XCII. THE CHEMISTRY OF HEXOSE-
DIPHOSPHORIC ACID. PART I.
THE α - AND β - METHYLHEXOSIDEDIPHOSPHORIC
ACIDS¹.

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(*Received May 2nd, 1927.*)

THE application of the process of methylation in the sugar group has proved of such great value in the elucidation of the structure of these compounds that it seemed to afford a promising method of investigating the constitution of hexosediphosphoric acid.

The general problem of the chemical constitution of hexosediphosphoric acid divides itself naturally into two parts. The first concerns the exact nature of the hexose portion of the molecule, while the second deals with the position of the two phosphoric acid groups in the six carbon atom chain.

The earlier work of Young [1909] showed that hexosediphosphoric acid on hydrolysis with boiling hydrochloric acid yielded free phosphoric acid along with a laevo-rotatory solution containing fructose or possibly some other reducing sugar. The fact observed by Young that the ratio of the reducing power to the rotation of the product of hydrolysis was greater than corresponds with pure fructose was explained by Neuberg and Kretschmer [1911] as due to the formation of decomposition products owing to the prolonged boiling with acid. That this explanation was not in itself sufficient was shown later by Young who found that pure fructose after heating with phosphoric acid for some hours showed only a slight decrease in the ratio $\frac{\text{Rotation}}{\text{Reduction}}$.

The behaviour of hexosediphosphoric acid towards phenylhydrazine has been investigated both by Lebedev [1909, 1910, 1911] and Young [1911]. The latter worker found that a molecule of phosphoric acid was split off during the formation of the osazone, but that, if the reaction was carried out, using cold reagents, hexosediphosphoric acid reacts without loss of phosphorus to form a derivative which he regarded as a phenylhydrazine salt of hexosediphosphoric acid phenylhydrazone. These reactions led Young [1911] to put forward the following structures which show the probable position of one of the phosphoric acid groups in the molecule. Formula I assumes glucose and formula II fructose as the parent hexose in hexosediphosphoric acid.

¹ This paper was communicated to the Biochemical Society on Feb. 7th, 1927, and an abstract was published in *Chemistry and Industry*, 1927, 46, 129 (Feb. 11th).

The production of a fully methylated derivative of hexosediphosphoric acid offers a fresh method of attacking the constitution of this substance, since it has been shown by the work of Purdie and Paul [1907], Irvine and Patterson [1922] and Howarth and Hirst [1926] that the methylation and subsequent oxidation of fructose has yielded sufficient evidence for the determination of the constitution of this sugar.

Some early experiments on the production of a methylated hexosediphosphoric acid showed that considerable oxidation occurred during the methylation. When the silver oxide was added to the hexosediphosphoric acid dissolved in methyl alcohol and methyl iodide, a vigorous reaction ensued, but only a small yield of the methylated compound was obtained. Even when the alkylating agent was used in considerable excess, the methyl derivative was produced in very small quantity. As has been explained by Purdie and Irvine [1903] in the case of glucose, the poor yield is partly due to the fact that the alkylation extends to the solvent methyl alcohol and that much of the alkylating agent is thus lost. Examination of the silver residues after the methylation showed that there had been a production of metallic silver, thus indicating that oxidation had occurred. Previous work on the production of tetramethylglucose and tetramethylfructose showed that to avoid oxidation during methylation it was necessary that the aldehydic or ketonic group of the sugar should be protected, and that instead of the free sugar, the methylglucoside or methylfructoside should be used. The application of these principles to hexosediphosphoric acid has led to the preparation of the methyl-hexosediphosphates.

Since hexosediphosphoric acid is a derivative of a hexose, most probably fructose, it must necessarily, from stereochemical considerations, exist in α - and β -modifications, corresponding to the α - and β -forms of the parent sugar. Obtained as a result of yeast fermentation, hexosediphosphoric acid has an optical activity, $[\alpha]_D + 3.4^\circ$, which suggests that it is a mixture of two isomeric acids. In order to detect the production of the two methyl-hexosediphosphoric acids during the condensation of hexosediphosphoric acid with methyl alcohol, this process, and subsequent hydrolysis of the hexoside, were followed by polarimetric observations.

Assuming that hexosediphosphoric acid is a derivative of the ordinary *d*-fructose, the methylhexosediphosphate corresponding to β -methyl-*d*-fructoside, $[\alpha]_D - 172^\circ$, should possess the greater laevo-rotation of the two hexosides. α -Methylfructoside, $[\alpha]_D - 10^\circ$, is much less laevo-rotatory than the corresponding β -compound, that is, the more strongly dextro- or less laevo-rotatory methylhexosediphosphate should correspond to α -methylfructoside and would itself be α -methylhexosediphosphate. Since the methylhexoside-

diphosphate as obtained by Fischer's method is dextro-rotatory, one component of the mixture must possess a dextro-rotation and this, from the above reasoning, would be the α -methylhexosidediphosphate. It has been shown by Purdie and Paul [1907] that α -methylfructoside (β -methylfructoside in their paper) is more rapidly formed than the corresponding β -compound when *d*-fructose is methylated by Fischer's method. Applying these considerations to hexosediphosphoric acid, it would be expected that the α -methylhexosidediphosphate would be formed more rapidly at the commencement of the experiment and thus give rise to an increase in dextro-rotation; the slower production of the β -compound causing the optical rotation of the solution to fall towards the end of the experiment. Exactly the reverse of these optical changes should take place on complete hydrolysis of the mixed methylhexosidediphosphates by acid. There should be a fall in optical rotation, corresponding with the more rapid hydrolysis of the α -compound, followed by a diminished negative rotation towards the end of the hydrolysis. It will be seen from the experimental portion of this paper (Figs. 1 and 2) that there is complete qualitative correspondence between the predicted and experimental changes in optical rotation.

Preparation of barium methylhexosidediphosphate.

The hexoside was prepared from pure barium hexosediphosphate according to Fischer's method [1895]. The methyl alcohol used in all the experiments was free from acetone [see Menzies, 1922] and was fractionally distilled over sodium immediately before use, the portion boiling between 64.5° and 65.5° being collected.

The barium hexosediphosphate (20 g.) was added in the form of a fine powder to 400 cc. of methyl alcohol containing 6.75 g. of dry hydrogen chloride. These quantities give, after complete solution of the barium salt, a solution containing 0.5 % of free hydrogen chloride, which is the concentration of acid used by Fischer throughout his preparations of the α - and β -methylglucosides. In order to obtain the barium hexosediphosphate in solution as rapidly as possible the reaction was carried out in a thick glass bottle containing a number of glass beads which served to grind up any large particles of the salt formed during the rapid addition of the barium salt to the acid methyl alcohol. Under these conditions 5 or 6 minutes' shaking completed the solution of the salt and a clear pale yellow methyl alcohol solution resulted. As soon as this stage was reached a portion of the solution was filtered into a 4 dm. polarimeter tube and the rotation measured. If the precautions mentioned were observed it was possible to obtain a polarimeter reading 12–15 minutes after the barium hexosediphosphate had first come into contact with the acid methyl alcohol. The optical rotation, reducing power and inorganic phosphorus of the solution were determined at intervals until methylation was complete. The values obtained for one particular preparation are shown in Table I.

For the first 2 or 3 hours the solution of the barium hexosediphosphate in acid methyl alcohol remained perfectly clear. The barium chloride after this time was rapidly thrown out of solution as a crystalline mass, only about half of the barium, however, being deposited as chloride during the course of the methylation. During the methylation the temperature was kept at 25° since higher temperatures caused a certain degree of hydrolysis of one of the phosphoric acid groups.

Table I.

Time		Observed* rotation	Total phosphorus g.	Inorganic phosphorus g.	Sugar as glucose per 1 cc. of solution mg.
15 mins.		+0.70°	2.01	0.28	5.29
1 hour	5 mins.	+0.74	—	—	—
4 hours	30 "	+0.78	—	—	—
†7	" 30 "	+0.83	2.16	0.31	2.64
24	"	+0.88	—	—	1.80
32	"	+0.79	—	—	—
48	"	+0.66	—	—	—
56	"	+0.57	—	—	—
70	"	+0.54	—	—	1.12
78	"	+0.54	—	—	—
92	"	+0.53	—	—	0.70
100	"	+0.53	2.11	0.37	0.51
114	"	+0.53	1.96	0.34	0.48
144	"	+0.53	—	—	—
168	"	+0.525	2.02	0.33	—

* Mercury green line.

† Temperature raised to 25° until methylation was complete.

Fig. 1. Curve showing the change in optical rotation during the methylation of hexosediphosphoric acid.

It will be seen from the curve that there is a definite rise followed by a fall in the optical rotation. Since theoretically hexosediphosphoric acid can exist in α - and β -modifications, the rise and fall in optical rotation at once suggest the methylation of two compounds, one of which methylates to give a hexoside with a more strongly positive rotation than barium hexosediphosphate, while, more slowly, a hexoside with a negative rotation is formed. On arresting the reaction after 24 hours, that is, when the maximum positive rotation was obtained, a methylhexosidediphosphate was recovered which possessed a specific rotation of $+8.3^\circ$ which is more than twice the value of the original barium hexosediphosphate.

Table I shows that during methylation there was a small but definite increase in the inorganic phosphorus showing that slight hydrolysis of one of the phosphoric acid groups of the hexosediphosphoric acid had taken place. In order to be certain that any phosphorus hydrolysed was not converted to methyl phosphate, thus escaping detection as inorganic phosphate, an experiment was made using orthophosphoric acid in methyl alcohol and hydrogen chloride, the conditions of concentration, time and temperature being similar to the main hexoside preparation. No change, however, in the concentration of inorganic phosphate could be detected.

When the hexoside preparation showed no further fall in optical rotation and caused no reduction of Fehling's solution, the barium chloride formed during the methylation was filtered off and the clear methyl alcoholic solution was shaken with a slight excess of barium carbonate in order to remove the excess hydrogen chloride. The solution was again filtered and neutralised to phenolphthalein with a methyl alcoholic solution of baryta. The crude hexoside was precipitated under these conditions as a white amorphous powder.

Separation of the α - and β -barium methylhexosidediphosphates.

Fractionation by alcohol. The crude barium salt, which contained small quantities of barium phosphate, unchanged barium hexosediphosphate and barium chloride, was ground with 5 parts of ice-cold water for about 30 minutes. The mixture was then filtered and the residue again ground with the same quantity of water. This extraction operation was continued until 20 or 25 parts of water had been used. The residue still contained a small amount of organic phosphorus, the last traces of which were exceedingly difficult to remove by extraction with water. The combined filtrates were then fractionally precipitated by the addition of ethyl alcohol.

For the purpose of following the fractionation, the details are given of one particular experiment in which 40 g. of crude, air-dried barium hexosidediphosphate were used.

				Weight of fraction
				g.
A.	1st fraction.	Final alcohol concentration 15 % (by volume)		16.0
B.	2nd	Alcohol concentration increased from 15-35 %		7.7
C.	3rd	Alcohol concentration increased from 35-80 %		5.5

The first fraction (A) was dissolved in 10 parts water and increasing quantities of alcohol were added, when the following sub-fractions were obtained:

		$[\alpha]_{\text{Hg green}}^{18-19^\circ}$	Weight of fraction g.
(1)	Fraction precipitated with alcohol up to 5 % concentration	+ 3.0°	5.0
(2)	" " increasing from 5-10 % conc.	+ 0.3	5.8
(3)	" " " 10-15 % "	- 1.3	1.8
(4)	" " " 15-70 % "	- 2.4	1.8

The result of this fractionation showed at once that at least two compounds, one possessing a positive and the other a negative rotation, were present in the crude hexoside preparation.

The second main fraction (B), obtained by increasing the alcohol concentration from 15 to 35 % as shown above, was also dissolved in 10 parts of water and fractionally precipitated with alcohol.

		$[\alpha]_{\text{Hg green}}^{18-19^\circ}$	Weight of fraction g.
(1)	Fraction precipitated with alcohol up to 10 % concentration	- 1.1°	3.6
(2)	" " increasing from 10-15 % conc.	- 1.9	2.1
(3)	" " " 15-35 % "	Slightly positive	About 1 g.

The third main fraction (C), which was only precipitated by alcohol above 35 % concentration, dissolved readily in 10 parts of 20 % alcohol, thus differing considerably in solubility from the first and second fractions. The process of fractional precipitation was carried out as for the previous two fractions but no indication of separation into two different optically active substances was obtained. The specific rotation of the fractions remained constant at about + 0.7°.

The combined fractions from the first main fraction, which possessed a positive rotation, were again dissolved in 10 parts of water and fractionally precipitated with alcohol, the progress of the separation being followed by the determination of the specific rotation of the various fractions. As a result of several fractionations a barium hexosidediphosphate was obtained which possessed a specific rotation of + 4.6°.

Similar methods of separation were then applied to those fractions which possessed negative rotation, when a barium salt possessing a specific rotation of - 2.3° was obtained.

The occasional occurrence of fractions possessing higher specific rotations for example, values such as - 5.5° or + 5.0°, at once suggested that the resolution into optical isomers was far from complete.

Fractionation by brucine. The optically active fractions obtained were then converted into brucine salts. The barium salt was dissolved in water and a slight excess of the theoretical quantity of sulphuric acid carefully added in the form of a 2 *N* solution. Brucine in 10-20 % excess of the calculated quantity was then added in a warm 50 % alcoholic solution, the mixed solutions possessing a pale yellow colour.

In the case of the brucine salt prepared from the β -methylhexosidediphosphate crystals separated within a few minutes of mixing the solutions. After standing overnight, the crystals were filtered off, dissolved in 10 parts of hot 50 % alcohol and allowed to crystallise. The process of recrystallisation was carried out several times, the mother-liquors from each recrystallisation being concentrated to about half their original bulk and mixed together.

A portion of the sparingly soluble salt so obtained was dried to a constant weight at 78° and 15–20 mm. pressure over phosphorus pentoxide, no discoloration taking place.

Phosphorus, estimated by modified Briggs-Doisy method	Found P 3.1 %
Calculated for $C_6H_3O_3(PO_4H_2)_2(OCH_3)(C_{23}H_{26}N_2O_4)_4$	P 3.2 %
Specific rotation of the brucine salt in 10 % alcohol (conc. 0.38 %) $[\alpha]_{H_g}^{18^\circ}$	green - 38.4°

The isolation of the isomeric α -brucine salt in a pure condition proved a much more difficult process. The preparation of the brucine salt was carried out exactly as has been described above for the β -compound. The β -compound present could be slowly removed by concentrating the alcoholic solution of brucine salt and allowing it to stand in the cold room (0°) overnight. The α -compound remained in solution. A micro-crystalline mass of the brucine α -methylhexosidediphosphate could, however, be obtained by pouring this concentrated alcoholic solution into 3 volumes of dry acetone. Under these conditions the excess of brucine remained in solution. This process of acetone precipitation was repeated several times until the brucine salt was free from excess of brucine. It was then fractionally crystallised from hot 96 % alcohol, the process of separation being followed by converting small portions of the fractions into barium salts and determining the specific rotation. The final brucine salt, after drying at 78° (20 mm.) over phosphorus pentoxide, gave the following analytical figures:

Found	P 2.9 %
Calculated for $C_6H_3O_3(PO_4H_2)_2(OCH_3)(C_{23}H_{26}N_2O_4)_4$	P 3.2 %

Barium β -methylhexosidediphosphate.

The brucine β -methylhexosidediphosphate was converted into the barium salt by treatment with a 50 % alcoholic solution of baryta until the mixture was pink to phenolphthalein. The liberated brucine passed into solution while the barium β -methylhexosidediphosphate was precipitated. It was filtered off, washed with warm 96 % alcohol to remove any adhering brucine, and dried in a vacuum desiccator over sulphuric acid. The salt was then dissolved in 10 parts of ice-cold water and filtered to remove any barium carbonate, the barium salt being again precipitated with alcohol and dried. This process of solution in water and reprecipitation with alcohol must be carried out until the barium salt is perfectly free from brucine, otherwise the observed specific rotation will be much more laevo than the true value.

The final barium salt was obtained as a white amorphous substance which was distinctly more soluble in cold than hot water, thus resembling in solubility the original barium hexosediphosphate.

Analysis. For analysis the salt was dried to constant weight over phosphorus pentoxide. After heating for 2 to 3 hours at 100° (20 mm.) it remained perfectly white, in this respect differing from hexose mono- and di-phosphate.

The estimations of methoxyl groups and barium were carried out by Pregl's micro-methods.

	OCH ₃	P	Ba
Found	4.93 %	9.76 %	43.75 %
Calculated for C ₆ H ₉ O ₃ (OCH ₃) (PO ₄ Ba) ₂	4.97 %	9.92 %	43.91 %

Specific rotation of the salt in water (conc. 3.16 %) $[\alpha]_{\text{Hg green}}^{18^\circ} - 10.4^\circ$

The specific rotation of the β -methylhexosidediphosphoric acid (conc. 1.66 %):

$$[\alpha]_{\text{Hg green}}^{18^\circ} - 23.2^\circ$$

Molecular weight determination by the cryoscopic method yielded the following results, samples from two distinct preparations being used:

(1) 0.0942 g. barium salt in 9.95 g. water gave $\Delta = 0.074^\circ$, whence apparent molecular weight = 236

(2) 0.0807 g. barium salt in 9.95 g. water gave $\Delta = 0.053^\circ$, whence apparent molecular weight = 284

C₆H₉O₃(OCH₃) (PO₄Ba)₂ requires molecular weight = 624; if 100 % dissociated apparent molecular weight = 208

The experimental results are, therefore, consistent with this formula.

The free β -methylhexosidediphosphoric acid, liberated by the addition of the theoretical quantity of sulphuric acid to the pure barium salt, decomposed rapidly. Concentration of the aqueous solution of the acid in a vacuum desiccator led to rapid elimination of the methyl group. By working with ice-cold solutions, it was possible to obtain the acid as a colourless syrup free from inorganic phosphate, but the aqueous solution of this syrup, after standing at room temperature for 36 hours, showed a considerable reducing power, the estimation of which showed that 23 % of the methyl group had been removed.

Barium α -methylhexosidediphosphate.

This salt was prepared by exactly the same method as has been described for the β -compound and was analysed in the same way.

Analysis.

	OCH ₃	P	Ba
Found	5.11 %	9.89 %	43.55 %
Calculated for C ₆ H ₉ O ₃ (OCH ₃) (PO ₄ Ba) ₂	4.97 %	9.92 %	43.91 %

Specific rotation of the salt in water (conc. 3.65 %): $[\alpha]_{\text{Hg green}}^{18^\circ} + 8.2^\circ$

Specific rotation of the free α -acid (conc. 1.96 %): $[\alpha]_{\text{Hg green}}^{19.5^\circ} + 19.7^\circ$

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

(I) 0.0780 g. barium salt in 9.95 g. water gave $\Delta = 0.062^\circ$, whence apparent molecular weight = 235

(II) 0.0557 g. barium salt in 9.95 g. water gave $\Delta = 0.054^\circ$, whence apparent molecular weight = 193

Barium methylhexosidemonophosphate.

During the preparation of the α - and β -methylhexosidediphosphates by Fischer's method, there always occurred a slight increase in the inorganic phosphorus present, thus indicating that hydrolysis of the phosphoric acid groups in the original hexosediphosphoric acid had occurred. The actual amount of inorganic phosphorus so formed varied slightly in each experiment but never exceeded 3.0 % of the total organic phosphorus.

Since one phosphoric acid group is removed by hydrolysis much more readily than the other in hexosediphosphoric acid, it would seem that a hexosemonophosphoric acid, or methylhexosidemonophosphoric acid as it would become after methylation, would be the most likely product. From the crude barium methylhexosidediphosphates a very soluble barium compound was isolated and proved to be a methylhexosidemonophosphate.

As has already been stated (p. 680), fractional precipitation of the barium salt gave no indication of the presence of two optical isomers and fractional crystallisation of the brucine salt from 50 % alcohol also failed to give any separation. The solubility of the salt in 50 % alcohol, however, was such that crystals of the brucine salt could only be obtained from very concentrated solutions. The crystalline brucine salt could, however, be readily obtained from alcohol-acetone solution.

The specific rotation of the brucine salt (conc. 1.45 %) $[\alpha]_{\text{Hg green}}^{19} - 31.7^\circ$

Calculated for $\text{C}_6\text{H}_{10}\text{O}_4(\text{PO}_4\text{H}_2)(\text{OCH}_3)(\text{C}_{23}\text{H}_{26}\text{N}_2\text{O}_4)_2$	P	2.91 %
Found	P	3.06 %

The brucine salt was converted to barium salt as has been described for the α - and β -methylhexosidediphosphates.

Analysis.

	OCH_3	P	Ba
Found	7.48 %	7.50 %	35.12 %
Calculated for $\text{C}_6\text{H}_{10}\text{O}_4(\text{OCH}_3)(\text{PO}_4\text{Ba})$	7.57 %	7.57 %	33.52 %

The specific rotation: $[\alpha]_{\text{Hg green}}^{19.5} + 0.92$ (conc. 1.08 %)

The hydrolysis of α - and β -methylhexosidediphosphate with acid.

Considerable difficulty has been encountered in attempting to remove the methyl group from each of these hexosides while leaving both phosphoric acid groups intact. With complete removal of the methyl group by acid hydrolysis there always occurred a slight removal of the phosphoric acid groups. Under these conditions too rigid a value cannot be placed on the determination of reducing power, as a means of distinguishing the end-point of the elimination of the methyl group. The barium salt of the methylhexosidediphosphoric acid was dissolved in normal hydrochloric acid and kept at 25° until the solution possessed a reducing power which agreed with the complete removal of the methyl group. The optical rotation of the solution was measured at intervals during the hydrolysis, details of which are shown in Tables II and III and Fig. 2.

Fig. 2. Optical changes during hydrolysis of α -, β - and $\alpha\beta$ -methylhexosidediphosphate with *N* HCl at 50°.

Table II.

Approximately 0.30 g. of barium β -methylhexosidediphosphate dissolved in 10 cc. of *N* HCl. Temperature 25°. Rotation for mercury green line.

Time (in hours)	Observed rotation	Total P mg.	Inorganic P mg.	Reducing power as glucose mg.
15 mins.	-1.07°	35	Nil	0.28
2 hours	-1.02	34	—	—
3.5 "	-1.00	—	Nil	0.80
24 "	-0.70	—	Nil	12.1
40 "	-0.54	—	0.5	16.8
62 "	-0.30	—	2.1	27.2
86 "	-0.19	—	2.8	33.0
110 "	-0.15	35	3.4	39.0
134 "	-0.08	—	—	—

Table III.

Approximately 0.35 g. of barium α -methylhexosidediphosphate dissolved in 10 cc. of *N* HCl. Temperature 25°. Rotation for mercury green line.

Time (in hours)	Observed rotation	Total P mg.	Inorganic P mg.	Reducing power as glucose mg.
15 mins.	+0.78°	34.3	Nil	0.19
3 hours	+0.67	—	—	—
18 "	+0.57	—	—	13.0
24 "	+0.36	35.0	0.5	14.9
42 "	+0.14	—	—	21.5
66 "	+0.01	35.0	1.4	29.2
90 "	-0.08	—	—	41.3

Both α - and β -hexosidediphosphoric acids are produced as a result of the removal of the methyl group but as both forms are unstable in solution, the observed rotation in the case of the α -acid falls while that of the β -acid rises until both values are approximately equal and should, in absence of any hydrolysis of phosphoric acid groups, be equal to the rotation of the commonly known hexosediphosphoric acid in which mutarotation is complete. It will be seen from Tables II and III and Fig. 2, that the final optical value is slightly laevo-rotatory. Presumably this can be explained as due to the production of a small quantity of a laevo-rotatory decomposition product since in both cases a slight production of inorganic phosphate had taken place.

In order to determine the relative rates of hydrolysis of the α - and β -methylhexosidediphosphates, equal quantities of these two substances were

dissolved in the same volume of $N/10$ hydrochloric acid and kept at 50° until the reducing power of each corresponded to complete removal of the methyl group. Estimation of the inorganic phosphate at the end of the experiment showed that not more than 2% of the total organic phosphate had been hydrolysed. The relative rates of hydrolysis of these two compounds are shown in Fig. 3.

Action of emulsin on the α - and β -methylhexosidediphosphates.

β -Methylfructoside is not hydrolysed by emulsin although this enzyme is generally considered specific in its action on all β -hexosides. Whether emulsin attacks β -methylhexosidediphosphate will, therefore, probably depend on whether or not fructose is the carbohydrate part of the molecule.

Fig. 3. Hydrolysis of α - and β -methylhexosidediphosphate by $N/10$ HCl at 50° .

Two solutions containing 50 mg. of each of the hexosides in 10 cc. water were treated with 0.2 cc. of concentrated emulsin extract prepared from sweet almonds. The solutions were incubated at 37 – 38° in the presence of toluene. As a control on the activity of the enzyme preparation, solutions of α -methylglucoside and salicin were treated, under the same conditions, with an equal quantity of enzyme solution. The results are shown in Table IV.

Table IV.

	Reducing power of the solution (mg. glucose) Time in hours					Rotation in 1 dm. tube Time in hours			
	0	3	16	40	64	0	16	40	88
α -Methylhexoside-diphosphate	0.50	0.50	0.48	0.44	0.50	+0.05°	+0.04°	+0.04°	+0.02°
β -Methylglucoside	0.38	0.41	0.38	0.38	0.38	-0.06	-0.07	-0.07	-0.09
Salicin	1.36	4.11	14.30	17.20	—	-0.39	-0.24	-0.19	-0.19
Boiled enzyme	0.36	0.36	0.31	—	—	-0.01	-0.01	-0.01	—

Emulsin has, therefore, no hydrolytic action on the methoxyl group of the α - and β -methylhexosidediphosphates. There is, however, a considerable hydrolysis of organic phosphate since the inorganic phosphate present at the end of the experiment was 5.2 % and 7.2 % of the total organic phosphorus for the α - and β -hexosides respectively. That the liberation of this quantity of phosphorus did not in itself rapidly increase the reducing power of the solution showed that the methyl groups remained attached to the reducing groups of the hexosides.

Action of invertase on the α - and β -methylhexosidediphosphates.

50 mg. of each of the hexosides were dissolved in 10 cc. water and treated with 0.5 cc. of invertase solution. The activity of the enzyme was followed by using control solutions of sucrose, α -methylglucoside and salicin. The solutions were kept at 37° throughout the experiment. Complete hydrolysis of the α - or β -compound would yield a reducing solution equivalent to 5.0 mg. glucose. The results of the experiment are shown in Table V.

Table V.

Substance	Reducing power of solutions (as glucose, mg.)				
	Time in hours				
	0	4	20	44	68
α -Methylhexosidediphosphate	1.22	—	2.64	2.64	2.64
β - " "	1.25	1.28	1.58	1.59	1.59
α -Methylglucoside	1.25	1.30	1.30	1.62	1.58
Salicin	2.30	2.28	2.34	2.34	2.64
Sucrose	1.27	32.1	58.0	59.0	59.0
Boiled enzyme	1.20	1.30	1.25	1.39	1.39

There has apparently been some hydrolysis in the case of the α -hexoside (25 %). In another experiment 32 % of the α -hexoside was hydrolysed, thus indicating that invertase slowly attacks the α -, but not the β -compound. Removal of the phosphate groups also occurred to the extent of 10 and 13 % of the total organic phosphorus in the α - and β -hexoside respectively. Harding [1912], using a crude invertase preparation, found that the phosphoric acid groups of hexosediphosphate were rapidly removed by hydrolysis.

The hydrolytic removal of the phosphate groups in the experiments described above was no doubt due to hexosephosphatase contained in the invertase, which had been prepared from autolysed yeast.

The methylation of hexosediphosphoric acid.

As has been described in the introduction, a few preliminary experiments were made, during the earlier part of this investigation, on the methylation of the free hexosediphosphoric acid in methyl alcohol solution.

Barium hexosediphosphate (20 g.) in the form of a fine powder was slowly added to 50 cc. ice-cold methyl alcohol containing sufficient sulphuric acid to precipitate the whole of the barium as barium sulphate. After standing for

a few hours in the cold room (0°) the whole of the barium sulphate was precipitated. The clear methyl alcohol solution of the hexosediphosphoric acid was then concentrated to a volume of about 10–15 cc. and methylated according to the method of Purdie and Irvine [1903]. The methylation of the compound was carried out in three stages and a large excess of the methylating agents was used. The methyl alcohol solution was mixed with 12 mols. of methyl iodide and warmed on a water-bath. Silver oxide (6 mols.) was then slowly added over a period of 5 hours and the heating continued for some hours after the last addition of silver oxide. The product was then filtered and the residue of silver oxide and silver iodide extracted with boiling methyl alcohol. The filtrate together with the methyl alcohol extracts was evaporated in a desiccator, when a pale yellow syrup was obtained: yield 2 g. This syrup was again methylated using the minimum quantity of methyl alcohol possible to effect solution (about 5 cc.). A non-reducing colourless syrup was obtained which was free from inorganic phosphate but contained 14.5 % of organically combined phosphorus (7OCH_3 requires 14.1 % P). Estimation of methoxyl groups showed that the substance contained 42.5 % as against 49.5 % OCH_3 for the fully methylated hexamethyl-methylhexosidediphosphoric acid. Further methylation produced a small quantity of a colourless syrup which contained 47.8 % OCH_3 , thus indicating a mixture of compounds containing 6 and 7 OCH_3 groups. An attempt to distil this syrup under 1 mm. pressure only gave rise to decomposition. Further work on the production of methylated derivatives of hexosediphosphoric acid is in progress.

The hydrolysis of β -methylhexosidediphosphate with bone enzyme.

The discovery by Robison [1923] of an enzyme in ossifying cartilage, which will rapidly remove the phosphoric acid groups of hexosediphosphate by hydrolysis, suggests a further method of attacking the constitution of the hexose portion of the hexosediphosphoric acid molecule. A method of hydrolysing barium hexosediphosphate with the enzyme, the optimum p_{H} of which is well on the alkaline side of neutrality, is discussed in a recent paper by Martland and Robison [1927] who point out the difficulty of identifying the liberated hexose after it has remained in alkaline solution for several days. The application of this method to β -methylhexosidediphosphate has given more promising results, as the liberated β -methylhexoside does not undergo the Lobry de Bruyn transformation owing to the substituent methyl group attached to the reducing group.

An experiment using 0.3 g. of barium β -methylhexosidediphosphate after treatment with bone enzyme gave, on concentrating the aqueous solution, a syrup which contained 9.9 % OCH_3 (β -methylfructoside requires 15.9 %). A solution of the syrup was non-reducing and strongly laevo-rotatory, $[\alpha]_{\text{Hg}}^{18^{\circ}} - 133^{\circ}$. After boiling a small quantity of the syrup with dilute hydrochloric acid there was a considerable increase in reduction. Control experiments with solutions containing the enzyme together with phosphate

were made and correction made for any optical activity or reducing power due to the enzyme. The few results so far obtained support the evidence of Young and Neuberg that fructose is the hexose part of the hexosediphosphoric acid. Further work on this subject is in progress.

SUMMARY.

1. Two isomeric methylhexosidediphosphoric acids have been prepared from yeast hexosediphosphoric acid.

2. A method is described for the separation of these new compounds.

3. The α -methylhexosidediphosphoric acid is dextro-rotatory

$$[\alpha]_{\text{Hg green}}^{20^\circ} + 19.0^\circ,$$

while the isomeric β -compound is strongly laevo-rotatory

$$[\alpha]_{\text{Hg green}}^{18^\circ} - 23.0^\circ.$$

4. The barium and brucine salts of both of these compounds are described.

5. The methyl group in α -methylhexosidediphosphoric acid is more rapidly removed by hydrolysis than that in the corresponding β -compound.

6. Neither compound is hydrolysed by emulsin.

7. Invertase causes a partial removal of the methyl group from the α -hexoside. The β -methylhexosidediphosphoric acid is not attacked.

8. The methylation of hexosediphosphoric acid yielded a non-reducing syrup consisting largely of the hexamethyl-methylhexosidediphosphoric acid.

9. Bone enzyme rapidly removes the phosphoric acid groups from β -methylhexosidediphosphate, at the same time producing a strongly laevo-rotatory non-reducing substance possessing the properties of a fructoside.

10. A methylhexosidemonophosphoric acid, which was produced in small quantities during the methylation of hexosediphosphoric acid by Fischer's method, has been isolated in the form of its barium salt.

The author wishes to express his most sincere thanks to Prof. A. Harden, F.R.S., and Dr R. Robison for their constant advice and criticism throughout the whole of this investigation.

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