CCXLIV. MANNOSEMONOPHOSPHATE.

III. PHOSPHOMANNONIC ACID AND ITS LACTONES.

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(Received August 19th, 1934.)

ON the basis of the investigations of Young [1909; 1911], Morgan [1927; 1929], Morgan and Robison [1928] and Levene and Raymond [1928] it appears probable that the hexosediphosphoric ester of Harden and Young has the constitution (I).

The occurrence of the Robison ester (hexosemonophosphates) side by side with the Harden and Young ester in the products of fermentation was an indication that some structural relationship existed between these compounds; and the method of preparation of the Neuberg ester (II) by partial hydrolysis of hexosediphosphate was a proof of its genetic relation with the Harden and Young ester.

Robison and King [1931] succeeded by repeated crystallisation of the brucine salts of the mixed monophosphates of fermentation in isolating pure glucose-monophosphate for which they proposed the structure (III).

They also isolated from the same source a fructosemonophosphate identical with the Neuberg ester.

Robison [1932] isolated another ester from the products of fermentation of glucose and fructose with yeast preparations, which he showed to be a derivative of mannose. It gave the same osazone as the three esters previously mentioned and very probably in this case too the phosphate radical occupies position 6 in the hexose molecule.

The experimental facts on which these formulae are based are summarised later in the paper, and additional evidence has been obtained which forms the subject of the present communication.

Neuberg, I. S. and Ostendorf [1930] observed that in the fermentation of mannose with fresh yeast and toluene at 37° large amounts of monophosphates

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were formed. Their crude monophosphate fraction showed a high specific rotation which was possibly due to the presence of trehalosemonophosphate. After the isolation of pure mannosemonophosphate by Robison [1932] from the products of fermentation of glucose and fructose by dried yeast, Jephcott and Robison [1933; 1934] were able to show that at 37° the fermentation of mannose with dried yeast yields a large proportion of monophosphate which is mostly mannosemonophosphate. From these fractions they isolated the mannosemonophosphate in pure condition by the formation of the phenylhydrazone and its subsequent decomposition by benzaldehyde followed by the crystallisation of the barium salt of the ester. Their conclusions have been confirmed in the present investigation and large amounts of mannosemonophosphate have been prepared by a method based on these observations.

EXPERIMENTAL.

Experiments which will be described in a subsequent paper have shown that mannose when fermented with yeast juice or zymin at 37° does not yield a monophosphate fraction rich in mannosemonophosphate. Dried brewer's yeast is the only type of yeast preparation that is known to give rise to such large amounts of the mannose ester. When mannose was fermented with dried baker's yeast at 37° the yield of the monophosphate fraction was low and the nature of this fraction differed considerably from that of the product obtained from the fermentation of mannose with dried brewer's yeast under similar conditions. Table I shows the difference between the crude monophosphate fractions obtained by fermenting mannose with different yeast preparations at 37°.

Table I. *Fermentation of mannose with different yeast preparations at 37°. Analyses of the monophosphate fractions (barium salts).*

Yeast preparation	Monophosphate-P as % total ester-P	P %	Selivanoff fructose %	Reducing power as hexose %		[α] ₅₄₆₁
				H. and J.	Iodine	
Dried baker's yeast	23.2	6.30	6.3	16.5	9.1	+ 29.4°
"	29.5	6.51	6.1	17.5	9.7	+ 39.6
Dried brewer's yeast	85.5	7.25	0.8	29.0	19.4	+ 3.9
Yeast juice	63.8	6.17	4.7	24.8	21.0	+ 19.7
Zymin	39.8	6.65	5.0	21.7	19.4	+ 14.2

Preparation of mannosemonophosphate.

Mannosemonophosphate was prepared by fermenting mannose at 37° with dried English brewery yeast, as described by Jephcott and Robison [1934]. 50 g. dried yeast were mixed with 25 g. mannose and 250 ml. water; after the preliminary period of fermentation a 0.5M solution of Na₂HPO₄ containing 18 % mannose was added at such a rate (10 ml. every 5 minutes) that a nearly constant high rate of fermentation was maintained until a total of 300 ml. solution had been introduced: trichloroacetic acid was then added to give a 4 % concentration. The monophosphate fraction represented 74 % of the esterified P; yield of air-dry crude barium hexosemonophosphate, 43.3 g. The analysis of this salt (Table II) indicated that it probably contained nearly 90 % of mannosephosphate.

Purification through the phenylhydrazone. 12 g. of the crude barium mannosemonophosphate were converted into the free ester and from this the phenylhydrazine salt of phosphomannosephenylhydrazone was prepared. It began to

separate within a few minutes and, after 3 hours at room temperature, was filtered and washed with ice-cold 10 % methyl alcohol in ether and dried *in vacuo*; yield, 9.4 g., nearly 80 % of the amount calculated on the P content¹. It was decomposed by shaking for 3 hours with 25 g. benzaldehyde and 30 ml. water, extracting with ether, again shaking with benzaldehyde and re-extracting with ether. The free mannosemonophosphoric acid in the aqueous layer was converted into barium salt and precipitated with alcohol. The dry salt was redissolved in 10 parts of water and one of alcohol and filtered; crystallisation of the barium salt started at once but was allowed to proceed overnight at 0°. It was further purified by three or four crystallisations from 20–30 % aqueous alcohol.

The mother-liquors obtained after filtering off the sparingly soluble phenylhydrazones were also decomposed with a large excess of benzaldehyde and converted into the barium salts.

Purification by direct crystallisation from the crude monophosphate fractions. Barium mannosemonophosphate was also found to crystallise directly from aqueous solutions of the crude monophosphate fractions when present in concentrations higher than about 40 %. Crystallisation was induced by seeding a 10 % aqueous solution of the crude monophosphate with a few crystals of barium mannosemonophosphate. The salt was recrystallised from aqueous alcohol and, as the following analyses show, was of the same degree of purity as the recrystallised barium salt obtained by the decomposition of the phenylhydrazones. For purposes of comparison the analyses of the residues recovered from the phenylhydrazones mother-liquors and from the mother-liquors after direct crystallisation of the barium mannosemonophosphate fractions are also given in Table II.

Table II. *Analyses of the monophosphate fractions during purification.*

Ester	P %	Selivanoff fructose %	Reducing power as glucose %		[α] _{D461}
			H. and J.	Iodine	
(A) Crude monophosphate; starting material	7.18	1.5	33.9	23.4	+ 2.6°
(B) Crystalline Ba salt by direct crystallisation from (A)	7.80	0.3	35.8	26.6	$\left\{ \begin{array}{l} \text{Free acid} \\ + 16 \\ (c = 1.68) \end{array} \right.$
(C) Crystalline Ba salt from the hydrazones prepared from (A)	7.84	0.3	36.3	24.5	
(D) Ba salt from the mother-liquor of (B)	6.88	4.3	25.8	15.9	+ 2.2
(E) Ba salt from the mother-liquor of phenylhydrazones	6.62	5.4	19.6	19.0	+ 12.5

Monophosphomannonic acid. The oxidation of mannosemonophosphate.

5 g. of the crystalline barium mannosemonophosphate were dissolved in water, and bromine and baryta were added in three portions at intervals of 10 minutes so that both reagents were finally present in 33 % excess [Macleod and Robison, 1933]. The mixture was kept at room temperature, and 40 minutes after the first addition of bromine and baryta it was acidified with dilute HCl. Excess of bromine was driven off by aeration and the acid barium salt of

¹ Starting with pure barium mannosemonophosphate, the yield of hydrazone obtained by the same procedure varied from 70 to 80 % of the calculated weight.

phosphomannonic acid was precipitated at p_H 4.0 by 3 volumes of 96 % alcohol. The precipitate was filtered, washed with absolute alcohol and dried *in vacuo* over sulphuric acid. The dry acid salt was redissolved in a large volume of water and the solution was brought to p_H 8.0 with baryta and poured into twice its volume of alcohol; the neutral salt separated out as an amorphous mass which was filtered off and dried. It was further purified by redissolving in water and reprecipitating with alcohol. (Found for the dry neutral salt: P, 6.48; Ba, 43.24 %; $(C_6H_{10}O_{10}PBa)_2Ba$ requires P, 6.47; Ba, 43.01 %.)

Transformation of phosphomannonic acid into its lactones.

Phosphomannonic acid in aqueous solution undergoes a spontaneous transformation into the lactones, a change which can be followed by polarimetric observations. This property is common to all hydroxycarboxylic acids in which the hydroxyl is in the γ - or δ -position with respect to the carboxyl. The following experiments illustrate such a change; the isolation of the lactones is described later.

(a) *In aqueous solution.* 88.6 mg. of the barium phosphomannonate were decomposed with the calculated amount of *N* sulphuric acid, the volume was made up to 5 ml. and the $BaSO_4$ separated by centrifuging. The clear centrifugate was kept at 24° and polarimetric readings were taken at intervals. The results are given in Table III and Fig. 1, curve *C*, the rotations being calculated for the free acid.

Table III. *Lactone formation in aqueous solution at 24°.*

Time	(α)	$[\alpha]_{5461}$
20 min.	- 0.005°	- 1.0°
1 hour	+ 0.026	+ 5.2
1½ hours	+ 0.030	+ 6.1
2½ "	+ 0.040	+ 8.2
20 "	+ 0.065	+ 13.3
2 days	+ 0.105	+ 21.4
4 "	+ 0.116	+ 23.7

(b) *In dilute HCl.* 0.1267 g. barium phosphomannonate was dissolved in water with 1 ml. of *NHCl* and the volume made to 10 ml. The excess of *HCl* over that required for the complete liberation of phosphomannonic acid was such that the final concentration of free *HCl* was 0.025 *M*. The solution was kept at 24°, and the polarimetric observations were made in a 1 dm. tube. The numbers in Table IV and curve *A*, Fig. 1, show the change in rotation due to lactone formation, the rotations being calculated for the free acid.

Table IV. *Lactone formation in 0.025M HCl at 24°.*

Time	(α)	$[\alpha]_{5461}$
0 hour	- 0.010°	- 1.4°
1 "	+ 0.046	+ 6.6
4 hours	+ 0.075	+ 10.7
9 "	+ 0.100	+ 14.4
25 "	+ 0.125	+ 17.9
7 days	+ 0.204	+ 29.5

After 4 hours a portion of the liquid was heated in a sealed tube at 100° for 1 hour. The rotation rose to +34.6° and remained unchanged after further

heating (curve *B*, Fig. 1). Fig. 1 illustrates the course of lactone formation under different conditions; it appears from curve *A* that two reactions are proceeding simultaneously, one slow and the other rapid, a fact which indicates the formation of two lactones.

Fig. 1. Lactone formation from 6-phosphomannonic acid. *A*, in 0.025 *M* HCl at 24°; *B*, at 100°; *C*, in aqueous solution at 24°.

Preparation of phosphomannono-γ- and phosphomannono-δ-lactone.

It is known [Haworth, 1929] that heating a hexonic acid at 100° for a short time leads to the formation of γ -lactone, while heating at lower temperatures for prolonged periods favours the formation of δ -lactone. This observation has been made use of in the isolation of the phosphomannonolactones.

2 g. of the barium phosphomannionate were decomposed with the calculated amount of $N H_2SO_4$, and the free acid was separated from $BaSO_4$ by centrifuging and washing the residue repeatedly with water. The combined centrifugates were divided into two portions, one being heated in a sealed tube at 100° for one hour and then evaporated to dryness *in vacuo* over sulphuric acid, while the other portion was evaporated to dryness *in vacuo* at room temperature without previous heating. In both cases the lactones separated in star-shaped clusters of small needles after the evaporation of the solvent. Attempts to recrystallise them from alcoholic solutions failed; hence they were purified by washing with cold anhydrous acetone, redissolving in water, filtering and evaporating to dryness *in vacuo* over sulphuric acid and finally over phosphorus pentoxide. The properties and analyses of the pure compounds are given below.

Phosphomannono- γ -lactone: M.P. 125–125.5°; P, 12.10%; $[\alpha]_{5461} + 54.1^\circ$.

Phosphomannono- δ -lactone: M.P. 127.5–128.5°; P, 12.07%; $[\alpha]_{5461} + 60.6^\circ$.

$C_6H_{11}O_9P$ requires P, 12.01 %.

Hydrolysis of the phosphomannonolactones in aqueous solution.

34.7 mg. of the lactone were dissolved in 5 ml. water and the polarimetric readings taken in a dm. tube at 24° (Table V). The amount of free acid present in aqueous solution at any given moment is calculated from the expression [Drew *et al.*, 1927]

$$\frac{(R - R_l)}{100(R_a - R_l)},$$

where R is the specific rotation at any given time, R_a and R_l the specific rotations of the substance in form of the acid and the lactone respectively. The value of R_a is arrived at from the expression

$$r_a \cdot \frac{M_a}{M_l},$$

where r_a is the true specific rotation of the acid and M_a and M_l the molecular weights of the acid and the lactone respectively.

Table V. *Hydrolysis of lactones in aqueous solution at 24°.*

Time in days	γ -lactone		δ -lactone	
	$[\alpha]_{5461}$	Free acid %	$[\alpha]_{5461}$	Free acid %
0	+54.1°	0.0	+60.6°	0.0
1	+53.6	1.0	+58.2	3.8
2	+53.4	1.4	+55.2	8.3
4	+50.5	6.5	+50.9	15.0
6	+48.6	10.0	+47.0	20.9
8	—	—	+43.6	30.7
15	+43.1	19.8	—	—
19	—	—	+43.6	—
30	+43.1	—	—	—

The δ -lactone is more readily hydrolysed in aqueous solution than the γ -lactone; ultimately, however, an equilibrium would be attained between the two lactones and the free acid. It is true that the percentages of free acid calculated are different in the two cases, but the close agreement between the final values of the specific rotations suggests that equilibrium has been reached. Clearly the above formula by which these values for the free acid were calculated is not strictly applicable to this case since three variants instead of two are present in the solution.

Hydrolysis of monophosphomannonic acid by NHCl at 100°.

49.7 mg. of barium phosphomannonate together with 26.1 mg. anhydrous potassium sulphate were dissolved in 10 ml. $NHCl$. Barium sulphate was separated by centrifuging and the clear solution measured into small ampoules, 1.2 ml. in each. The ampoules were then sealed and immersed in a boiling water-bath. At definite intervals one ampoule was taken out, cooled in water and the inorganic phosphate content of the solution determined. Table VI shows the rates of dephosphorylation of the phosphomannonic acid and also of phosphogluconic acid under similar conditions. The latter was a pure sample kindly supplied by Prof. Robison [Robison and King, 1931]. The constant k was calculated according to the equation for unimolecular reactions

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

where x_1 and x_2 represent the amounts hydrolysed at the times t_1 and t_2 respectively, a being the total concentration of the unhydrolysed acid at the beginning of the hydrolysis.

The higher values of k for the first 8 hours are peculiar in that they seem to be common to both phosphomannonic and phosphogluconic acid. This suggests that the compounds undergoing hydrolysis during that period are different from those being hydrolysed in the later stages. It is possible that the free phosphohexonic acids are hydrolysed at faster rates than the corresponding

Table VI. *Hydrolysis of phosphomannonic and phosphogluconic acids in NHCl at 100°.*

Time in hours	Phosphomannonic acid		Phosphogluconic acid	
	Hydrolysis %	$k \times 10^3$	Hydrolysis %	$k \times 10^3$
4	10.4	0.199	13.8	0.267
8	19.2	0.187	23.9	0.228
24	38.6	0.124	45.9	0.154
48	60.8	0.136	67.7	0.155
72	72.9	0.111	—	—
96	82.4	0.131	—	—

lactones which are formed by heating the acids in *NHCl* at 100°. Only when all the acid is converted into the lactone is a constant rate attained. That such is possibly the case is shown by the hydrolyses of phosphomannono- γ - and phosphomannono- δ -lactones which are set out in Table VII. In both cases the rates of hydrolysis of the phosphoric ester linkage were constant from the beginning.

Table VII. *Dephosphorylation of phosphomannonolactones in NHCl at 100°.*

Time in hours	γ -lactone		δ -lactone	
	Hydrolysis %	$k \times 10^3$	Hydrolysis %	$k \times 10^3$
4	6.9	0.129	6.3	0.117
8	12.7	0.116	12.4	0.122
24	32.6	0.117	32.2	0.116

12.9 mg. of the lactone were dissolved in 5 ml. of *NHCl* and the hydrolysis investigated by the procedure already described.

DISCUSSION.

Mannosemonophosphoric ester on mild oxidation yields phosphomannonic acid which in aqueous solution or in presence of dilute *HCl* is transformed into its lactones. By heating the free acid in aqueous solution at 100° for one hour a lactone has been isolated which is probably the γ -lactone; on the other hand slow evaporation of the aqueous solution at room temperature has led to the isolation of another derivative which is probably the δ -lactone. The properties of the two compounds are given below; the figures for the corresponding unsubstituted mannonolactones, quoted from Haworth and Nicholson [1926], are shown for comparison.

Lactone	M.P.	$[\alpha]_D$	$[\alpha]_{5461}$
Phosphomannono- γ -lactone	125–125.5°	—	+54.1°
Phosphomannono- δ -lactone	127.5–128.5	—	+60.6
γ -Mannonolactone	151	+51.8°	—
δ -Mannonolactone	156	+114	—

The transformation of phosphomannonic acid into its lactones and the rates of hydrolysis of these lactones in aqueous solution appear to be in general agreement with the known behaviour of the corresponding substituted and unsubstituted sugar acids and their lactones. Hence it is probable that the designations γ -lactone and δ -lactone for the two isolated phosphomannonolactones are correct, although further confirmation would be desirable.

Assuming then that these two lactones are the 1:4- and 1:5-lactones of phosphomannonic acids the further conclusion would follow that the positions 4 and 5 in the acid and therefore in the parent mannosemonophosphoric ester must be free, as otherwise only one lactone should have been formed. The evidence which supports the conclusion that the phosphate radical in mannosemonophosphate occupies position 6 can be summarised as follows.

(1) Hexosediphosphoric acid yields a phenylhydrazone containing two phosphate radicals, but during the formation of the osazone one molecule of phosphoric acid is split off [Young, 1911]. One of the two phosphoric acid groups must therefore occupy position 1 in the hexosediphosphate molecule.

(2) Hexosediphosphoric acid on methylation forms the α - and β -methylglycosides [Morgan, 1927], which on dephosphorylation with bone-phosphatase yield the methylglycosides identified as derivatives of fructofuranose [Morgan and Robison, 1928].

(3) Hexosediphosphoric acid on partial hydrolysis with dilute hydrochloric acid [Neuberg, C., 1918] yields a hexosemonophosphoric acid which is a derivative of fructose and which yields an osazone identical with the osazone from the hexosediphosphoric acid [Neuberg, C. and Reinfurth, 1924]. Fructosemonophosphate (Neuberg ester) therefore does not contain the phosphate radical which occupied position 1 in hexosediphosphoric acid.

(4) Glucosemonophosphate isolated by Robison and King [1931] from fermentation hexosemonophosphate yields on methylation α - and β -methylglycosides of the furanose and pyranose type [King *et al.*, 1931] which indicates that positions 4 and 5 in the glucosemonophosphate molecule are free.

(5) An ester, probably glucose-6-phosphate, has been synthesised by Levene and Raymond [1931] and appears to be identical with the glucosemonophosphate of Robison and King.

(6) Glucosemonophosphate and mannosemonophosphate yield the same osazone [Robison, 1932], which is also identical with that obtained from fructosemono- and fructosediphosphate. The phosphate radical therefore must occupy identical positions in all the three monophosphoric esters.

(7) An enzyme from yeast (kinase) converts any of these three monophosphoric esters in aqueous solution into equilibrium mixtures containing all the three esters [Lohmann, 1933; Patwardhan and Robison, unpublished experiments]. This points to the same conclusion as arrived at in the preceding paragraph.

(8) Mannosemonophosphate on mild oxidation yields phosphomannonic acid which forms two lactones whose properties correspond with those of the γ - and δ -lactones of the sugar acids.

The whole of this evidence put together points to the conclusion that the phosphoric acid group in mannosemonophosphate occupies position 6 in the mannose chain. This confirms the conclusions previously arrived at not only with regard to the constitution of mannosemonophosphate but also to that of the glucose and fructose esters isolated from the products of fermentation.

SUMMARY.

1. Large amounts of mannosemonophosphate have been prepared by the fermentation of mannose with dried brewer's yeast at 37° followed by crystallisation of the barium salt from the crude monophosphate fractions.

2. Mannosemonophosphate has been oxidised with bromine in presence of baryta to yield phosphomannonic acid which in aqueous solution passes into the lactone form.

3. Phosphomannono- γ - and phosphomannono- δ -lactone have been isolated and their rates of hydrolysis in aqueous solutions studied.

4. The rate of dephosphorylation of the phosphomannonic acid in $NHCl$ at 100° falls after the first 8 hours and then attains a constant value which is equal to the rate of dephosphorylation of the corresponding lactones under comparable conditions. Hence it is suggested that the free acid is dephosphorylated by $NHCl$ at 100° at a higher rate than its lactones.

5. The formation of these two lactones furnishes evidence that positions 4 and 5 in the mannose chain are free, and this taken in conjunction with previous findings points to the correctness of the conclusion that phosphate group occupies position 6 in mannosemonophosphate.

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