raising the proportion of aldosemonophosphate from 68 % to 79 % (based on the Willstätter number) and the $[\alpha]_D$ of the free ester from + 28·5° to + 35·7°. Lohmann concluded that either his residual ester must still contain non-aldose components or the aldehyde oxygen must be linked in two different rings, one of which is not split by hypoiodite.

Similar difficulties have been encountered in our own attempts, continued over several years, to separate the components of hexosemonophosphoric

[1] Research Associate of the Department of Medical Research, University of Toronto.

ester. A knowledge of the constitution of these components was much to be desired but it was first of all essential to isolate them in reasonably pure condition. The methods employed for this purpose have included the fractional crystallisation of the brucine salt, fractional precipitation of normal and basic barium and lead salts and fractional hydrolysis. Two years ago we had obtained a crystalline brucine salt, which, after many recrystallisations, had the appearance of homogeneity. The corresponding barium salt had $[\alpha]_{5461} + 21°$, but the iodimetric reducing power was still 9 % below that required for a pure aldosemonophosphate. Evidence was obtained that this ester was a derivative of *d*-glucose [Robison and King, 1929] and its methylation was commenced in order to determine the position of the phosphoric acid group [King, McLaughlin and Morgan, 1931]. Work on the further purification of the ester was, however, continued and at length yielded a compound whose iodimetric value corresponded exactly with that required for a pure aldosemonophosphate. The preparation and investigation of this ester and of the other components of hexosemonophosphate are here described.

Preparation of the hexosemonophosphoric esters.

The methods now employed for the preparation of the phosphoric esters and for their preliminary separation according to the solubility of their barium salts have been described in another communication [Robison and Morgan, 1930] in which a general account was given of the quantitative examination of the separate fractions. The material for the present investigation of the monophosphoric esters was provided by the soluble-B fractions from the fermentation of glucose or fructose with yeast juice. These fractions were obtained by addition of basic lead acetate to the 10 % alcohol filtrate after removal of the sparingly soluble barium salts, reconversion of the basic lead salt into the barium salt and extraction of the latter with 10 % alcohol. The soluble-B fractions were in some cases converted directly into the brucine salt, but usually a further purification was first carried out by dissolving them in 10 % alcohol and treating the solution with mercuric acetate as previously described [Robison, 1922]. The monophosphate was reprecipitated as the basic lead salt, which was converted back into the barium salt and purified by solution in 10 % alcohol and reprecipitation with alcohol.

Isolation of the aldosemonophosphoric ester.

Crystallisation of the brucine salt. The barium salt was dissolved in 5 times its weight of water and treated with the amount of $5N$ H_2SO_4 required to precipitate the whole of the barium. The barium sulphate was removed by centrifuging and washed once with water and 3 times with 96 % alcohol. The acid solution was treated with a slight excess of brucine (2·2 g. for each cc. $5N$ H_2SO_4), rather less than half of this amount being added in solid form before centrifuging, the remainder, dissolved in hot 50 % alcohol, after the

final washing was completed. Crystallisation of the brucine salt usually commenced immediately, but the solution was allowed to remain at 0° for at least a week before filtration. The salt was recrystallised by dissolving it in 7 or 8 times its weight of 20 % ethyl alcohol at 40° and allowing the filtered solution to remain at 0°. Two types of crystals were obtained, the first fractions consisting of hard, translucent needles, while later fractions, which separated on allowing the mother-liquors to evaporate spontaneously, consisted partly of needles and partly of hexagonal plates. The separate fractions were repeatedly recrystallised from 20 % alcohol and portions of each were then converted into barium salts by dissolving in 20 % alcohol, adding a solution of barium acetate in slight excess and precipitating the barium hexosemonophosphate with absolute alcohol. The salts were purified several times by solution in water and reprecipitation with absolute alcohol. Analyses showed that the barium salts obtained from the needle fractions contained an increased proportion of aldosemonophosphate while those obtained from the hexagonal plates contained less than 50 % of the aldose component. It was noticed that on recrystallisation of the platelets some clusters of needles were usually obtained, and it was concluded that these two types of crystals represented mixed brucine salts of the aldose- and ketose-monophosphates in different proportions. One of the needle fractions obtained after exhaustive fractionation gave a barium salt, which, by iodimetric estimation, contained 91 % of aldose ester, but, in general, recrystallisation from 20 % ethyl alcohol failed to yield so pure a product. A brucine salt recrystallised 4 times from this solvent, the solution being left at 0° for at least a week at each recrystallisation in order to obtain the maximum crop of crystals, contained only 79 % of the aldose component. The next stage in the purification was effected by recrystallisation of this brucine salt from boiling 90 % or 95 % methyl alcohol, from which it slowly separated, at room temperature or at 0°, in large clumps of soft, opaque, silky needles. After two further recrystallisations a portion of the salt was converted to barium salt, the analysis of which showed that the proportion of aldose ester had increased from 79 % to 90 %. The specific rotation was, however, somewhat lower than that of the 91 % salt mentioned above. The final stage in the purification of the aldosemonophosphate was achieved by recrystallisation from boiling absolute methyl alcohol in which the salt is only sparingly soluble. A small amount of sparingly soluble residue was filtered off and a small fraction of hard, minute crystals which separated rapidly on cooling to room temperature was also removed. The clear solution was then left in a closed vessel at 0° for some days, during which almost the whole of the remaining brucine salt crystallised out. These operations were repeated twice but in the last recrystallisation the crystals were removed by filtration after 24 hours. This fraction and the salt remaining in the mother-liquors were converted into the barium salts which were purified as already described and analysed. The iodimetric value of the salt prepared from the crystals was found to be equal to that required for 100 % aldosemonophos-

phate, while the barium salt prepared from the mother-liquors was of almost equal purity.

Fractional hydrolysis. Lohmann's method of separation of the esters by fractional hydrolysis was also tried but, as in his experiments, with only partial success. For this attempt we used a sample of hexosemonophosphate that had already been purified by recrystallisation of the brucine salt from 20 % ethyl alcohol and contained 80 % of the aldose derivative. The barium salt was hydrolysed with *N* H_2SO_4 for 6 hours at 100°, 14 % of the phosphate being set free. The residual monophosphate now contained 84 % of the aldose ester. After a further 6 hours in *N* H_2SO_4 at 100° the proportion of aldose ester had increased to 88 % and rose to 91 % after a third hydrolysis of similar duration. During the 18 hours 35 % of the original ester had been hydrolysed.

Other components of hexosemonophosphoric ester.

The dual nature of the platelet fractions obtained during the crystallisation of the brucine salt from 20 % ethyl alcohol was confirmed by submitting the barium salts derived from these fractions to bromine oxidation. The salts, dissolved in water, were treated with bromine and barium carbonate at room temperature. The flask was shaken at intervals during 24 hours and the excess of bromine then removed by aeration. The filtered solution was made acid to methyl orange and treated with 4 times its volume of alcohol by which the acid salt of the oxidation product (phosphohexonic acid) was precipitated. The filtrate was neutralised to phenolphthalein with baryta and the precipitated barium hexosemonophosphate filtered off, purified by solution in 10 % alcohol and reprecipitation, and analysed. After a second treatment with bromine the purified barium salt had a very low iodimetric value and specific rotation, and was very similar in most respects to Neuberg's fructosemonophosphate. For the purposes of comparison a specimen of the latter ester was prepared by partial hydrolysis of hexosediphosphate which had been very carefully purified by precipitation of the barium salt from its aqueous solution by heating the latter to 75°. This purification was very essential since the diphosphate is likely to be contaminated with small amounts of aldosemonophosphate carried down as the double barium salt [*v.* Robison and Morgan, 1930]. Such aldosemonophosphate is not appreciably hydrolysed during the short treatment with acid and therefore becomes concentrated in the fructosemonophosphate produced. The chief differences between the ester derived from the platelet fraction and the Neuberg ester lay in the lower Hagedorn and Jensen reducing power of the former, which may indicate that it was contaminated with the unknown ester referred to below.

The final mother-liquors from the crystallisation of the brucine salts in 20 % alcohol still contained a considerable amount of phosphoric ester, very soluble in this solvent and even in a much higher concentration of alcohol. The analyses of the barium salts prepared from these mother-liquors indicated the presence of some unknown ester since the analytical data could not be

reconciled with any possible mixture of the four known esters. The specific rotations of these salts were higher than that of the aldosemonophosphate, but this might be due to the presence of a very small proportion of trehalosemonophosphate. Whether these salts contain the new ester in small or large proportion cannot be decided until it has been isolated and its properties investigated. In spite of much work this has not yet been achieved. A comparison of the Hagedorn and Jensen values found for the original hexosemonophosphate (*cf.* Table I, salts 1–4) with those of the pure aldose and ketose esters suggests, however, that the unknown ester is present in significant amounts.

Analyses of the various barium salts obtained in these operations and referred to in the above paragraphs are shown in Table I. One of the original specimens of hexosemonophosphate prepared 10 years ago from glucose [Robison, 1922, Table II, No. 7], has also been analysed by the micro-methods now employed and the results are included in Table I. Details of these methods have already been given, but some notes may be added here. The estimations were carried out on the substance dried over sulphuric acid, the moisture being separately estimated in Pregl's micro-drying apparatus. The results are calculated for the anhydrous salt. The values quoted for "fructose" were found by estimating the colour developed with the Selivanoff reagent and do not represent the actual percentage of this hexose in the sample. Thus, the value for barium fructosemonophosphate is only half the percentage of hexose in the salt.

The estimation of reducing power by the Hagedorn and Jensen method was carried out with addition of 0·5 cc. *N*/2 sodium hydroxide to each tube. Without this addition the curve of reducing power for these esters deviates so widely from the curve determined for glucose that very inconsistent values are obtained by reference to the glucose table. Even with this extra alkali the percentage reducing power is found to vary a little according to the amount of ester taken. For both glucose and fructosemonophosphates the values lie between 35 % and 37 %, that is, the reducing power is about 80 % of that of the unsubstituted hexose.

In the iodimetric estimations, carried out by the method of Macleod and Robison [1929], it was found that the aldosemonophosphate was even more sensitive than glucose to various conditions, particularly to the concentration of alkali. Low results were obtained unless the iodine was in very large excess or if the amount of 5 % sodium carbonate solution was increased from 0·2 cc. to 0·4 cc., an amount shown to be permissible in the estimation of glucose.

The specific rotation of barium aldosemonophosphate varies slightly with the concentration of the salt and still more with changes in p_H of the solution. Thus, the value of $[\alpha]_{5461}$ for salt No. 11 in 0·5 % solution (p_H *circ.* 8·3) was + 19·6°, at p_H 9·8 it was + 18·0°, while at p_H 5·8 it was + 23·3°. The free acid corresponding to this salt had $[\alpha]_{5461}$ + 40·0°, but in 0·07 *N* H_2SO_4 this

became + 43·6°. These differences are possibly due to changes in the equilibrium between the un-ionised ester and its ions.

Table I. *Analyses of barium salts of hexosemonophosphoric acids obtained from the products of fermentation by yeast juice.*

No. of salt	Source of the barium salt	P %	Fructose (Seliwanoff) %	Reducing power as glucose (%) H. and J.	Reducing power as glucose (%) Iodine	Aldose-monophosphate %	$[\alpha]^{20°}_{5461}$	$[\alpha]^{20°}_{5461}$ of free acid
1	"Hexosemonophosphate" [Robison, 1922]	7·85	6	30·0	25·2	55	+14·4°	+29·5°
2	Soluble-B fraction	7·37	9	27·4	21·1	46	+11·3°	—
3	„	7·45	—	30·8*	27·7	61	+15·0°	—
4	Soluble-B after further purification by mercuric acetate and basic lead precipitation	7·90	5	31·9	23·6	52	+16·3°	—
5	Brucine salt after repeated fractional crystallisation from 20 % ethyl alcohol. Hard, translucent needles	7·80	—	31·8*	41·4	91	+21·0°	—
6	Brucine salt recrystallised 4 times from 20 % ethyl alcohol. Hard needles	7·84	0·5	34·9	36·1	79	+17·9°	—
7	Brucine salt recrystallised 4 times from 20 % ethyl alcohol, 3 times from 90 % methyl alcohol. Opaque, silky needles	7·86	0·5	36·1	41·0	90	+19·2°	—
8	Brucine salt recrystallised twice from 20 % ethyl alcohol, 4 times from 95 % methyl alcohol, 3 times from absolute methyl alcohol. Opaque, silky needles	**7·86**	**0·5**	**35·5**	**45·7**	**100**	**+20·6°** (c=0·84 %) +21·2° (c=8·4 %)	**+41·4°** (c=0·74 %)
9	Mother-liquors in final recrystallisation of above brucine salt	7·87	0·5	35·3	44·9	99	+20·1° (c=1·4 %)	—
10	Brucine salt recrystallised from 20 % ethyl alcohol	7·90	2·4	35·1	36·6	80	+16·2°	—
11	Residual salt after 18 hrs. hydrolysis of No. 10, with N H_2SO_4 at 100°	7·90	? 0	34·8	41·4	91	+19·6°	+40·0°
12	Brucine salt fractionally crystallised from 20 % ethyl alcohol. Hexagonal plates	7·76	—	25·4	18·9	41	+ 8·2°	—
13	Residual salt after oxidation of No. 12 with bromine and removal of phosphohexonate	7·75	22	27·6	2·6	6	+ 2·1°	—
14	Residual salt after oxidation of another similar fraction	7·60	20	29·0	1·3	3	+ 3·3°	—
15	Fructosemonophosphate (Neuberg) prepared from highly purified hexosediphosphate	7·80	22	36·2	3·0	7	+ 0·7°	—
16	Final mother-liquors in the recrystallisation of brucine salts	7·05 6·90	6·8 5·7	25·3 25·0	16·7 21·1	37 46	+21·4° +22·2°	— —

$C_6H_{11}O_5PO_4Ba$ requires P = 7·85 %; $C_6H_{12}O_6$ = 45·6 %.

* Determined without addition of NaOH.

Osazones of hexosemonophosphoric esters.

The osazone of hexosemonophosphoric ester was originally described [Robison, 1922] as melting at 139°, and it was concluded that this osazone was not identical with that prepared by Young [1911] from Harden and Young's fructosediphosphoric ester. Should the aldose ester prove to be a derivative of glucose, or of mannose, the non-identity of the osazones would show that the phosphoric acid group is not in the same position in the two esters. The demonstration of the composite nature of the original hexosemonophosphate had, however, thrown doubt on the purity of the osazone

prepared from it and a re-examination of this point was, therefore, necessary. Osazones were prepared from the pure aldosemonophosphate (No. 8, Table I) and from the Neuberg fructosemonophosphate (No. 15) prepared from hexosediphosphate. Solutions of the free acids, prepared from the barium salts, were treated with as much phenylhydrazine as they would dissolve before adding the calculated amount of the base in acetic acid. The solutions were heated in a boiling water-bath for short periods (½ hour), cooled to 0°, filtered and again heated. The osazones were recrystallised by dissolving in boiling alcohol and adding chloroform to the filtered solution. For the determination of the melting points a short thermometer, with the mercury thread completely immersed in the bath, was used and the melting points are, on this account, higher than that given by Young (151–152°). The tube containing the osazone was introduced when the temperature of the bath was 4° below the melting point and was rising at the rate of 6–8° per minute. The osazones from the aldosemonophosphate and from fructosemonophosphate both melted with decomposition at 154–154·5° and an intimate mixture of the two melted at the same temperature. The yield of osazone obtained from the pure aldosemonophosphate was equal to 48 % of the weight theoretically possible. More than half of this yield was obtained from the first period of heating and the melting point of this sample before recrystallisation was identical with that of the recrystallised osazone. The successive products melted within one or two degrees of this temperature.

Analyses. The P content corresponded with that of a phenylhydrazine salt of the osazone of hexosemonophosphoric acid.

Osazone from aldosemonophosphate gave P = 5·63 %.

,, fructosemonophosphate (Neuberg) gave P = 5·61 %.

Calculated for $C_{24}H_{31}O_7N_6P$ = 5·68 %.

The specific rotation of the osazone from aldosemonophosphate was determined in pyridine-alcohol (2:3).

$[\alpha]^{21°}_{5461}$ − 60° after 15 min., − 38° after 85 min., − 35° after 24 hrs.

Neuberg and Reinfurth [1924] give $[\alpha]_D$ − 51° after 15 minutes and − 36° after 1 hour (equilibrium) for the phenylhydrazine salt of the osazone of fructosemonophosphate and also for that obtained from hexosediphosphate.

Further evidence was obtained of the identity of the two osazones by measuring the rate of hydrolysis in *N* H_2SO_4 at 100°. The results are shown below.

	Aldosemonophosphoric ester	Fructosemonophosphoric ester %	Osazone of aldosemonophosphoric ester %	Osazone of fructosemonophosphoric ester %
Hydrolysis in 1 hour	—	—	47	44
Hydrolysis in 2 hours	0	41	80	75

The two osazones were hydrolysed at approximately equal rates, which were twice as great as that for fructosemonophosphoric ester. The hydrolysis

of aldosemonophosphoric ester was too small to be estimated with the quantities taken.

An osazone was also prepared from the ketose component of the platelet fraction (No. 14) and this also, after recrystallisation, melted at 154° and at the same temperature when mixed with the osazone of fructosemonophosphoric acid.

Hydrolysis of aldosemonophosphoric ester.

Hydrolysis by acids. The hydrolysis of aldosemonophosphoric ester was studied by heating a 0·03 *M* solution of the free acid at 100° and also by heating the ester, in similar concentration, with 0·1 *N* and with *N* H_2SO_4 at 100°. The results of two experiments are shown in Fig. 1, the actual rates

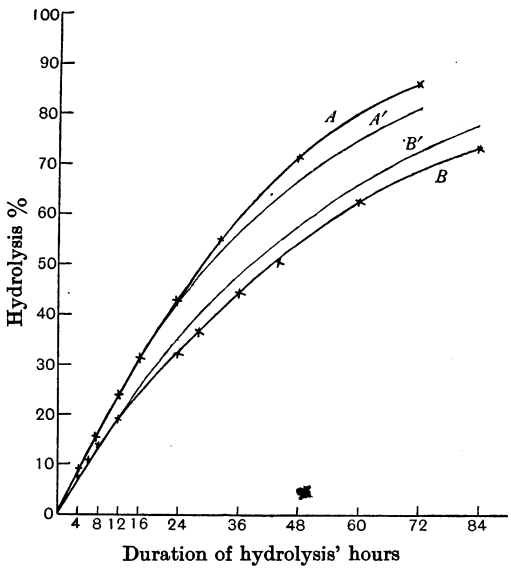

Fig. 1. *A*. Autolysis of 0·03 *M* aldosemonophosphoric ester at 100°.
A'. $k = 0{\cdot}167 \times 10^{-3}$. *B'*. $k = 0{\cdot}13 \times 10^{-3}$.
B. Hydrolysis of 0·03 *M* aldosemonophosphoric ester in *N* H_2SO_4 at 100°.

of hydrolysis being shown by the thick lines while the thin lines are theoretical curves of unimolecular equations drawn for the velocity constants calculated from the results of the first 16 hours' hydrolysis ($k = 1/t \log a/(a - x)$, t being time in minutes). No sharp deviation from these theoretical curves is shown in either case, but a gradual, though slight, falling off in the rate is shown for the hydrolysis in *N* H_2SO_4, while a slight increase is shown in the rate of hydrolysis of the free ester alone. The results appear to support the view that we are dealing with a homogeneous ester. It was very surprising, however, to find that the hydrolysis proceeded more rapidly in the solution containing aldosemonophosphoric acid alone than in presence of *N* H_2SO_4, but this fact was confirmed by a repetition of both experiments. Further, with 0·1 *N* H_2SO_4

the rate of hydrolysis was very slightly higher (66 % in 60 hours) than with *N* acid (63 % in 60 hours). Is it possible that these results are due to the presence of two ring systems, one of which, favoured by the presence of sulphuric acid, hinders the removal of the phosphoric acid group? The increased specific rotation of the free ester in presence of sulphuric acid might possibly be connected with the formation of this ring and the sensitiveness of the ester towards alkali, noted in reference to the iodimetric estimation, may also be related to such an equilibrium (*cf.* Lohmann's [1928] suggestion quoted on p. 323 of this paper).

The sugars produced from aldosemonophosphoric ester by hydrolysis with 0·1 *N* H_2SO_4 and heating the solution of the free acid alone were isolated and examined. The solutions were neutralised with baryta and treated with 4 times their volume of alcohol. The precipitated barium salts were removed by filtration and thoroughly washed with 80 % alcohol. The filtrate was evaporated on the water-bath to a small bulk and then in an evacuated desiccator over sulphuric acid. The results of the examination of the products are shown in Table II. The properties of the sugar obtained from hydrolysis with 0·1 *N* H_2SO_4 corresponded very closely with those of glucose. That obtained from the partial hydrolysis of the ester without H_2SO_4 had a somewhat lower specific rotation and was less pure. A control experiment was carried out by heating a solution of glucose for 60 hours with 0·1 *N* H_2SO_4 and then recovering the sugar in the manner described above; it is seen from the results that very little decomposition or alteration of the glucose occurred.

Hydrolysis by bone phosphatase. Two experiments were carried out on the hydrolysis of the aldosemonophosphoric ester by the bone phosphatase. 0·4 g. of the pure barium salt was converted into the free acid and the solution (15 cc.) treated with sodium hydroxide to bring the p_H to 7·0. A purified preparation of bone phosphatase (50 mg.) was added and the solution kept at room temperature in presence of chloroform, the p_H being checked and the liberated inorganic phosphate estimated at intervals. Hydrolysis was practically complete in 4 days. The solution was evaporated to dryness *in vacuo* over sulphuric acid and the residue was extracted repeatedly with hot 80 % ethyl alcohol. The filtrate was evaporated, first on the water-bath and finally in an evacuated desiccator and the residue dissolved in 30 cc. water for examination. The hydrolysis was carried out in neutral solution and at room temperature in order to minimise the risk of the Lobry de Bruyn transformation taking place. The slower rate of hydrolysis at room temperature is compensated for by the fact that no destruction of enzyme occurs (as it does at 37°) so that hydrolysis will continue smoothly even during several weeks. In spite of these mild conditions it was evident that the sugar product contained a considerable proportion of ketose. The analytical results and the preparation of an osazone, which was identified by its melting point (206°) with glucosazone, justified the conclusion that the product contained glucose and fructose. Whether mannose was also present is uncertain, but an attempt to obtain the

phenylhydrazone was not successful. This transformation of the sugar component of monophosphoric ester on hydrolysis with phosphatase is in agreement with the results found by Martland and Robison [1929] for the enzymic hydrolysis of hexosediphosphoric ester. Control experiments carried out in a similar manner with bone phosphatase + distilled water, and with phosphatase + 0·5 % glucose solution showed that only a very small amount of reducing and optically active material could be derived from the phosphatase and that glucose itself suffered no transformation under the conditions of the hydrolysis.

Table II.

Method of hydrolysis	Duration of hydrolysis hrs.	Hydrolysis %	Wt. of hexose equivalent to P liberated mg.	Wt. of syrup dried over H_2SO_4 mg.	Reducing power as glucose (mg.) H. and J.	Reducing power as glucose (mg.) Iodine	Fructose (Selivanoff) mg.	$[\alpha]_{5461}^{20°}$ calculated on the H. and J. value
1. 0·1 *N* H_2SO_4 at 100°	60	66	86	83	62	64	6	+60·5°
2. Free acid alone at 100°	44	73	92	118	88	92	7	+54°
3. Bone phosphatase at room temperature and p_H 7·0	96	99	144	155	133	116	24	+33°
4. Bone phosphatase at room temperature and p_H 7·0 (Results of 3 and 4 corrected for phosphatase blank)	80	97	82	—	82	67	13	+29°
Controls:			Wt. of glucose taken					
20 cc. 0·5 % glucose solution + 80 cc. alcohol evaporated to dryness and extracted with 80 % alcohol			100	100	96	97	1	+63°
27 cc. 0·5 % glucose solution heated with 0·1 *N* H_2SO_4 60 hrs.; neutralised and treated as above			135	155	125	133	6	+62°
30 cc. 0·5 % glucose solution + 50 mg. bone phosphatase evaporated to dryness in desiccator and extracted with 80 % alcohol. (Results corrected for phosphatase blank)			150	160	143	147	2	+63°
30 cc. H_2O + 50 mg. bone phosphatase evaporated to dryness in desiccator and extracted with 80 % alcohol			0	5	0·4	1·3	0	α_{5461} −0·04° (l = 4 dm.)

Preparation of a phosphohexonic acid from hexosemonophosphoric ester.

A preparation of hexosemonophosphate, which had been purified by recrystallisation of the brucine salt from 20 % ethyl alcohol and contained about 80 % of the aldose ester, was oxidised with bromine in order to convert this ester into the corresponding phosphohexonic acid. To 10 g. of the barium salt, dissolved in 80 cc. water, 1·4 cc. bromine and 12 g. barium carbonate were added, a further 1·4 cc. bromine being added after 24 hours. The solution was kept at room temperature and shaken at intervals. Iodimetric estimations carried out on 0·2 cc. portions, after removal of the bromine, indicated that oxidation was complete after 48 hours. The excess of bromine was removed by aeration and the barium carbonate by filtration. The filtrate (p_H 3·5) was poured into 5 times its volume of alcohol upon which the acid salt of the phosphohexonate was precipitated. This was filtered off, washed thoroughly with 85 % alcohol and dried. It was then dissolved in 20 cc. water with the aid of 2 cc. *N* HCl and, after filtration, treated with a solution of barium

hydroxide till pink to phenolphthalein. The precipitate was filtered off, washed with water and absolute alcohol and dried over sulphuric acid *in vacuo*. It was non-reducing and gave a negative Selivanoff reaction.

P found 6·42 %; calculated for $(C_6H_{10}O_{10}P)_2Ba_3$, P = 6·48 %.

$[\alpha]^{21°}_{5461} = -1·5°$.

Solubility in water at 100°. 100 cc. solution contained 0·71 g.

The acid barium salt was prepared by dissolving 1 g. of the neutral salt in sufficient dilute hydrochloric acid to make the solution just acid to methyl orange and adding 5 times the volume of alcohol. The precipitate was filtered off, washed thoroughly with 85 % and absolute alcohol and dried *in vacuo*.

P found 7·40 %; calculated for $C_6H_{11}O_{10}PBa$, P = 7·54 %.

$[\alpha]^{21°}_{5461} = +0·2°$.

A solution of the free phosphohexonic acid was prepared by decomposing 0·25 g. of the barium salt with the exact amount of sulphuric acid and removing the barium sulphate by centrifuging. The change in rotation due to lactone formation is shown below, the times being measured from the addition of the sulphuric acid. After heating to 70° the solution was rapidly cooled.

Time	$\alpha^{21°}_{5461}$ (l=4 dm.)	$[\alpha]^{21°}_{5461}$
10 minutes at 21°	0	0
2 hours at 21°	+0·03°	+ 2°
18 hours at 21°	+0·08°	+ 5°
1 hour at 70°	+0·26°	+16°
2 hours at 70°	+0·285°	+18°

The solution was finally heated for an hour at 70° with 0·1 *N* HCl and cooled rapidly when the $[\alpha]_{5461}$ was + 21°. The yield of phosphohexonate was equivalent to 62 % of the original hexosephosphate, but a further quantity of less pure salt was recovered from the first acid-alcohol filtrate together with a small amount of fructosemonophosphate.

Preparation of hexonic acid by hydrolysis of the phosphohexonic acid with bone phosphatase.

The hydrolysis of the phosphohexonate was carried out by means of bone phosphatase in two separate experiments.

1st method. 1 g. of the neutral barium salt in 20 cc. water was treated with 0·5 g. of purified phosphatase at 37° and p_H 8·6–8·8. The flask was shaken at frequent intervals and barium hydroxide solution added as required to maintain the stated p_H. Hydrolysis was complete in about 9 hours and the solution was then heated at 100° and filtered. The residue was well washed with hot water and the filtrate and washings were concentrated on the water-bath. The barium hexonate was converted into the calcium salt by treatment with a solution of calcium sulphate. The filtered solution was again concentrated and poured into absolute alcohol to precipitate the salt, which was then filtered off, washed with alcohol and dried *in vacuo*. The salt was free from phosphate but estimation of the calcium indicated that it contained a little calcium sulphate which could not be easily removed.

2nd method. 1·35 g. barium phosphohexonate was treated with the exact quantity of sulphuric acid required to precipitate the barium and the sulphate removed by centrifuging and thoroughly washed. The acid solution was heated with pure calcium carbonate, filtered and treated with sufficient calcium hydroxide to raise the p_H to 6·0. (The addition of more calcium hydroxide caused the salt to be precipitated.) After cooling the solution was treated with 0·5 g. of purified bone phosphatase and a few drops of chloroform and was left in a stoppered flask at room temperature. Each day the p_H was noted but required no adjustment and the progress of the hydrolysis was determined by estimating the inorganic phosphate in 0·2 cc. Hydrolysis was complete in 4 days, after which the solution was heated on the water-bath and treated with calcium hydroxide until pink to phenolphthalein. It was filtered and the residue washed with boiling water. The filtrate was concentrated on the water-bath and poured into absolute alcohol; the precipitate was filtered, washed with alcohol, dried, and purified by solution in water and reprecipitation with alcohol. The salt was free from phosphorus and did not reduce hypoiodite. By the Hagedorn and Jensen method a very slight reduction (1 %) was found.

Ca found 9·4 %; calculated for $(C_6H_{11}O_7)_2Ca$, 9·30 %.
$[\alpha]^{20°}_{5461}$ + 6·8° (l = 4 dm., c = 0·8 %).

The recorded values for the $[\alpha]_D$ of calcium gluconate vary between + 6° and + 10·5°.

A solution of the free acid was produced by treating the calcium salt with the calculated quantity of oxalic acid and filtering. The change in rotation due to lactone formation is shown below.

Time	$\alpha^{20°}_{5461}$ (l=4 dm.)	$[\alpha]^{20°}_{5461}$
20 minutes at room temperature	−0·08°	− 2·9°
18 hours at room temperature	+0·22°	+ 8·6°
2 hours at 70°	+0·54°	+21·0°
3 hours at 70°	+0·59°	+22·9°
In 0·1 *N* HCl 2 hours at 70°	+0·71°	+27·8°
3 hours at 70°	+0·71°	+27·8°
66 hours at room temperature	+0·52°	+20·4°

The results agree with the recorded values for *d*-gluconic acid if the factor 1·18 is used to convert $[\alpha]_D$ into $[\alpha]_{5461}$. Rehorst [1928] gives the following values for $[\alpha]^{20°}_D$ in 2·8 % aqueous solution: after 5 mins. − 6·72°; after 20 mins. − 2·75°; after 24 hrs. + 7·02°; after 5 days + 11·9°. In Tollen's "Handbuch der Kohlenhydrate" (3rd edition) the equilibrium mixture obtained on heating the free acid is stated to have $[\alpha]_D$ + 23·4°, which, multiplied by 1·18 gives $[\alpha]_{5461}$ + 27·6°.

Oxidation of aldosemonophosphoric acid with nitric acid.

It was considered that if the phosphoric acid group is in position 3 of the aldose molecule, or in any position other than 6, it should be possible to obtain

a phosphodicarboxylic acid by oxidation of the ester itself or of the phosphohexonate with nitric acid. Accordingly, a number of attempts to prepare such a compound were made by treating the pure aldosemonophosphate and phosphohexonate described above with nitric acid of different concentrations, at different temperatures, and for various periods of time. The phosphohexonate manifested a considerable resistance to further oxidation under mild conditions and when oxidation did occur it was invariably accompanied by hydrolysis. The products obtained from this and from the aldose ester were always mixtures containing dicarboxylic acids, but in no case could the analytical results be interpreted as providing evidence of the presence of a dicarboxylic acid in which the phosphoric group was still present.

Discussion.

The results we have described lead us to conclude that one, and possibly the chief, constituent of hexosemonophosphoric ester of fermentation has been isolated in pure homogeneous condition and that this compound has the constitution of an aldosemonophosphoric ester. The properties of the sugar obtained from this ester by acid hydrolysis as well as those of the hexonic acid formed from it by oxidation with bromine followed by enzymic removal of the phosphate group, provide evidence of the identity of the aldose with *d*-glucose. Hydrolysis of the ester by phosphatase has been shown to involve partial transformation of the hexose as already demonstrated for the hydrolysis of fructosediphosphate. This transformation is in itself of interest but the properties of the sugar produced obviously cannot be accepted as proving the nature of the aldose present in the ester.

For the position of the phosphoric acid group in this ester 2 is excluded by the formation of a phosphohexosazone, while the formation of both stable and unstable methylhexosides [King and Morgan, 1929; King, McLaughlin and Morgan, 1931] very probably excludes positions 4 and 5. Position 6 appeared at first to be excluded by the differences between the melting points of the osazone originally obtained from hexosemonophosphoric ester and of the osazone prepared by Young from fructosediphosphoric ester and later by Neuberg and Reinfurth [1924] from fructosemonophosphoric ester. This difficulty has disappeared since the osazone now prepared from the pure aldosemonophosphate has been found to have the same melting point and rate of hydrolysis as the osazone of fructosemonophosphoric ester in which the phosphoric acid group is considered to occupy position 6.

Our failure to obtain a phosphodicarboxylic acid by oxidation of the aldosemonophosphoric ester with nitric acid further supports the view that the ester is a glucose-6-phosphate. The properties of the pure ester do not, however, entirely agree with those of any of the synthetic glucosephosphates which have been prepared by a number of workers [Komatsu and Nodzu, 1924; Nodzu, 1926; Raymond and Levene, 1929; Josephson and Proffe, 1930; Levene and Raymond, 1930]. In their last paper Levene and Raymond [1930]

report a new examination of the ester synthesised from di-*iso*propylideneglucose and conclude, in agreement with Josephson and Proffe, that this ester is glucose-3-phosphate, and that it differs from the hexosemonophosphate of fermentation in its specific rotation and its reaction with phenylhydrazine, with which it forms a 3:6-anhydrohexosazone. They also find that its rate of fermentation is much slower than that of the natural ester. Levene and Raymond have further synthesised a phosphoric ester from *iso*propylideneglucose which they consider to be glucose-6-phosphate and have shown that it gives a phosphohexosazone identical with that obtained from the Harden and Young and Neuberg esters, and that its rate of fermentation is identical with that of hexosemonophosphate. The specific rotations given for this synthetic ester and its barium salt do not, however, agree with those of the pure aldosemonophosphoric ester described in the present communication.

By multiplying Levene and Raymond's values for $[\alpha]_D$ of their synthetic esters by the factor 1·18 the following values for $[\alpha]_{5461}$ are obtained for comparison with those of the aldose ester here described:

	$[\alpha]_{5461}$ of barium salt	$[\alpha]_{5461}$ of free acid
Synthetic ester from di-*iso*propylideneglucose (glucose-3-phosphate)	+31·3°	+46·6°
Synthetic ester from *iso*propylideneglucose (glucose-6-phosphate)	+15·3°	+30·6°
Pure aldosemonophosphoric ester	+21·2°	+41·4°

Although Levene and Raymond state that the ester prepared from monoacetone glucose may not have been quite pure, the differences in the specific rotations are sufficiently great to cause hesitation in concluding that this synthetic ester is identical with the aldosemonophosphate of fermentation.

Apart from this discrepancy all the evidence at present available goes to show that the natural ester whose isolation and properties we have described is glucose-6-phosphate. From the iodimetric estimations it would appear that this ester cannot normally form more than about 50–70 % of the hexosemonophosphate isolated from the products of fermentation, while it may well be that part of this percentage represents other esters, *e.g.* a disaccharide-diphosphate, which would account for the low Hagedorn and Jensen reducing power of some of the other fractions isolated during the investigation of the mixed hexosemonophosphate. That the latter contains also the Neuberg fructosemonophosphate seems to be certain, but whether this ester accounts for the whole of the non-aldose fraction, as estimated iodimetrically, will not be settled until the properties of the unknown ester are determined.

Trehalosemonophosphoric ester has not been isolated from these yeast juice products, although the high specific rotation of certain small fractions obtained in the crystallisation of the brucine salt might be accounted for by its presence. Monophosphoric esters (not yet obtained in pure condition) of higher specific rotation than that of aldosemonophosphate have been described by Euler, Myrbäck and Runehjelm [1928] and by I. S. Neuberg

and Ostendorf [1930] (from the products of fermentation of mannose by fresh yeast and toluene), but in the case of these compounds also the presence of trehalosemonophosphate in small proportion might possibly account for the high specific rotation recorded by the authors. It does not, however, necessarily follow that this is the true explanation and further information with regard to these esters will be awaited with interest.

Summary.

1. An aldosemonophosphoric ester has been isolated in pure condition from the hexosemonophosphoric ester produced by fermentation of hexoses with yeast juice.

2. The $[\alpha]^{20°}_{5461}$ of the free ester is + 41·4°, and of the barium salt + 21·2° ($c = 8{\cdot}4$ %). The reducing power of the ester by an iodimetric method is equal to that of the equivalent amount of glucose. Its reducing power by the Hagedorn and Jensen method is 80 % of that of glucose. The Selivanoff reaction is not appreciably greater than that given by glucose.

3. The phenylhydrazine salt of the osazone of aldosemonophosphoric ester has been prepared and shown to be identical both by its melting point and its rate of hydrolysis with the corresponding salt of the osazone of fructosemonophosphoric ester; M.P. 154–154·5°.

4. The ester is very resistant to hydrolysis by acids at 100°. Hydrolysis proceeds more rapidly when the free aldosemonophosphoric acid is heated alone than in presence of sulphuric acid. The sugar product of acid hydrolysis has the character of *d*-glucose.

5. Hydrolysis by bone phosphatase proceeds rapidly at room temperature and p_H 7·0, but the sugar product contains both glucose and fructose.

6. Oxidation of the aldosemonophosphoric ester with bromine yields a phosphohexonic acid from which gluconic acid has been obtained by hydrolysis with bone phosphatase.

7. Oxidation with nitric acid failed to yield any evidence of the production of a dicarboxylic acid containing a phosphoric acid group.

8. These experiments in conjunction with those reported by King, McLaughlin and Morgan point to the constitution of the ester being that of a glucose-6-phosphate.

9. The specific rotation of the free ester and of its barium salt differ considerably from those of the synthetic ester prepared by Levene and Raymond from *iso*propylideneglucose, and considered by them to be glucose-6-phosphate.

10. A ketosemonophosphoric ester similar in most respects to Neuberg's fructosemonophosphoric ester has also been isolated from the fermentation products, while evidence has been obtained of the presence of another ester as yet unidentified.

REFERENCES.

Euler, Myrbäck and Runehjelm (1928). *Arkiv Kemi Min. Geol.* **9**, 1.
Harden and Robison (1914). *Proc. Chem. Soc.* **30**, 16.
Josephson and Proffe (1930). *Liebig's Ann.* **481**, 91.
King, McLaughlin and Morgan (1931). *Biochem. J.* **25**, 310.
—— and Morgan (1929). *Chem. Ind.* **48**, 143.
Komatsu and Nodzu (1924). *Mem. Coll. Sci., Kyoto*, **7**, 377.
Levene and Raymond (1930). *J. Biol. Chem.* **89**, 479.
Lohmann (1928). *Biochem. Z.* **194**, 306.
Macleod and Robison (1929). *Biochem. J.* **23**, 517.
Martland and Robison (1929). *Biochem. J.* **23**, 237.
Meyerhof and Lohmann (1927). *Biochem. Z.* **185**, 113.
Neuberg, I. S. and Ostendorf (1930). *Biochem. Z.* **221**, 154.
Neuberg and Reinfurth (1924). *Biochem. Z.* **146**, 589.
Nodzu (1926). *J. Biochem.* (*Japan*), **6**, 31.
Raymond and Levene (1929). *J. Biol. Chem.* **83**, 619.
Rehorst (1928). *Ber. deutsch. chem. Ges.* **61**, 163.
Robison (1922). *Biochem. J.* **16**, 809.
—— and King (1929). *Chem. Ind.* **48**, 143.
—— and Morgan (1930). *Biochem. J.* **24**, 119.
Sobotka (1926). *J. Biol. Chem.* **69**, 267.
Willstätter and Schudel (1918). *Ber. deutsch. chem. Ges.* **51**, 780.
Young (1911). *Biochem. Z.* **32**, 178.