CCXLIII. MANNOSEMONOPHOSPHATE.
II. THE FERMENTATION OF MANNOSE BY DRIED YEAST.

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The isolation of mannosemonophosphate from the products of fermentation of glucose and fructose by yeast juice has been described in a previous communication [Robison, 1932]. Three components of the complex hexosemonophosphate of fermentation have now been identified as the monophosphoric esters of glucose, fructose and mannose; a fourth ester of low reducing power is present but has not yet been separated in pure condition. The disaccharide ester, trehalosemonophosphate [Robison and Morgan, 1928], may also occur in varying proportions among these products.

From the properties of the mannose ester and particularly from the identity of its osazone with that obtained from glucose- or fructose-monophosphate, it was inferred that the phosphoric acid group occupies the same position in all three compounds and that the new ester is therefore mannose-6-phosphate.

The experiments described in this and subsequent papers were planned to obtain further evidence as to the homogeneity and character of this substance and particularly to study the fermentation and esterification of mannose, in order to discover whether this sugar would yield a mixture of monophosphoric esters similar to that obtained from glucose and fructose. An answer to this question might help us to decide whether phosphorylation is preceded by a change in the hexose molecule or is itself the first stage in the fermentation process.

It was shown by Slator [1908] that mannose is fermented by almost all yeasts that ferment glucose but that the relative rates of fermentation vary with the type of yeast and especially with the treatment which the yeast has undergone. Yeast which is kept for some time or is heated loses its power to ferment mannose to a much greater extent than its power to ferment glucose. On the other hand, glucose and fructose are fermented under all conditions at approximately equal rates.

Harden and Young [1909] showed that mannose behaves towards yeast juice, both in the presence and the absence of added phosphates, in the same manner as glucose; the initial rates of fermentation were similar and on the addition of phosphate a rapid rise in this rate occurred, the extra amounts of carbon dioxide and alcohol produced being equivalent to the phosphate added. The fermentative power of the juice, however, fell off more rapidly with mannose than with glucose or fructose.

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From the products of the fermentation of mannose Young [1909] isolated a hexosediphosphoric ester identical with that obtained from glucose and fructose and now known to be γ-fructose-1:6-diphosphate.

I.S. Neuberg and Ostendorf [1930] fermented mannose and glucose at 37° with fresh yeast in presence of toluene and phosphate. From mannose they obtained two to three times as much monophosphate as from glucose, the proportion of hexosediphosphate being correspondingly low. In one experiment no diphosphate at all was found. The monophosphate fractions obtained from mannose showed high dextrorotations ($[\alpha]_D$, +45°, +34° for the free acids) but had low phosphorus contents. It seems likely that they contained trehalosemonophosphate.

Our own experiments were carried out with dried yeast prepared from English mild ale top yeast. We compared the fermentation and esterification of mannose, glucose and fructose at different temperatures, varying also the concentration of sugar and the amount of added phosphate.

Experimental.

Method of experiment.

The dried yeast was prepared from fresh brewery yeast, pressed to half a ton per square inch and dried for 18–20 hours at 37°.

2 g. dried yeast + 10 ml. H_2O + 1–5 g. sugar were placed in a flask, immersed in a thermostat and connected to a nitrometer. Three or more fermentations were carried out at the same time with different sugars or different concentrations of the same sugar. The evolution of carbon dioxide was measured at intervals of 5 minutes and, when the rate had fallen to an approximately constant level, 2 ml. of a phosphate-sugar solution (0·5 *M* Na_2HPO_4 + *M* sugar, saturated with CO_2) were added. Further additions of this solution were made when the evolution of carbon dioxide had again fallen to the basal rate, the maximum number of additions being 6, each addition representing 1 millimol phosphate and 2 millimols sugar. At the end of the fermentation 3 ml. 25 % trichloroacetic acid were added, together with sufficient water to bring the total volume to 25 ml., and the solution was filtered.

Estimation of phosphates. Inorganic and total phosphates were estimated in the protein-free filtrate by the modified Briggs's method. Monophosphoric and diphosphoric esters were estimated in the following way. To 2 ml. of the filtrate, 1 ml. of a 50 % solution of barium acetate was added, and a solution of baryta was run in until the solution turned phenolphthalein faintly pink; 1 ml. absolute alcohol was added and the volume made up with water to 10 ml. The solution was heated in a water-bath at 70° for 5 minutes and filtered; it was then kept at 0° for 1 hour and again filtered if any further precipitate had formed. The total P in this filtrate represented the monophosphate fraction, the diphosphate being obtained by difference.

Separation of the monophosphate fraction. The main bulk of the trichloroacetic acid filtrate was treated with barium acetate, in amount equal to ten times the total P present, and then with baryta until the p_H was brought to 8·0. The precipitate (crude hexosediphosphate + inorganic phosphate) was removed by filtration and the filtrate treated with a solution of basic lead acetate in slight excess. The precipitated lead salt was separated by centrifuging, washed twice with water, decomposed with the minimum excess of sulphuric acid and again centrifuged to remove the lead sulphate, this being also washed several times with water. The solution was brought to p_H 8·0 with baryta, on which a small

amount of sparingly soluble barium salt was usually precipitated and this was removed by filtration (intermediate fraction). The filtrate was poured into three times its volume of alcohol and the precipitate (crude hexosemonophosphate) was filtered off, washed with alcohol and dried in an evacuated desiccator. After a further purification by solution in ten parts of water and re-precipitation with alcohol these barium salts were analysed by the methods described in previous papers.

Phosphoric esters produced from mannose, glucose and fructose under various conditions.

The effect of varying the initial concentration of sugar on the proportion of mono- and di-phosphoric esters produced during fermentation was investigated in a series of six experiments, the sugars used being mannose and glucose. In a further series of experiments the nature of the monophosphoric esters produced from mannose, fructose and glucose at 25° and 38° was examined more closely. Details of these experiments with the percentages of monophosphates produced are set out in Table I, while the analyses of the barium monophosphate fractions are given in Table II.

Two different preparations of dried yeast were used; the first, Y 9, was 3½ months old at the date of the first experiment, while the second, Y 10, was prepared shortly before use in Exp. 10. The duration of the preliminary period of fermentation, before the first addition of phosphate, ranged from about 60 minutes at 38° to 4 hours at 17°; the duration of the experimental period, from the first addition of phosphate to the addition of trichloroacetic acid, varied from 50 to 615 minutes according to the age of the yeast and the temperature of fermentation, as shown in Table I. The rates of evolution of carbon dioxide shown in the next two columns are (*a*) the basal rate at the end of the preliminary period, (*b*) the maximum value observed for a 5-minute interval between the first and second additions of phosphate. Owing to the rapid change in the rate these observed maxima have a limited significance but serve to show the marked falling off in the mannose-fermenting power of the yeast Y 9 which occurred during the course of the experiments. In Exp. 2, 6 millimols of phosphate were almost completely esterified in 180 minutes, the maximum rate of gas evolution being 13·0 ml. per 5 minutes. In Exp. 9, 5 millimols of phosphate were added during a period of 605 minutes and only 82% of this phosphate was esterified, the maximum rate of gas evolution being 5·2 ml. per 5 minutes. No such marked falling off was observed in the fermentative power of this yeast towards glucose, for in Exp. 9, 5 millimols of phosphate became esterified to the extent of 96% in 185 minutes, the maximum rate being 13·5 ml. per 5 minutes.

The total phosphate in the trichloroacetic acid filtrate included an amount, equivalent to about 40 mg. P, derived from the yeast; part of this was originally present as inorganic phosphate, becoming esterified during the preliminary fermentation; the remainder consisted of organic phosphates not necessarily identical with the esters formed during the fermentation.

In the fermentation of glucose at 34° (Exp. 1) the proportion of monophosphate was increased on raising the initial concentration of sugar, and reduced on increasing the total amount of added phosphate. On the other hand, in the fermentation of mannose at 38° (Exps. 2–4) the proportion of monophosphate was highest when the amount of sugar initially added was relatively small, 0·5–1 g., and was markedly reduced when this amount was increased to 3 or 5 g. Much smaller amounts of monophosphate were produced from mannose at 17° (Exp. 5), but in this case the proportion rose slightly with increasing concentration of sugar.

Table I. *Rates of fermentation and amounts of mono- and di-phosphoric esters produced from mannose, fructose and glucose under various conditions.*

Exps. 1–9: dried yeast, Y9, prepared 2. x. 31. Exps. 10–14: dried yeast, Y10, prepared 30. iii. 32.

Exp.	Date	Temp. 0° C.	Sugar	Additions: Initial sugar g.	Additions: Total sugar millimols	Additions: Total phosphate millimols	Duration of experimental period min.	Rate of evolution of CO_2 ml. per 5 min.: Basal	Rate of evolution of CO_2 ml. per 5 min.: Maximum	Ester-P as % of total P	Monophosphate-P as % of total ester-P
1	21. i. 32	34	Glucose	1	10	2	65	3·0	15·5	98	21
,,	,,	,,	,,	3	21	2	55	,,	10·2	99	28
,,	,,	,,	,,	5	32	2	50	,,	9·2	98	34
,,	,,	,,	,,	3	25	4	105	,,	10·4	97	20
,,	,,	,,	,,	3	29	6	155	,,	11·3	95	19
2	26. i. 32	38	Mannose	1	10	2	60	3·0	11·8	99	66
,,	,,	,,	,,	3	21	2	60	,,	13·9	98	50
,,	,,	,,	,,	5	32	2	60	,,	10·0	97	42
,,	,,	,,	,,	3	25	4	120	,,	12·5	97	42
,,	,,	,,	,,	3	29	6	180	,,	13·0	95	38
3	2. ii. 32	38	Mannose	1	10	2	60	3·0	11·0	97	68
,,	,,	,,	,,	3	21	2	50	,,	16·5	97	44
,,	,,	,,	,,	5	32	2	60	,,	11·6	97	48
,,	,,	,,	,,	3	25	4	120	,,	13·8	96	44
,,	,,	,,	,,	3	29	6	180	,,	14·9	94	36
4	5. ii. 32	38	Mannose	0·5	7	2	85	2·5	8·1	98	60
,,	,,	,,	,,	1	10	2	85	,,	8·1	97	64
,,	,,	,,	,,	2	15	2	70	,,	15·5	97	50
,,	,,	,,	,,	3	25	2	65	,,	13·0	97	48
5	9. ii. 32	17	Mannose	0·5	7	2	210	0·7	2·9	99	27
,,	,,	,,	,,	1	10	2	210	,,	2·8	99	28
,,	,,	,,	,,	2	15	2	225	,,	2·2	99	30
,,	,,	,,	,,	3	25	2	210	,,	1·9	98	33
6	12. ii. 32	38	Mannose	1	10	2	65	2·5	10·1	99	58
,,	,,	,,	,,	3	21	2	55	,,	12·7	98	51
,,	,,	,,	,,	1	18	6	190	,,	9·5	96	74
7	1. iii. 32	38	Mannose	1	18	6	305	2·5	7·7	98	68
,,	,,	,,	,,	1	18	6	305	,,	6·6	98	79
,,	,,	,,	,,	1	18	6	305	,,	5·8	98	80
8	4. iii. 32	38	Mannose	1	16	5	615	2·0	3·7	75	51
,,	,,	,,	,,	1	16	5	615	,,	5·1	73	50
,,	,,	,,	,,	1	16	5	615	,,	4·0	76	54
9	9. iii. 32	38	Mannose	1	16	5	605	2·0	5·2	82	58
,,	,,	,,	Fructose	1	18	6	185	,,	13·2	96	29
,,	,,	,,	Glucose	1	18	6	185	,,	13·5	96	22
10	8. iv. 32	38	Mannose	1	18	6	180	3·0	15·5	97	65
,,	,,	,,	Fructose	1	18	6	170	,,	27·0	96	27
,,	,,	,,	Glucose	1	18	6	180	,,	19·0	96	21
11	20. iv. 32	38	Mannose	1	18	6	175	3·0	14·2	97	62
,,	,,	,,	,,	1	18	6	175	,,	14·7	97	66
,,	,,	,,	,,	1	18	6	175	,,	15·6	97	60
12	28. iv. 32	38	Mannose	3	29	6	160	3·0	15·2	96	51
,,	,,	,,	Fructose	3	29	6	160	,,	24·4	96	36
,,	,,	,,	Glucose	3	29	6	160	,,	19·5	96	21
13	9. v. 32	25	Mannose	3	29	6	350	2·0	13·1	97	37
,,	,,	,,	Fructose	3	29	6	350	,,	14·8	97	25
,,	,,	,,	Glucose	3	29	6	350	,,	13·4	97	18
14	14. v. 32	25	Mannose	3	29	6	350	2·0	12·8	95	36
,,	,,	,,	Fructose	3	29	6	350	,,	14·9	95	22
,,	,,	,,	Glucose	3	29	6	350	,,	13·3	95	18

Both at 38° and at 25° mannose yielded a much higher proportion of monophosphate than either fructose or glucose; and this result is in agreement with the findings of I. Neuberg and Ostendorf [1930] for fermentations at 37° with fresh yeast in presence of toluene. The highest value recorded in Table I is 80 %,

but values approaching 100 % have been obtained in this laboratory. With all three sugars the proportion of monophosphate was higher when the fermentation was carried out at 38° than at 25°. The highest yields were obtained from mannose at 38° with a relatively low concentration of sugar (initially 1 g. to 10 ml.); and under these conditions the difference between mannose and the other sugars was most pronounced. Fructose gave a little more monophosphate than glucose both at 38° and at 25°.

Nature of the monophosphate fractions. Analyses of the monophosphate fractions (crude soluble barium salts) revealed considerable variations in their composition (Table II). Those obtained from the fermentations of fructose and

Table II. *Analyses of monophosphate fractions (barium salts) isolated from Exps. 7–14.*

Exp.	Temp. °C.	Sugar	Monophosphate-P as % of total ester-P	Analysis of barium salt: P %	Fructose (Selivanoff) %	Reducing power as glucose %: H and J*	Reducing power as glucose %: Iodine	$[\alpha]_{5461}$
4 and 6	38	Mannose	58	7·53	3·5	30·5	24·9	+ 4·3°
7	„	Mannose	76	7·42	1·2	33·0	27·5	+ 3·0
8	„	Mannose	52	6·70	2·1	28·7	22·8	+ 8·4
9	„	Mannose	58	7·37	3·2	29·1	26·3	+ 8·4
	„	Fructose	29	7·12	5·3	24·7	23·6	+12·2
	„	Glucose	22	7·21	6·0	23·1	20·9	+12·4
10	„	Mannose	65	7·70	1·7	30·2	23·7	+ 3·9
	„	Fructose	27	7·79	8·0	23·0	17·2	+ 9·2
	„	Glucose	21	7·67	6·8	20·8	14·8	+ 6·8
11	„	Mannose	63	7·69	1·4	30·0	27·0	+ 3·0
12	„	Mannose	51	7·65	2·3	30·6	24·4	+ 4·7
	„	Fructose	36	7·57	7·8	27·7	19·2	+10·8
	„	Glucose	21	7·36	9·5	22·7	16·7	+13·8
13	25	Mannose	37	7·38	4·3	30·4	20·3	+15·0
	„	Fructose	25	7·14	7·1	22·8	16·7	+18·9
	„	Glucose	18	7·11	6·7	21·6	15·2	+26·2
14	„	Mannose	36	7·00	4·5	25·0	20·4	+16·8
	„	Fructose	22	6·95	6·9	21·7	15·3	+23·7
	„	Glucose	18	6·93	6·3	18·1	15·1	+26·5
	Glucosemonophosphate			7·86	0·5	35·5	45·7	+20·6
	Fructosemonophosphate			7·80	22·0	36·2	3·0	+ 0·7
	Mannosemonophosphate			7·87	0·3	36·0	28·5	+ 3·6

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

* Determined with addition of 0·5 ml. of 0·5 *N* NaOH.

glucose were of the mixed type, containing 25–43 % of ketose esters, judged by the Selivanoff values, and a rather high proportion of the unknown ester of low reducing power. The high specific rotations of the products from fructose and glucose at 25° (Exps. 13, 14) pointed to the presence of trehalosemonophosphate, though in lower proportions than had been given by other samples of dried yeast [Robison and Morgan, 1928]. Very little, if any, trehalosemonophosphate was formed in the fermentations at 38°.

The monophosphate fractions obtained from mannose at 38° differed notably from those just considered and in their properties approximated more closely to mannosemonophosphate. The triple mannose fermentations of Exp. 7 yielded

monophosphate to the extent of 76 % of the total esterified phosphorus; and the analytical data suggested that over 90 % of this fraction consisted of the mannose ester. Very similar products, though of slightly lower reducing power, were obtained in Exps. 10 and 11.

Apart from the specific rotations, the low Selivanoff values are especially to be noted, as showing that ketose esters were present in very small amounts, probably not exceeding 5 %. Other mannose fermentations at 38° (Exps. 4–6, 8, 9, 12), in which the yields of monophosphate were a little lower, gave products containing higher proportions of fructose and glucose esters, though still consisting for the most part of mannosemonophosphate.

Fermentation of mannose at 25° yielded monophosphate fractions of intermediate character, whose composition was more difficult to judge from the analytical data. The Selivanoff values indicated about 20 % of ketose esters; the specific rotations were similar to those of the usual mixed ester, but would also be consistent with the presence of much mannosemonophosphate and a little trehalosemonophosphate. It is shown later that the latter explanation is more probably correct.

Separation of mannosephosphate from the products of mannose fermentations at 38°.

(*a*) *By the brucine salt.* The products from Exps. 7 and 11 which had apparently yielded the highest proportions of mannose ester were converted into the brucine salt which was recrystallised five times from 80 % methyl alcohol. The main fraction, after re-conversion into barium salt, gave analytical values in agreement with those previously obtained for mannosephosphate but the Selivanoff value had not been lowered; there was indeed evidence that unduly prolonged contact with the solvent had led to some increase of ketose esters.

(*b*) *By the phenylhydrazone.* Formation of the phenylhydrazone provided a simpler and more satisfactory method of isolation of the mannose ester and one that could be directly applied to mixtures containing this ester in relatively small proportions. It was found possible to remove both phenylhydrazine groups from this derivative by treatment with benzaldehyde at room temperature, the benzaldehydehydrazone and excess of benzaldehyde being subsequently removed by extraction with ether. In this way the recovery of the free mannose ester was effected under conditions involving no likelihood of intramolecular change.

The combined soluble barium salts from the mannose fermentations at 38° of Exps. 4, 6, 8, 9, 10, 12, were converted into the free esters by treatment with the minimum excess of sulphuric acid, the barium sulphate being removed by centrifuging and washed four times with acidified water. The ester solution was treated with pure phenylhydrazine (4 mols.) and the equivalent amount of acetic acid and was then left in an evacuated desiccator for some hours. The phenylhydrazine salt of phosphomannosephenylhydrazone which separated out was filtered off and washed with cold methyl alcohol; the yield corresponded with 60 % of the total P present in the barium salt. The hydrazone was recrystallised from 50 % ethyl alcohol, the operation being conducted as rapidly as possible. This recrystallisation, which involved considerable reduction in the yield, was later found to be unnecessary as a stage in the isolation of the pure ester.

The hydrazone was decomposed by treating it with about ten times its weight of benzaldehyde, in which it dissolved almost completely. After leaving for an hour at room temperature, an equal volume of water and twice the volume of ether were added, and the mixture was gently shaken in a separating

funnel. The aqueous layer was removed and shaken with a further quantity of benzaldehyde during one hour at room temperature; it was then extracted with ether until the extract was colourless and gave a negligible residue on evaporation. The aqueous solution, which now contained the free mannosemonophosphoric ester, was brought to p_H 8·0 by addition of baryta and poured into three times its volume of alcohol. The precipitated barium salt was purified by solution in 10 % alcohol and reprecipitation in 70 % alcohol.

Analyses of the salts obtained in this way agreed well with those of mannosemonophosphate but the Selivanoff value was still between 1 and 2 %.

(*c*) *By the crystalline barium salt.* During purification of the barium salt obtained from the phenylhydrazone it was observed to separate from dilute aqueous alcohol in a crystalline and sparingly soluble form. As in the case of barium trehalosemonophosphate, when once the crystalline form had been obtained it separated readily from any solution in which the ester was present in moderate concentration. Thus 1·2 g. of the amorphous barium salt, which had been several times dissolved in 10 parts of water, suddenly separated out as a mass of crystals which thereafter required 150–200 ml. of water for complete solution. From this solution, on gradual addition of alcohol up to 20 % concentration, the barium salt separated slowly at room temperature in clusters of fine needles; they were recrystallised in similar manner from 20 % alcohol and were analysed.

(The crystalline salt lost 8·4 % at 110° in the Pregl drier. Required for $2H_2O$, 8·4 %. Found for the dry salt: C, 18·02, 17·90; H, 3·2, 2·90; Ba, 34·5, 34·7; P, 7·87, 7·85 %. $C_6H_{11}O_5PO_4Ba$ requires C, 18·20; H, 2·81; Ba, 34·75; P, 7·85 %.) C and H were determined by the Pregl method, the finely divided substance being mixed with potassium dichromate and copper oxide; as with other hexosephosphates, complete combustion was very difficult to effect. Other analytical data for this salt are shown at the foot of Table II. The values for specific rotation and reducing power do not differ significantly from those previously recorded [Robison, 1932]; but the Selivanoff value has been reduced from 1 to 0·3 % which is no more than that given by pure mannose. Recrystallisation of the barium salt had therefore removed the last traces of ketose esters. The H. and J. reducing power, determined with addition of NaOH, is the same as that of glucose- and fructose-6-phosphates and is 80 % of that of the equivalent amount of mannose. The iodimetric reducing power is only about 60 % of that theoretically required for an aldosemonophosphate. As previously stated, it can be raised to about 70 % by increasing the concentration of potassium iodide in the iodine solution. Further tests showed that, under the normal conditions of this iodimetric method, mannose itself gives somewhat low values, which are slightly raised if the concentration of potassium iodide is increased. By extending the period of oxidation, still higher values were obtained; but the oxidation of the ester proceeded more slowly and was less complete than that of the free sugar. Thus in two hours the oxidation of mannosemonophosphate corresponded with 87 % of the theoretical value, while that of mannose reached 100°. Under similar conditions fructose was oxidised only to the extent of 2 %. The typical results, shown in Table III, suggest that the ester exists in solution in two forms, only one of which is rapidly oxidised by iodine.

Although the separation of mannose ester in these operations was not quantitative, the conclusions deduced from the analyses of the crude fractions were definitely confirmed—namely that these fractions consisted to a very large extent of mannosemonophosphate, which therefore constitutes the main phosphorylated product of the fermentation of mannose by dried yeast at 38°.

Table III. *Iodimetric reducing power of mannosemonophosphate.*

3 ml. 0·02*N* I; 0·2 ml. 5 % Na_2CO_3; $t=22°$; normal time, 30 min.

Substance	Weight of substance mg.	Time min.	Extra KI mg.	Reducing power as aldose: % of substance	Reducing power as aldose: % of equivalent hexose
Barium mannose-phosphate	3	30	—	28·5	62
	„	15	30	26·9	59
	„	30	„	32	70
	„	60	„	36·4	80
	„	120	„	40·6	87
Mannose	1	30	—	92	92
	„	15	30	89	89
	„	30	„	96	96
	„	60	„	97	97
	„	120	„	100	100
Fructose	5	30	—	0·5	0·5
	„	30	30	0·5	0·5
	„	60	„	1·2	1·2
	„	120	„	2·0	2·0

Separation of mannosemonophosphate from the products of mannose fermentations at 25° and of glucose and fructose fermentations at 25° and 38°.

The analyses of the monophosphate fractions obtained from mannose fermentations at 25° and from the fermentations of glucose and fructose at 25° and 38° had given no clear evidence as to the presence of mannosemonophosphate in these mixtures of aldose and ketose esters. The fractions were therefore dissolved separately in 10 parts of 10 % alcohol and the filtered solutions were seeded with a minute crystal of barium mannosephosphate and left for some days at 0°. Separation of crystalline barium salt was observed to take place from the products obtained from mannose at 25° (Exps. 13 and 14) but not from any of the products obtained from glucose or fructose. Analysis of the crystalline product from Exp. 13, mannose, showed that this was barium mannose-monophosphate; the residual salt, precipitated from the mother-liquors by alcohol, had an increased specific rotation ($[\alpha]_{5461}+23°$) and most probably contained trehalosemonophosphate. The weight of crystalline mannosephosphate recovered amounted to 18 % of the fraction, but almost certainly this did not represent the whole of the mannose ester present. The phenylhydrazone was also prepared, and after recrystallisation from 75 % alcohol, melted at 144–144·5°, both when heated alone and when mixed with pure mannosephosphate hydrazone.

Formation of the hydrazone was also used to demonstrate the presence of mannosemonophosphate in the monophosphate fractions from glucose and fructose fermentations at 38° (Exps. 9, 10 and 12) and at 25° (Exp. 13). Although these products failed to yield the crystalline barium mannosephosphate, all gave small amounts of sparingly soluble hydrazones which, after recrystallisation, melted at 144–145° and did not lower the melting-point of pure mannosephosphatehydrazone.

It appears, therefore, that mannosemonophosphoric ester is a normal product of the fermentation of glucose, fructose and mannose by dried yeast both at 25° and at 38°, but that it occurs in considerably larger amounts in the products obtained from mannose than in those from glucose and fructose.

Intermediate fractions obtained from the fermentation products.

Analysis of the intermediate fractions obtained in the separation of the barium salts gave results consistent in all cases with the view that these fractions might be composed chiefly of hexosediphosphate, mixed with a little hexose-monophosphate, inorganic phosphate and, possibly, also with phosphoglycerate. It was noted, however, that such intermediate fractions were not obtained in appreciable amounts from mannose fermentations at 38°, which suggests that some unknown products of intermediate solubility may be formed from glucose and fructose and also from mannose at 25° but not from mannose at 38°.

Phenylhydrazine salt of mannosemonophosphatephenylhydrazone.

A specimen of the phenylhydrazone prepared from pure mannosemono-phosphate and recrystallised from 75 % alcohol was very pale yellow in colour, slightly soluble in water and methyl alcohol, less soluble in ethyl alcohol, ether, chloroform and acetone; M.P. 144–144·5°. (Found for the dry substance: P, 6·80; N (micro-Dumas), 12·2 %. $C_{18}H_{27}O_8N_4P$ requires P, 6·78; N, 12·23 %.)

Discussion.

If the simplified methods for the isolation of pure mannosemonophosphate which were evolved during the course of this investigation could have been applied throughout the whole series of fermentation experiments, the quantitative value of the results would have been increased. Nevertheless these results show that mannosemonophosphate is normally formed in the fermentation of glucose, fructose and mannose by dried yeast in presence of phosphate at 25° and at 38° but that it occurs in considerably larger amounts in the products from mannose than in those from glucose and fructose. This difference is apparent in fermentations at 25° but is very much exaggerated when the temperature of fermentation is raised to 38°; in such cases mannosemonophosphate may form almost the whole of the monophosphate fraction and the chief part of the total phosphorylated products.

Such conclusions make it necessary to reconsider the scheme put forward in a previous paper [Robison, 1932] according to which the first stage in the fermentation of glucose, fructose and mannose is the formation of the enol compound common to all three sugars; the phosphoric acid group then enters position 1 of this enol compound. Clearly this view is inconsistent with the production of mannosephosphate to such preponderating extent in the fermentation of mannose. Rather it would seem that the phosphoric group may enter position 6 in the molecule of unchanged hexose and that enolisation of the hexosemonophosphate then takes place. Enolisation of mannose-6-phosphate may occur less readily than that of the corresponding esters of glucose and fructose at 25° and may be inhibited almost completely at 38°. Since fructosediphosphate could obviously not be formed unless enolisation first took place, this explanation would also account for the very small proportion of diphosphate in the phosphorylated products from mannose fermentations at 38°.

Before this argument can be continued, however, it is necessary to know whether the formation of mannosemonophosphate in large amount occurs as a normal stage in the fermentation cycle or as a result of a side reaction, a simple phosphorylation, unconnected with the carbohydrate breakdown. This can be investigated by determining the ratio of carbon dioxide evolved to phosphate esterified under conditions involving the formation of mannosemono-

phosphate in large as well as in small amounts. In the present series of experiments, although carbon dioxide evolution was measured, the corrections necessary for the accurate determination of this ratio could not be applied. A further series of experiments has since been carried out in order to study this aspect of the question; and these will be discussed in a subsequent communication (Patwardhan and Robison, unpublished results).

The preparation of the phenylhydrazine salt of the phenylhydrazone of mannosemonophosphate and its decomposition by benzaldehyde have supplied a satisfactory method of separating this ester from other monophosphates; while the sparingly soluble crystalline barium salt has furnished a simple means of obtaining the ester in pure condition. By a combination of these two processes the detection and approximate estimation of mannosemonophosphate in mixed phosphorylated products becomes a relatively simple operation which may be usefully applied in future investigations.

Summary.

1. The monophosphoric esters formed in the fermentation of mannose, glucose and fructose by dried yeast at various temperatures have been studied.

2. A method for the isolation of mannosemonophosphoric ester by formation of its phenylhydrazone and decomposition of the latter with benzaldehyde at room temperature is described.

3. The barium salt of mannosemonophosphate has been obtained in a sparingly soluble crystalline form which has furnished a simple means of obtaining the ester in pure condition.

4. The properties of this pure mannosemonophosphoric ester are described; they agree in nearly all respects with those recorded in the previous communication.

5. Mannosemonophosphate is formed in the fermentation of glucose, fructose and mannose by dried yeast in presence of phosphate at 25° and at 38°.

6. It is formed in considerably larger amounts from mannose than from glucose or fructose. This difference is apparent in fermentations at 25° but is greatly exaggerated when the fermentation is carried out at 38°.

Mannosemonophosphate may thus constitute almost the whole of the monophosphate fraction and the chief part of the phosphorylated products formed in the fermentation of mannose at 38°.

7. The bearing of these facts on previous views regarding the first stages in the fermentation of sugars is discussed.

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