CLVIII. CONSTITUTION OF HEXOSE-DIPHOSPHORIC ACID. PART II.

THE DEPHOSPHORYLATED α - AND β -METHYLHEXOSIDES¹.

BY WALTER THOMAS JAMES MORGAN (*Beit Memorial Research Fellow*)
AND ROBERT ROBISON.

From the Biochemical Department, Lister Institute, London.

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THE researches of Harden and Young [1909, 1910] showed that the three monosaccharides glucose, mannose and fructose behave in the same way towards yeast juice in the presence of added phosphate. The identity of the hexosediphosphoric acid produced in each case was established by Young [1909], who showed that this ester on acid hydrolysis yielded free phosphoric acid and a laevo-rotatory solution from which fructose could be isolated. The rotation of this solution, however, was always lower than that of a fructose solution of equal reducing power, and this caused Young to avoid drawing definite conclusions as to the nature of the sugar in hexosediphosphoric acid. Later Neuberg and Kretschmer [1911] succeeded in isolating crystalline fructose from the products of hydrolysis and they concluded that this was the only sugar present and that the original acid was fructosediphosphoric acid.

At this time the reactive γ -sugars had not been described, for it was not until some years later that Fischer [1914] prepared γ -methylglucoside, the first representative of the structural isomerides containing an unstable oxide ring. The recognition that inulin and sucrose are derivatives of γ -fructose followed from the work of Haworth [1920] and Irvine and Steele [1920] and in 1922 Menzies [1922] isolated γ -methylfructoside from the products of esterification of ordinary fructose by Fischer's method.

In the light of this more recent knowledge of structural isomerism in the sugar group the further investigation of the constitution of hexosediphosphoric acid was undertaken in order to determine the type of oxide ring in the hexose molecule as well as the position of the phosphoric acid groups. Of these two groups the position of one followed with considerable certainty from the work of Young [1911] on the reaction of hexosediphosphoric acid with phenylhydrazine. Using cold reagents he obtained the phenylhydrazine salt of a

¹ The conclusions reached in this paper were communicated to the Biochemical Society on December 9th, 1927, and an abstract was published in *Chemistry and Industry*, 1927, 46, 1183.

phenylhydrazone in which both phosphoric acid groups were still present. On heating, the osazone was formed, one phosphoric acid group however being simultaneously eliminated. From these facts Young concluded that if the parent hexose is fructose one phosphoric acid group is attached to the terminal carbon atom adjacent to the carbonyl group. No evidence was forthcoming as to the position of the second group.

The preparation of methylhexosidediphosphoric acid and the separation of the α - and β -isomerides by crystallisation of their brucine salts has already been described [Morgan, 1927] and in the present paper experimental proof is given that the methylhexosidediphosphoric acids are derivatives of γ -fructose and the bearing of this conclusion on the constitution of the free phosphoric ester is discussed.

The removal of the phosphoric acid groups from the α - and β -methylhexosidediphosphoric acids by the bone phosphatase.

By the action of the bone phosphatase it has been possible to eliminate the phosphoric acid groups from both the α - and β -methylhexosidediphosphoric acids without removing the glucosidic methyl group, thus confirming and extending the preliminary experiment of Morgan [1927]. For most of the experiments here described we have used active preparations of the bone phosphatase purified by precipitation from the aqueous extract of bones of young rabbits by a mixture of ether and alcohol [Martland and Robison, 1928] and have in this way avoided contamination of the reaction products with alcohol-soluble material derived from the bones.

The barium salts of the methylhexosidediphosphoric acids were dissolved in water and treated with the bone phosphatase preparation. The flasks containing the mixture were immersed in a water-bath at 38° and the p_H of the solution during the hydrolysis was maintained at 8.6–8.8 by the addition of cold saturated baryta at short intervals. Under these conditions the inorganic phosphate liberated by the action of the enzyme was at once precipitated as barium phosphate. The progress of the hydrolysis was roughly indicated by the amount of baryta added but in practice this amount was usually considerably less than the equivalent of the inorganic phosphate set free, probably owing to the precipitation of a portion of the latter as $BaHPO_4$ in place of $Ba_3(PO_4)_2$. For example, 0.6 g. bone enzyme acting on 4 g. barium methylhexosidediphosphate set free about 95 % of the phosphoric acid in 12 hours while the amount of baryta added corresponded with no more than 70 %. At the close of the hydrolysis the enzyme and any barium salts remaining in solution were precipitated by the addition of three volumes of alcohol and the alcoholic filtrate was evaporated to dryness *in vacuo*. The syrupy residue was dissolved in absolute alcohol and the filtered solution again evaporated to dryness. A blank experiment was in all cases carried out with the enzyme preparation alone but the amount of the alcohol-soluble matter so obtained did not exceed 2 % of the original weight of the enzyme pre-

paration. The small residues obtained in these blank experiments were optically inactive and possessed only very slight reducing properties either before or after acid hydrolysis.

The results of a number of experiments carried out on various specimens of α - and β - and the mixed $\alpha\beta$ -methylhexosidediphosphates are shown in Table I.

Table I.

Barium methylhexoside- diphosphate		Dephosphorylated methylhexoside						[α] ₅₄₆₁ of hexose formed by acid hydrolysis
		Hydro- lysed by enzyme %	Reducing power mg. glucose	Reducing power after acid hydro- lysis mg. glucose	Equiv. weight of hexoside mg.	MeO in dried hexoside syrup %	[α] ₅₄₆₁ of hexoside	
Isomer	[α] ₅₄₆₁							
α	+ 8.3°	—	4.0	83	89	—	+ 64°	- 103°
α	+ 8.9°	98	0.0	233	251	—	+ 54°	—
α	+ 8.3°	—	7.0	298	321	—	+ 48°	—
β	- 10.4°	—	0	280	302	14.7	- 51°	- 109°
β	- 8.3°	98	0	274	295	—	- 44°	—
β	- 9.7°	—	2.0	65	70	—	- 47°	- 101°
β	- 9.0°	—	1.2	85	92	—	- 44°	—
$\alpha\beta$	—	91	3.6	782	848	15.2	—	- 109°
$\alpha\beta$	—	96	8.4	846	910	15.6	—	—
Methyl fructose requires			—	—	—	15.9	—	- 110.8° (fructose)

A small portion of the syrupy hydrolysis product was dried to constant weight *in vacuo* at 78° over phosphorus pentoxide and determinations of the methoxy-group were made on this material by the micro-Zeisel method. The specific rotations were determined on aqueous solutions of the partially dried syrups, the weight of hexoside being calculated from the reducing power (Hagedorn and Jensen) after acid hydrolysis. A small error is introduced by this method of calculation owing to the slight destruction of sugar which occurs during the hydrolysis in spite of the rapidity with which the latter takes place. This is shown by the following figures obtained for the reducing power after heating the α - and β -methylhexosides with 0.1 *N* hydrochloric acid at 100° for varying periods.

Reducing power as mg. glucose.

	Time of heating			
	0 min.	3 min.	5 min.	10 min.
α -Methylhexoside	0	233	227	215
β -Methylhexoside	0	274	271	264

The small reducing power shown by some of the syrups before acid hydrolysis and due in part to material derived from the enzyme preparation has also been neglected in these calculations. In view of these possible errors and of the variations in the specific rotations of the original barium methylhexosidediphosphates (see Table I) the values for the specific rotation of the methylhexosides show fair agreement.

The sensitiveness of the methylhexosides to acids is more clearly seen in Table II which shows the progress of hydrolysis of the α - and β - and of the mixed $\alpha\beta$ -isomers by 0.01 *N* acid at room temperature. The final values obtained for the specific rotation of the hexose thus formed are in satisfactory agreement with that of $\alpha\beta$ -fructose (normal), $[\alpha]_{5461} = -110.8^\circ$. The extreme ease of hydrolysis and the specific rotation of the methylhexosides suggested, however, that the latter were not derivatives of the normal but of γ -fructose.

 Table II. *Hydrolysis of methylhexosides with 0.01 N HCl.*

α -Methylhexoside vol. 10 cc., temp. 18°			β -Methylhexoside vol. 10 cc., temp. 18°		Mixed $\alpha\beta$ -hexoside vol. 15 cc., temp. 22–23°		
Time (hours)	α observed ($l=1$)	Reducing power as glucose	α observed ($l=1$)	Reducing power as glucose	Time (hours)	α observed ($l=2$)	Reducing power as glucose
		mg.		mg.			mg.
0	+0.19°	1.2	-0.13°	0.17	0	+0.05	0
4	+0.16°	—	—	—	4	+0.01	1.35
20	+0.12°	5.4	-0.16°	2.5	23	-0.08	6.8
27	+0.05°	6.3	-0.17°	3.1	49	-0.15	—
44	+0.02°	9.3	-0.18°	4.8	72	-0.19	12.3
92	-0.04°	14.8	-0.19°	8.5	96	-0.225	—
116	-0.09°	—	-0.19°	—	120	-0.245	—
140	-0.13°	—	-0.20°	—	168	-0.29	19.8
208	-0.19°	—	-0.215°	—	192	-0.29	19.8
280	-0.25°	26.4	-0.23°	17.5			
336	-0.29°	29.2	-0.24°	22.0			
[α] ₅₄₆₁ of hexose = -99°			[α] ₅₄₆₁ of hexose = -109°		[α] ₅₄₆₁ of hexose = -109°		

Irvine and Patterson [1922] and Menzies [1922] showed that β -methylfructoside when dissolved in 0.009 *N* hydrochloric acid at room temperature possessed a constant rotation for several days, while under parallel conditions the rotation of methyl- γ -fructoside changed from dextro ($[\alpha]_D + 22^\circ$) to laevo in 30 hours and reached the value $[\alpha]_D - 64^\circ$ in 27 days. The specific rotations of the various methylfructosides recorded in the literature so far as we have been able to ascertain are given in Table III. The figures in brackets give the values of $[\alpha]_{5461}$ calculated from those for $[\alpha]_D$ by means of the factor 1.18.

Table III.

	$[\alpha]_D$	$[\alpha]_{5461}$	Reference
α -Methylfructoside (normal)	+45°	(+53°)	Schlubach and Schröter [1928]
β -Methylfructoside (normal)	-172°	(-203°)	Hudson and Brauns [1916]
α -Methyl- γ -fructoside	+65 to +70*	—	Schlubach and Rauchalles [1925]
β -Methyl- γ -fructoside	-17°*	—	Schlubach and Rauchalles [1925]
$\alpha\beta$ -Methyl- γ -fructoside	+26°	(+31°)	Menzies [1922]
α -Methylhexoside from methylhexosidediphosphoric acid	—	+55° (mean)	Present communication
β -Methylhexoside from methylhexosidediphosphoric acid	—	-47° (mean)	Present communication

* These values were calculated from results of experiments on the hydrolysis of $\alpha\beta$ -methyl- γ -fructoside by invertase and are not claimed by the authors as more than approximations to the correct figures.

Tetramethylmethylhexosides.

Further confirmation that the α - and β -methylhexosides possessed the γ or butylene oxide ring was obtained by converting them into the corresponding fully methylated tetramethyl- α - or - β -methylhexoside by the action of silver oxide and methyl iodide [Purdie and Irvine, 1903]. These methylated derivatives were then converted into the tetramethylhexose by removal of the glucosidic methyl group by hydrolysis with dilute acid.

The very small quantities of the original methylhexosides, the poor yields obtained in the methylation and the unstable nature of the products added considerably to the difficulty of obtaining the tetramethylhexose in sufficiently pure condition for identification. Five successive methylations were carried out, in the first two of which a small quantity of methyl alcohol was added as an extraneous solvent. In the subsequent methylations the material was soluble in methyl iodide.

We did not succeed in raising the percentage of methoxyl in the crude methylated product above 50 % against a theoretical value of 62 % for tetramethylmethylhexoside. It was, however, found possible to purify these syrups by fractional distillation under pressures of about 1 mm., and although, when applied to such small quantities, this method could not be expected to yield perfectly pure products, a considerable improvement was effected, as can be seen from the percentage of CH_3O in the distillate (Table IV).

Table IV.

Hexoside methylated	CH_3O before distillation %	CH_3O after distillation %	Fraction collected	Weight of distillate mg.
α	44.6	50.3	—	38
β	49.6	56.8	100–110° (1–1.5 mm.)	88
β	49.6	54.8	—	54
$\alpha\beta$	49.8	61.7	87–100° (0.5 mm.)	180

Hydrolysis of the tetramethylmethylhexosides.

The fully methylated non-reducing hexosides were converted into the tetramethylhexose by hydrolysis with dilute mineral acid. The compounds were dissolved in water and concentrated hydrochloric acid was added to give a 0.1 *N* solution. The rotation became constant after heating for 3–5 minutes at 100°, showing that hydrolysis was complete. Table V gives the values of the specific rotations of the tetramethylmethylhexosides together

Table V.

Methylated product derived from	Before hydrolysis (tetramethyl- methylhexoside) [α] _{D461}	After hydrolysis (tetramethylhexose) [α] _{D461}
α -Hexoside	+ 15.5°	+ 25.6°
β -Hexoside	– 6.8°	+ 31.9°
$\alpha\beta$ -Hexoside	– 1.3°	+ 40°

with those of the tetramethylhexose produced on hydrolysis. The three values for the tetramethylhexose should be identical since on removing the glucosidic methyl group the equilibrium mixture of α - and β -forms would be obtained, but the purity of the products probably varied with the amount of the tetramethylmethylhexoside distilled, this amount being least in the case of the α -compound and greatest in the case of the $\alpha\beta$ -mixture. The tetramethylhexose obtained from the latter gave a value of 52.1 % CH_3O against 52.5 % calculated for tetramethylfructose. The specific rotation of this product ($[\alpha]_{5461} + 40^\circ$) is in close agreement with that found by Irvine and Steele [1920] and Haworth [1920] for tetramethyl- γ -fructose ($[\alpha]_D + 31^\circ$) but widely different from the specific rotation of crystalline (normal) tetramethylfructose ($[\alpha]_D - 121^\circ$; [Purdie and Paul, 1907]). From these results we therefore conclude that the isomeric methylhexosides prepared from hexosediphosphoric acid were derivatives of γ -fructose.

Constitution of hexosediphosphoric acid.

The formation of methyl- γ -fructoside from hexosediphosphoric acid does not at once prove that the latter compound is itself a derivative of γ -fructose, since it has been shown by Purdie and Paul [1907] and Menzies [1922] that ordinary fructose, esterified by Fischer's method, yields a mixture of normal and γ -methyl derivatives. However, the $\alpha\beta$ -methylhexoside which yielded the purest tetramethyl- γ -fructose ($[\alpha]_{5461} + 40^\circ$) represented about 80 % of the theoretical yield from the original hexosediphosphoric acid and neither in this nor in other preparations was evidence obtained of other isomers of different properties which might correspond with the more stable normal derivatives.

This would seem to indicate that the free acid also possesses the γ -fructose structure but it is hoped that further work now in progress will yield more conclusive evidence on this point.

The presence of the butylene oxide ring in the methylhexosidediphosphates implies that neither of the phosphoric acid groups can be in position 5; while, if the presence of a similar oxide ring in the free hexosephosphoric acid is accepted, we may infer that one phosphoric acid group is in position 6 since if this terminal CH_2OH group were unsubstituted we should expect the stable amylenoxide ring to be formed. Accepting Young's conclusion as to the position of the other phosphoric acid group we suggest therefore that hexosediphosphoric acid is γ -fructose-1 : 6-diphosphoric acid.

If this structure proves to be correct hexosediphosphoric acid will fall into line with other naturally occurring fructose derivatives, sucrose, raffinose, inulin, in which the fructose has been shown to possess the unstable butylene oxide ring.

SUMMARY.

1. By the action of the bone phosphatase on the α - and β -methylhexoside-diphosphoric acids the corresponding α - and β -methylhexosides have been obtained as non-reducing syrups.
2. The properties of these compounds agree with those expected for α - and β -methyl- γ -fructosides.
3. These methylhexosides have been further converted into fully methylated derivatives from which tetramethyl- γ -fructose has been obtained by acid hydrolysis.
4. The bearing of these facts on the constitution of hexosediphosphoric acid is discussed and it is suggested that this compound is γ -fructose-1 : 6-diphosphoric acid.

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