CXVI. THE HYDROLYSIS OF HEXOSEDIPHOSPHORIC ESTER BY BONE PHOSPHATASE.

II. (a) THE PARTICIPATION OF PHOSPHOHEXOKINASE; (b) THE ISOLATION OF PURE FRUCTOSE-1-PHOSPHATE¹.

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THE study of the hydrolysis of phosphoric esters by bone phosphatase may serve two purposes, to gain knowledge of the chemistry of the esters and to throw light on specific properties of the enzyme. The present investigations were planned with both these objects in view.

When hexosediphosphoric ester (fructose-1:6-diphosphate) is hydrolysed by acids, the two phosphate groups are split off at very unequal rates. The velocity constants for the reactions in *N* HCl at 100° have been determined as 52×10^{-3} for the group in position 1 and 4.2×10^{-3} for that in position 6 [Macleod and Robison, 1933], the latter value giving the velocity after the first group has been removed. The marked difference in behaviour of the two groups towards acids enabled Neuberg [1918], by partial hydrolysis of the diphosphoric ester, to obtain a fructosemonophosphate in which the phosphate group is now known to occupy position 6.

The relative rates of hydrolysis of phosphoric esters by bone phosphatase may differ widely from those of their hydrolysis by acids. By the action of this enzyme on hexosediphosphoric ester Macleod and Robison [1933] obtained the second fructosemonophosphate containing the phosphate group in position 1. They did not succeed in freeing this compound entirely from the Neuberg ester. Its isolation in pure condition is described in the present paper.

A point of much interest had emerged from earlier experiments [Martland and Robison, 1929], namely, that in the hydrolysis of fructosediphosphate by preparations of bone phosphatase, some of the sugar undergoes intramolecular change, producing a mixture of aldoses and ketoses; similar changes were also observed in the hydrolysis of glucosemonophosphate [Robison and King, 1931].

The more detailed investigations of Macleod and Robison [1933] appeared to confirm these findings. The proportion of aldose formed during hydrolysis of hexosediphosphate varied with the phosphatase preparation employed and with the experimental conditions; but no explanation of this variation was, at the time, forthcoming. By stopping the hydrolysis when 50 % of the phosphate had been set free they obtained, in addition to unchanged diphosphate and free sugar, a mixture of glucose-6-phosphate (possibly containing mannose-6-phosphate), fructose-6-phosphate, and a new ester characterised by its strong laevorotatory power and its very rapid hydrolysis in *N* HCl at 100°. From the hydrolysis curve of the mixed monophosphates it was calculated that the above esters were present in the approximate proportions 3 : 2 : 5. After removal of the aldosephosphates by oxidation with bromine and separation of the sparingly

¹ A brief report of these results was communicated to the Hungarian Physiological Society in May 1934 and an abstract was published in *Ber. ges. Physiol.* (1934), 81, 563.

soluble barium phosphohexonates, the residual mixture contained 75 % of the new ester and 25 % of fructose-6-phosphate. Further purification was not attained at that time, but the properties of the new ester were calculated from those of the mixture and provided strong evidence that its constitution was that of fructose-1-phosphate. The suggestion was then made that the two phosphate groups of fructose-1,6-diphosphate are removed at equal rates by bone phosphatase but that, during removal of the group from position 1, intramolecular change occurs, resulting in the formation of a mixture of fructose-6-phosphate, glucose-6-phosphate and mannose-6-phosphate. These three esters would together constitute 50 % of the monophosphate fraction, the remaining 50 % being fructose-1-phosphate which could not undergo such intramolecular change.

The main objects of the work now to be described were (a) the discovery of factors controlling the conversion of ketose into aldose derivatives, (b) the isolation of fructose-1-phosphate in pure condition and the further study of its properties.

EXPERIMENTAL.

The fructosediphosphate used in these experiments was prepared by allowing a mixture of 50 g. dried yeast, 30 g. fructose and 100 ml. H_2O to ferment at 25° with gradual addition of a phosphate-sugar solution ($0.5\ M\ Na_2HPO_4 + M$ fructose); the hexosephosphates were separated by the method previously described [Robison and Morgan, 1930]. The fructosediphosphate obtained from five fermentations was purified by repeated precipitation of the acid barium salt from its aqueous solution by alcohol [Macfarlane and Robison, 1935] until a small portion, after conversion into the neutral barium salt, gave satisfactory figures on analysis (Table III). The phosphatase was prepared by the method of Martland and Robison [1929].

The methods of procedure for the hydrolysis and for the separation and examination of the products were similar to those employed in the later experiments of Macleod and Robison [1933] with slight modifications. All solutions were prepared with water which had been freed from dissolved air by boiling. The hydrolysis was carried out at 28° in presence of chloroform and was stopped by addition of H_2SO_4 to lower the p_H to 5; organic and inorganic phosphates were precipitated with basic lead acetate¹. The solution, after removal of lead as $PbSO_4$, was examined for the free sugars. The basic lead precipitate was decomposed with sulphuric acid and converted into barium salts, which were separated in the usual way.

It was first sought to discover whether the relative rates at which the two phosphate groups are set free or the extent to which the conversion of fructose into aldose occurs during hydrolysis could be influenced by such factors as differences in the proportion of phosphatase, the p_H of the solution, or the concentration of magnesium.

Controls. As controls for these experiments a series of 8 solutions was prepared containing sodium phosphate and fructose but no phosphoric ester; phosphatase was added to 4 of these. Two of each group were adjusted to p_H 7.0 and two to 8.4, while $MgSO_4$ was added to one of each pair. A similar series was also prepared containing glucose in place of fructose. The proportions of the various constituents were equivalent to those in Exp. 1. The solutions were kept at 28° during 10 days, estimations of total and inorganic phosphate and reducing power (Hagedorn and Jensen (H.J.) and iodimetric) being carried

¹ The very alkaline solution "25 % $Pb_2O(C_2H_3O_2)_2$ " (B.D.H.) was not used, since, as previously noted, it causes changes in the dissolved sugars.

out at intervals. No evidence was obtained of esterification of phosphate or of significant change in the amount or character of the sugar present in any of the solutions. Two of each series (p_H 8.4, without $MgSO_4$, with and without phosphatase) were finally evaporated to dryness and the residues were extracted with boiling 85 % alcohol; over 95 % of the sugar originally present was recovered in each case. The sugars recovered from each of the two fructose solutions had $[\alpha]_{5461} - 103^\circ$, calculated on the H.J. values, and I values of 4 %, calculated as glucose; those recovered from the glucose solutions had $[\alpha]_{5461} + 60^\circ$ and I values of 98 %. Fructose and glucose have $[\alpha]_{5461} - 110.8^\circ$ and $+62^\circ$ respectively. It was thus evident that very little, if any, conversion of free sugar occurs under these experimental conditions.

Exp. 1. Rapid hydrolysis. A solution (140 ml.) of sodium fructosediphosphate, prepared from the acid barium salt, was divided into 4 equal portions, each containing 200 mg. P; two of these were brought to p_H 7.0 and two to p_H 8.6; magnesium sulphate was added to two of the solutions, one at each p_H , to give a final concentration of 0.005 *M*. 5 ml. of a phosphatase suspension containing 200 mg. of the dry preparation A_1 were added to each of the four solutions; the volume was made up to 40 ml., and the p_H was adjusted to 7.0 and 8.4 respectively; at intervals, portions were removed for analysis. Hydrolysis proceeded more rapidly than had been anticipated and had reached approximately 90 % in the solutions at p_H 8.4 when tested after 24 hours. In the two solutions at p_H 7.0 hydrolysis was stopped after 51 and 68 hours respectively, when slightly more than 50 % of the phosphate had been set free. The H.J. reducing power increased in all the solutions proportionately to the amount of hydrolysis. The initial iodimetric reducing power, equivalent to about 75 mg. aldose and mainly attributable to the phosphatase, was not significantly changed in any of the solutions. Conversion of ketose into aldose derivatives could not, therefore, have occurred to an appreciable extent during the hydrolysis. Analyses of the products are given in Table I; none showed more than a small iodimetric reducing power and it was concluded that the free sugars consisted almost wholly of fructose and that the monophosphate fractions contained over 90 % of the two fructose esters in approximately equal proportions. The differences found between the products of hydrolysis at p_H 7.0 and p_H 8.4 with and without added magnesium salt were not very marked or consistent.

Exp. 2. Slow hydrolysis. A further hydrolysis was carried out at p_H 8.4, using only 20 mg. phosphatase per 200 mg. organic P, *i.e.* one-tenth of the amount of phosphatase employed in the previous experiment. Hydrolysis reached 50 % in 19 days and was then stopped; the monophosphate fraction gave analytical values corresponding closely with those calculated for a mixture of fructose-6-phosphate and fructose-1-phosphate in equal proportions. The amount of aldosephosphate did not exceed 6 % and was probably less¹.

While the results of these experiments justified the conclusion that the two phosphate groups of fructosediphosphate are removed by bone phosphatase at nearly equal rates, they showed that the conversion of fructose into aldose, previously observed, is not a necessary consequence of this hydrolysis.

It seemed possible that an explanation of these varying results might lie in the difference in purity of the phosphatase used. Preparations obtained by addition of alcohol and ether to aqueous extracts of bone had been employed for most of the earlier hydrolyses; but the phosphatase A_1 used in the above experiments had been purified further by re-extracting the first precipitate with 30 % aqueous alcohol and again precipitating the phosphatase with alcohol and

¹ Fructosephosphates have a small iodimetric reducing power.

ether¹. This process of purification might have brought about the destruction or removal of some agent present in the cruder phosphatase and responsible for the change in the molecule of fructose.

Further evidence on this point was obtained by hydrolysing fructose-diphosphate with other preparations of purified bone phosphatase (Exps. 3 and 4) and with crude bone extract (Exp. 5). These hydrolyses were carried out at 28° and p_H 7.6–7.8, without addition of magnesium salt. Analyses of the products and descriptions of the phosphatase preparations are given in Table I.

Table I. *Products obtained from partial hydrolysis of fructosediphosphate by bone phosphatase.*

Exp.	Phosphatase Prep. Amt.		Ester mg. P	Conditions of hydrolysis			Hydro- lysis %	Sugars [α] ₅₄₆₁ calc. on H.J. (°)	Monophosphoric esters (barium salts)					[α] ₅₄₆₁ (°)
				<i>p</i> _H	MgSO ₄ <i>M</i>	Dura- tion days			Reducing power as glucose			Aldose- phos- phate %	Fruc- tose Seli- vanoff %	
									T.P. %	H.J. %	I %			
1 <i>a</i>	A ₁	200 mg.	200	7.0	—	2.8	55	— 108	7.8	29	3.5	8	25	— 17.2
1 <i>b</i>	A ₁	200	200	7.0	0.005	2.1	52	— 105	7.8	30	3.5	8	25	— 16.3
1 <i>c</i>	A ₁	200	200	8.4	—	1	87	— 97	7.7	30	4.0	9	23	— 15.1
1 <i>d</i>	A ₁	200	200	8.4	0.005	1	92	— 99	7.6	30	4.5	10	22	— 14.4
2	A ₁	20	200	8.4	—	19	50	— 97	7.8	30	2.9	6	23	— 17.5
3	B ₁	45	155	7.7	—	4	50	— 103	7.8	28	3.0	6	26	— 18.7
4	C ₂	45	155	7.7	—	4	50	— 103	7.8	28	3.2	7	25	— 18.1
5	D	12 ml.	—	7.7	—	2	50	— 53	7.8	29	14.4	32	19	— 13.3
6	D	200	3921	7.8	—	3	47	— 61	7.8	28	13.0	29	19	— 13.4
7	E	200	4500	7.6	—	7	48	— 55	7.8	29	14.7	32	18	— 12.9

Fructose has [α]₅₄₆₁ — 110.8°; C₆H₁₁O₅PO₄Ba requires P 7.85 %.

Phosphatase preparations:

A, B, C, precipitates by addition of alcohol and ether to filtered aqueous extracts of rabbit bones.

A₁, purified by extraction with 30 % aqueous alcohol and reprecipitation with alcohol and ether in presence of potassium acetate.

B₁, purified by extraction with 50 % aqueous alcohol and reprecipitation with alcohol and ether.

C₂, purified twice by extraction with water and reprecipitation with alcohol in presence of sodium sulphate.

D, E, crude aqueous extracts of rabbit bones.

The monophosphate fraction produced by crude extract had an I value of 14.4 %, equivalent to 31 % of aldosemonophosphate; the free sugar appeared to contain about the same proportion of aldose². The monophosphates obtained with purified phosphatase had I values of only 3 % and probably contained less than 6 % of aldosephosphate, while the proportion of aldose in the free sugars was equally low. Clearly, therefore, the crude bone extract contained an active agent which was not present in the purified phosphatase.

The phosphohexokinase of bone and marrow.

Presence of the active agent in bone marrow. In preparing the phosphatase most, but not all, of the marrow was removed before the bones were extracted with water. Tests were made with two extracts, one prepared from the heads of long bones without removing the marrow, the other from specially cleaned fragments of hard bone from the shafts. When allowed to act on hexosediphosphate both extracts produced monophosphate mixtures containing about 30 % of aldo-esters. Experiments were next carried out with (a) an aqueous extract of marrow and (b) the precipitate obtained from such an extract by addition of

¹ A difficulty experienced in obtaining flocculation of the material at this stage was overcome by addition of potassium acetate.

² Calculated from the specific rotation, assuming fructose and glucose to be the principal components. The I values of these sugar fractions were vitiated by the presence of I-reducing substances derived from the crude enzyme preparation.

Table II. *Interconversion of aldo- and keto-hexosephosphates by preparations of bone phosphatase and marrow.*

Exp.	Substrate	Aldo. %	Phosphatase*		Marrow†		<i>t</i> ^o	<i>p</i> _H	Time hrs.	Hydr. %	Aldo. %	Time hrs.	Hydr. %	Aldo. %
			Prep.	mg./ mg. P	Prep.	ml./ mg. P								
10	Fructose-1:6-di-phosphate	4	C	0.4	—	—	28	7.5	17	32	18	48	81	29
		4	C	0.4	<i>a</i>	0.01	28	7.4	17	32	24	48	70	36
		4	C	0.4	<i>a</i>	0.04	28	7.2	17	32	25	48	67	36
		4	C ₂	0.9	—	—	28	7.5	17	27	4	48	64	6
		4	C ₂	0.9	<i>a</i>	0.01	28	7.3	17	29	20	48	57	33
		4	C ₂	0.9	<i>a</i>	0.04	28	7.2	17	30	24	48	59	35
11	Fructose-1:6-di-phosphate	4	C	0.2	—	—	23	8.4	—	—	—	40	71	29
		4	C ₂	0.4	—	—	23	8.4	—	—	—	40	63	5
	"	4	C ₂	0.4	<i>b</i>	0.3	23	8.4	—	—	—	40	65	30
		4	{C C ₂	{0.02 0.4}	—	—	23	8.4	—	—	—	40	74	21
	Fructose-6-phosphate	5	C ₂	1	<i>b</i>	1	23	8.4	—	—	—	40	98	57
	"	5	C ₂	—	<i>b</i>	1	23	8.4	—	—	—	40	9	55
12	Fructose-6-phosphate	5	—	—	<i>b</i>	1	23	7.7	17	2	56	40	5	56
	Fructose + inorganic phosphate	< 1	—	—	<i>b</i>	1	23	7.7	17	< 1	—	40	—	< 1
13	Fructose-6-phosphate†	5	—	—	<i>b</i>	3	23	6.4	4	< 1	61	20	2	62
	"	5	—	—	<i>b</i>	3	23	7.7	4	1	62	20	4	63
	"	5	—	—	<i>b</i>	9	23	7.7	4	2	62	20	7	62
	Fructose-1-phosphate	2	—	—	<i>b</i>	2	23	7.7	4	1	2	20	5	2
	"	2	C ₂	6	<i>b</i>	2	23	7.7	4	82	2	20	100	2
	Glucose-6-phosphate	92	—	—	<i>b</i>	3	23	7.7	4	1	63	20	4	63

* Phosphatase preparations. C, precipitate obtained by addition of alcohol and ether to aqueous extract of rabbit bones. C₂, C twice purified by extraction with water and reprecipitation with alcohol in presence of sodium sulphate.

† Marrow preparations. *a*, aqueous extract of bone marrow; marrow from the long bones of young rabbits was shaken with 3 times its weight of water, left 3 hours at 0° and centrifuged; 0.04 ml. ≡ 10 mg. fresh marrow. *b*, precipitate obtained by addition of alcohol and ether to unfiltered aqueous extract of bone marrow; 1 mg. was obtained from 11.6 mg. fresh marrow.

The amounts of these preparations used are given as mg. or ml. per mg. P of the substrate.

The figures for aldose and aldo-derivatives are based on the I values (less the amount due to the enzyme preparations) and are given as percentages of the total calculated hexose (not the H.J. value) in the substrate. The fructose esters reduce I to a small extent and are, therefore, shown as apparently containing 2.5 % aldosephosphate; fructose (Kerfoot) gives an I value of about 0.8 %. The specimen of glucose-6-phosphate used contained a small proportion of other esters, chiefly mannosephosphate.

The concentrations of the substrates varied between 0.015 *M* and 0.04 *M* in the different experiments.

‡ In Exp. 13, tests on fructose-6-phosphate + marrow were also done after 40 minutes and gave nearly the same percentages of aldo-derivatives as were found after 4 hours.

alcohol and ether. The results are shown in Table II. It was found (Exps. 10, 11) that either of the marrow preparations, when added to the purified phosphatase C₂ restored the power to produce aldo-compounds from fructosediphosphate. Addition of the marrow extract to the cruder phosphatase C led to a slight increase in the percentage of aldo-compounds formed on hydrolysis of the diphosphate; but larger quantities of marrow extract, added to either crude or purified phosphatase, did not raise the proportion of aldo-compounds beyond certain limits, namely, about 25 % of the total hexose when hydrolysis had reached 32 % and about 36 % when 60–70 % of the phosphate had been set free. Other experiments, not quoted in Table II, showed that the marrow preparation, like the crude phosphatase, lost its power to effect this change when left for some hours in aqueous alcohol. Exp. 11 also showed that the addition to phosphatase C₂ of 1/20 its weight of phosphatase C was sufficient to increase the proportion of aldo-compounds from 5 to 21 %, while 10 times the quantity of C alone produced 29 %. It was concluded that the same agent was present in both marrow and bone extracts but the results did not warrant the assumption that its presence in the latter was due solely to the marrow left in the bone.

In order to learn more about the properties of this agent its action on other phosphoric esters and on fructose + inorganic phosphate was investigated. By the use of the marrow preparation *b*, whose phosphatase activity was very low, and the highly purified phosphatase C_2 , the action of the two enzymes could be readily distinguished.

Fructose-6-phosphate was converted into aldo-esters to the extent of 55–63 % by marrow preparations alone at p_H 8.4, 7.7 and 6.4, very little hydrolysis taking place (Exps. 11, 12, 13). The amount of conversion was, in some cases, maximum within 40 minutes. When both phosphatase and marrow were allowed to act on this ester, 98 % hydrolysis occurred in 40 hours, but the percentage of aldoose was the same as that produced by marrow alone.

Fructose-1-phosphate, on the other hand, did not yield any aldo-compounds with marrow alone (5 % hydrolysis) or with marrow + phosphatase (100 % hydrolysis), nor did the marrow preparation produce any aldoose from a mixture of fructose + inorganic phosphate in periods up to 40 hours. The action of marrow on glucose-6-phosphate caused the aldoose content to fall to 63 %, the same proportion as that produced from fructose-6-phosphate under similar conditions.

It should be stated that the amounts of aldosephosphates present in the equilibrium mixtures may be somewhat higher than the maximum values recorded, for two reasons; (a) owing to the relatively large I-reducing power of the enzyme preparations the total reduction exceeded in some cases the limit recommended for this method [Macleod and Robison, 1929]; (b) the aldo-esters probably included some mannose-6-phosphate which gives an abnormally low I value.

The results of these preliminary experiments suggest that the active agent present in bone and marrow is a phosphohexokinase having the power to convert the hexose-6-phosphates into an equilibrium mixture of aldo- and keto-esters. It has no action on fructose-1-phosphate and, most probably, none on intact fructose-1:6-diphosphate. When the diphosphate is hydrolysed by bone phosphatase, fructose-1-phosphate and fructose-6-phosphate are formed in approximately equal proportions, so that if the kinase is also present the percentage of aldo-compounds in the products may rise to about half that given by fructose-6-phosphate under optimum conditions. The actual figure will depend on the relative activities of the phosphatase and kinase, that is, on how much of the fructose-6-phosphate is hydrolysed before conversion. The kinase does not effect the conversion of free sugar.

This phosphohexokinase is probably identical with the enzyme discovered by Lohmann [1933] in extracts of muscle, kidney, liver, brain and dried yeast. Lohmann showed that these extracts converted the Neuberg ester into a true equilibrium mixture which resembled, in its rates of hydrolysis by HCl and in its aldoose content, the Embden and Robison esters of muscle and yeast fermentations. The conversion was extremely rapid; at 20° equilibrium was reached in a few seconds with dialysed muscle extract and in about 5 or 10 minutes with the other tissue extracts. In our own experiments only a few tests were made after short periods, but these showed that fructose-6-phosphate was converted into the equilibrium mixture in about 40 minutes at 23° and p_H 7.7 by 3 mg. of marrow *b* ($\equiv$ 35 mg. fresh marrow) per mg. ester P. The proportion of animal tissue extract used by Lohmann corresponded with about 600 mg. fresh tissue per mg. ester P. It would seem, therefore, that the activity of the marrow is at least as great as that of brain, kidney and liver tissues.

Lohmann showed that a similar equilibrium was also reached in a few seconds when muscle extract was allowed to act on the aldosephosphate mixture resulting

from partial hydrolysis of the Embden ester by acids. The same result was obtained in Exp. 13 of the present series, starting with glucose-6-phosphate. Other experiments [Patwardhan and Robison, 1935] have shown that mannose-6-phosphate behaves in the same way when acted on by yeast extract. There can therefore be little doubt that each of these hexose-6-phosphates is converted by phosphohexokinase into an equilibrium mixture in which all three esters are present. Each has been isolated from the hexosemonophosphate of fermentation which, however, contains other esters whose precise nature is still unknown. It would be interesting to discover whether the kinase has any effect on these also or plays any part in their formation. It has been suggested [Robison and Rosenheim, 1934] that the calcifying mechanism of bone may consist of a complex enzyme system analogous to those in yeast and muscle. The presence of phosphohexokinase in association with the phosphatase of hypertrophic cartilage and osteoid tissue may have some significance in this connection and its location in the bone is therefore being further investigated.

The isolation of fructose-1-phosphate.

The high laevorotation of the mixed hexosemonophosphates obtained with purified phosphatase suggested that these would provide the most suitable material for the preparation of pure fructose-1-phosphate; but further consideration led to a different conclusion. The proportion of fructose-1-phosphate in these products was probably not greater than in those of lower specific rotation and high aldose content, and whereas aldosephosphates could be quantitatively removed by oxidation, a method for the elimination of fructose-6-phosphate had still to be found. It was therefore decided to use crude bone extract for the partial hydrolysis of fructosediphosphate on a larger scale. Two such hydrolyses (Table I, Exps. 6 and 7) were carried out and yielded 7.5 g. and 8.4 g. respectively of barium hexosemonophosphates, equivalent to about 15 % of the total P of the diphosphate. From their analytical values it was judged that these products, like that of Exp. 5, contained approximately 30 % of aldosemonophosphates and 50 % of fructose-1-phosphate¹. The residues from Exps. 1-7 contained considerable amounts of unchanged fructosediphosphate, which was separated from the inorganic phosphate by repeated precipitation of the acid barium salt; this was converted into sodium salt and partially hydrolysed with crude bone extract, whereby a further quantity (6.7 g.) of hexosemonophosphate of slightly lower aldose content was obtained.

Removal of aldosemonophosphates. 14 g. of the barium hexosemonophosphates were dissolved in 240 ml. of water and treated, at room temperature, with 1.5 ml. bromine; 70 ml. of a cold saturated solution of baryta (0.47 *N*) were added in 10 ml. portions at intervals during 20 minutes, with constant shaking; these quantities represented a 200 % excess of bromine and 50 % excess of baryta over the calculated amounts required for the oxidation of the aldosephosphate. After a further 5 minutes the solution was acidified with 12 ml. *N* HCl and the excess of bromine was removed by aeration for one hour. The p_H , which was then 4.2, was raised to 8.0 by addition of baryta and the precipitated barium phosphohexonate was filtered off; the fructosephosphates were recovered from the filtrate by precipitation with alcohol and were freed from phosphohexonate by repeated solution in small quantities of water and fractional precipitation with alcohol. The fraction finally precipitated between 18 and 66 % alcohol

¹ The figures are best satisfied by assuming the presence of both glucose and mannose esters, but the exact composition cannot be calculated without more information on this point.

weighed 7.2 g. and had $P\ 7.81\%$ and $[\alpha]_{5461} - 27^\circ$. 6.7 g. of hexosemonophosphate obtained from recovered diphosphate were similarly treated and yielded 3.1 g. barium salt having $P\ 7.63\%$ and $[\alpha]_{5461} - 23^\circ$. The product obtained by Macleod and Robison [1933] had $[\alpha]_{5461} - 25.8^\circ$.

Separation of the fructosemonophosphates. An attempt was first made to separate the two fructosemonophosphates by fractional precipitation of the barium salts ($[\alpha]_{5461} - 27^\circ$) from aqueous solutions by alcohol. Three fractions were obtained having $[\alpha]_{5461} - 28.7^\circ$, -27.1° , -24.2° respectively. The first, which amounted to 60 % of the total salt, contained the highest proportion of fructose-1-phosphate; but the degree of separation was small and it was thought well to try other methods before continuing this fractionation.

The next attempt was based on the possibility that in alkaline solutions fructose-6-phosphate might be converted into aldosephosphate (which could be oxidised with bromine) while fructose-1-phosphate remained unchanged. A number of tests were carried out with the mixed monophosphate (I value 3.5 %) obtained from Exp. 1 a; the results are briefly summarised below:

(a) In aqueous solution at $p_H\ 8.4$ and 28° no increase in the I value occurred in 11 days.

(b) In 0.01 *N* NaOH at 28° the I value slowly increased, reaching 6.6 % in 6 hours; but the H.J. value fell, in the same period, from 35 to 32 %; after 46 hours the I value had risen to 11 % and the H.J. value had fallen to 23 %, while 10 % of the total phosphate had been set free.

(c) In 0.05 *N* NaOH at 28° similar but more rapid changes occurred; in 1 hour the I value rose to 6.2 % and the H.J. value fell to 32 %; in 46 hours the H.J. value fell to 16 %, and 26 % of the phosphate was set free.

These results indicated that in 0.01 *N* and 0.05 *N* NaOH at 28° the fructose esters had undergone changes more profound than the simple conversion of ketose into aldose. In a final experiment 0.78 g. of the mixed monophosphates of low aldose content was treated with bromine in presence of a large excess of baryta (0.2 *N*) and the reaction was allowed to proceed for 100 minutes at room temperature. The amount of barium fructosephosphate recovered was only 0.12 g. and its specific rotation, -20° , was not very much higher than that of the original mixture. Destruction of both fructose esters had clearly taken place; the method was therefore abandoned as useless.

Recourse was now had to the fractional crystallisation of the brucine salts. It had been feared that this method might involve serious loss of the fructose-1-phosphate through hydrolysis during crystallisation or in the frequent inter-conversion of brucine and barium salts for analysis and further fractionation. Fortunately the brucine salts proved reasonably stable in aqueous and alcoholic solutions, while it was found possible to follow the course of their separation by measuring their specific rotations in aqueous solution; the loss in weight at 100° (Pregl micro-dryer) and P content of the salt were determined in each case. From time to time portions were converted into the barium salts and analysed; the amounts of hydrolysis occurring in 25 and 60 minutes in *N* HCl at 100° were also determined on a few mg. of the barium salts and furnished an indication of their purity.

The solution of the brucine salts in 80 % aqueous alcohol was prepared, as previously described [Robison and King, 1931], from 8.6 g. barium salts; it was cooled to -15° and, after crystallisation had begun, was left for a further 12 hours at 0° ; weight of crystalline salt 21 g., representing 80 % of the total P. It was recrystallised three times by dissolving it in a small volume of 40 % alcohol at 30° and adding absolute alcohol to give 90 % concentration; weight

after third recrystallisation 10.7 g. A small portion was converted into the barium salt for analysis; it had P 7.7 %, H.J. val. 28 %, I val. 1.5 %, $[\alpha]_{5461} -28^\circ$; hydrolysis 88 % in 60 minutes. It was thus evident that very little separation had been achieved. The brucine salt was next dissolved in 35 ml. of 40 % alcohol and allowed to crystallise at 0° ; four fractions, amounting to half the total salt, were removed and found to have an increased laevorotation, while the barium salt from one fraction had $[\alpha]_{5461} -31^\circ$. Still better results were obtained by recrystallisation from water, in which the brucine salt was much less soluble than in dilute aqueous alcohol. Solution was effected at 40° and crystallisation allowed to proceed at room temperature. Successive fractions of the brucine salt had specific rotations ranging from -47 to -34° . All the fractions obtained in previous operations were therefore crystallised from water, and fractions of high rotatory power were combined and recrystallised. In this way the specific rotation of the brucine salt was gradually raised to -52° , that of the corresponding barium salt to -39° and the hydrolysis, in *N* HCl at 100° , to 98 % in 25 minutes and 100 % in 60 minutes.

The barium salt was purified by repeated solution in 10 parts of water and precipitation by alcohol. Traces of a sparingly soluble substance persisted and the salt was therefore again purified by fractional precipitation with alcohol; the main fraction obtained between 20 % and 70 % alcohol was filtered off, washed with absolute alcohol and dried in an evacuated desiccator. The analytical values for this salt are shown in Table III; the rates of hydrolysis of the free acid

Table III. *Analyses of fructosephosphoric esters.*

Ester	Salt	Ba %	P %	Fructose (Seli- vanoff) %	Reducing power as glucose %		$[\alpha]_{5461}^{20^\circ}$
					H.J.*	I	
Fructose-1-phosphate	Barium	34.5	7.82	24	26	0.9	-39.0 (<i>c</i> = 6.1 %)
„	Brucine	—	2.95	—	—	—	-52.1
„	Free acid	—	—	—	—	—	-64.2 (<i>c</i> = 11.3 %)
Fructose-1-6-diphosphate (used in present exps.)	Barium	—	10.42	8	12	0.7	+ 1.9°
Fructose-6-phosphate (from fermentation hexosemono- phosphate)	„	—	7.73	22	35	1.6	+ 2.3
Fructosemonophosphate (from hydrolysis of fructosediphosphate in <i>N</i> HCl at 100°)							
5 mins. hydrolysis†	„	—	7.82	—	33	3.0	+ 2.6
30 mins. hydrolysis†	„	—	7.79	—	33	2.0	- 5.1
30 mins. hydrolysis, 20 % alcohol fraction	„	—	7.78	—	—	—	- 9.9

* Determined with addition of 0.5 ml. 0.5 *N* NaOH.

† These esters were hydrolysed to the same extent by *N* HCl at 100° in 25 and 60 min. $C_6H_{10}O_4(PO_4Ba)_2$ requires P 10.16 %; $C_6H_{11}O_5PO_4Ba$ requires P 7.85 %; Ba 34.75 %; $C_6H_{11}O_5PO_4$ ($C_{23}H_{26}O_4N_2$)₂ requires P 2.95 %.

in 0.01 *M* aqueous solution (autolysis) and of the potassium salt in 0.1 *N* and *N* HCl at 100° are given in Table IV. It is seen that the value of *k* increases with the *c_H* of the solution; in this respect the behaviour of the ester agrees with that of fructosediphosphate and differs from the very abnormal behaviour of the

Table IV. *Hydrolysis of fructose-1-phosphoric ester in acid solutions at 100°.*

Concentration of esters 0.01 <i>M</i> .								
Time mins.	Autolysis		0.1 <i>N</i> HCl		<i>N</i> HCl			
	Found %	<i>k</i> × 10 ³	Found %	Calc. % for <i>k</i> = 9 × 10 ³	Found %		Calculated %	
					(a)	(b)	(c)	(d)
5	—	—	10.4	9.9	55.7	55.8	55.3	56.7
10	—	—	18.1	18.7	80.7	80.2	80.0	80.8
15	—	—	—	—	88.8	90.6	91.1	90.9
20	—	—	32.8	33.9	—	—	—	—
25	—	—	—	—	98.5	97.6	98.2	97.1
30	—	—	47.0	46.4	—	—	—	—
40	—	—	—	—	100.0	100.0	99.8	98.5
60	—	—	72.6	71.2	—	—	—	—
120	15.8	0.62	90.9	91.7	—	—	—	—
300	33.0	0.55	—	—	—	—	—	—
600	51.7	0.47	—	—	—	—	—	—

The values of k are calculated from the equation for a unimolecular reaction

$$k = \frac{1}{t_2 - t_1} \log \frac{a - x_1}{a - x_2}$$

For the hydrolysis in N HCl the values in columns (a) and (b) are those determined in two separate experiments. The values in columns (c) and (d) are calculated, (c) for 100 % of an ester having $k = 70 \times 10^{-3}$ and (d) for a mixture containing 98 % of an ester having $k = 75 \times 10^{-3}$ and 2 % of an ester having $k = 4.2 \times 10^{-3}$. On the whole the observed values agree better with those in column (c) than with those in column (d)¹.

aldose-6-phosphates. The fall in the value of k during autolysis is probably due to the fall in c_H as phosphoric acid is set free.

The specific rotation of the barium salt is a little higher than the value previously calculated from the properties of the mixed fructosemonophosphates (-39° against -35°), while the value of k for hydrolysis in N HCl is a little lower (0.070 against 0.075). With such high rates of hydrolysis the possible errors of the micromethods employed may appreciably affect the calculated values of k , and the presence of 1 %, or even of 2 %, fructose-6-phosphate in our final product cannot be definitely ruled out. In Table IV the percentages of hydrolysis calculated for 100 % of an ester with $k = 0.070$ and of 98 % of an ester with $k = 0.075$ are shown side by side with the observed values. Another explanation is, however, suggested by the results noted in a later paragraph, which show that when hexosediphosphate is hydrolysed with N acid the fructosemonophosphate produced at first is dextrorotatory, but soon becomes laevorotatory as hydrolysis proceeds. The final value of k observed in this hydrolysis cannot, therefore, represent the value for the ester first produced, presumably fructose-6-phosphate, while the latter compound may conceivably be more dextrorotatory than has heretofore been supposed. The assumptions made for the second component of the fructosemonophosphate mixture were therefore not strictly correct. Further discussion of this interesting point will be postponed until the completion of experiments now in progress.

Reaction of fructose-1-phosphate with phenylhydrazine. 1.2 ml. of a solution containing 13 mg. of the free ester were treated with 0.2 ml. phenylhydrazine

¹ The sealed ampoules containing the acid solutions were immersed in a covered bath of vigorously boiling water. Variations in the temperature of the bath due to changes in barometric pressure might account for some of the observed differences in these rates. Hydrolyses (a) and (b) were carried out at 99.7° ($p = 752$ mm.); a subsequent hydrolysis of the same ester, at 100.7° ($p = 779$ mm.) gave k , 0.080.

and 0.2 ml. glacial acetic acid. A portion of this solution left at 0° for 48 hours deposited no crystalline phenylhydrazone. The remainder was heated in a boiling water-bath for 20 minutes, cooled to 0° and filtered after some hours; weight of dry osazone, 13 mg. The mother-liquor, which gave no weighable precipitate on heating for a further 45 minutes, contained 1.36 mg. P, almost entirely as inorganic phosphate. The crude osazone contained only a trace (0.1 %) of P and, after recrystallisation, melted at the same temperature as glucosazone, alone and when mixed with the latter; the yield of glucosazone was equivalent to about 90 % of the original ester. Since only a very small percentage of this ester would have been hydrolysed in 20 minutes at the p_H of the solution, the liberation of phosphoric acid must be attributed to the oxidising action of the phenylhydrazine on the terminal group, to which the phosphoric acid is therefore most probably attached.

Products of partial hydrolysis of fructosediphosphate by N H₂SO₄.

It had occasionally been noted that preparations of the Neuberg ester obtained in this laboratory by hydrolysis of fructosediphosphate with mineral acids at 100° were slightly laevorotatory; the possibility that this might be due to the presence of fructose-1-phosphate was investigated. A solution of the free diphosphoric ester in N H₂SO₄ was heated at 100° for 5 minutes and the barium hexosemonophosphate isolated in the usual way; the residual diphosphate was again heated in N H₂SO₄ for a further 25 minutes and the monophosphate again separated from the products. Both barium salts were purified by solution in water and reprecipitation by alcohol; their analyses are given in Table III. Unexpectedly, it was the second fraction, obtained after 30 minutes' heating, that proved to be laevorotatory (−5.1°), while the specific rotation of the 5-minute fraction (+2.6°) was close to that usually found for fructose-6-phosphate. Precipitation of the 30-minute product from aqueous solution by 20 % alcohol gave a fraction having $[\alpha]_{5461} - 9.9^\circ$. These dextro- and laevorotatory salts were each hydrolysed to approximately the same extent by N HCl at 100° in 25 minutes and 60 minutes. The laevorotatory substance cannot, therefore, have been fructose-1-phosphate. The presence of a derivative of fructopyranose, containing the phosphoric acid group in position 3, 4 or 5, might indicate either that the fructosediphosphate is not, after all, homogeneous or that migration of the phosphoric acid group from position 6 occurs under the conditions of hydrolysis. If the latter explanation be correct it might also account for other puzzling results noted in the past, *e.g.* the failure to obtain pure glucose-6-phosphate, in more than small proportion, from trehalosemonophosphate after long-continued hydrolysis in N H₂SO₄. It would also mean that the final rates of hydrolysis of the hexosephosphoric esters in N HCl at 100° may not be those of the ester supposedly present. Further work on this question is in progress and will shortly be reported.

SUMMARY.

1. In continuation of the experiments of Macleod and Robison [1933] the hydrolysis of fructose-1:6-diphosphate by preparations of bone phosphatase has been further investigated. It has been shown that the products of hydrolysis by specially purified phosphatase are fructose-1-phosphate and fructose-6-phosphate in approximately equal proportions; aldo-derivatives are not formed.
2. The production of aldo-derivatives which was previously observed is due to the presence of a phosphohexokinase in crude bone extracts and in phosphatase

preparations obtained from these by addition of alcohol and ether. During further purification of the phosphatase this kinase is destroyed.

3. The phosphohexokinase is present also in marrow extracts and can be precipitated from these extracts by alcohol and ether; it is inactivated if the precipitate is allowed to remain for some hours in aqueous alcohol.

4. This phosphohexokinase is probably identical with the enzyme found by Lohmann in extracts of muscle, kidney, liver, brain and yeast. It converts fructose-6-phosphate and glucose-6-phosphate into equilibrium mixtures of aldose and keto-esters. It has no apparent action on fructose-1-phosphate and none on fructose-1:6-diphosphate unless phosphatase is also present and first removes the phosphate group from position 1. It does not effect the conversion of free fructose into aldose.

5. Fructose-1-phosphate has been isolated in apparently pure condition from the products of hydrolysis of the diphosphate by fractional crystallisation of the brucine fructosemonophosphates from water.

The properties of the ester have been studied. The value of $[\alpha]_{5461}$ for the barium salt is -39.0° , slightly higher than that calculated by Macleod and Robison [1933]; $[\alpha]_{5461}$ of the free ester is -64.2° .

The rates of hydrolysis of the free ester in 0.01 *M* aqueous solution and in 0.1 *N* and *N* HCl at 100° have been determined. The values of *k* are for autolysis 0.62×10^{-3} , for 0.1 *N* HCl 9×10^{-3} , for *N* HCl 70×10^{-3} .

6. When heated with phenylhydrazine in acetic acid solution for 20 minutes at 100° the ester is converted almost quantitatively into glucosazone. This confirms the previous conclusion that the phosphate group occupies position 1 in the fructose molecule. The ester is probably 1-phosphofructopyranose.

7. Other laevorotatory fructosemonophosphates have been obtained as the result of heating fructosediphosphoric ester with *N* H_2SO_4 at 100° . Experiments are in progress to discover the nature of these esters and to explain their formation.

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