XVII. THE PHOSPHORIC ESTERS OF ALCOHOLIC FERMENTATION.

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SINCE the isolation of a hexosemonophosphoric ester from the products of the fermentation of glucose and fructose by yeast juice the further investigation of these products has been directed towards obtaining more information on certain questions then briefly discussed [Robison, 1922], namely:

(a) the proportions in which the hexosemonophosphoric and hexosediphosphoric esters are formed during fermentation with yeast juice and the significance of the variations observed in different experiments:

(b) the suggested dual nature of hexosemonophosphoric ester and the chemical constitution of this ester, or its components, and that of hexosediphosphoric ester:

(c) the possible occurrence of other phosphoric esters among the products of fermentation.

The investigation was extended to include the products of fermentation of glucose and of fructose by yeast juice, dried yeast and zymine. The esters, freed from yeast proteins and excess of sugar, were separated into fractions according to the solubility of their barium salts, and the nature of these fractions was investigated, making use of micro-analytical methods to avoid the rapid wastage of material. This quantitative examination included the estimation of total and inorganic phosphorus, specific rotation, reducing power by the Hagedorn and Jensen method and by an iodimetric method, and "fructose" by Selivanoff's reaction. The further separation of the esters was carried out by fractional precipitation of the barium salts, crystallisation of barium salts, and in other ways, the progress being followed at all stages by the aid of the above micro-estimations.

One result of this work, the isolation and identification of trehalosemonophosphoric ester has already been described [Robison and Morgan, 1928]. This ester was obtained from the fermentation of glucose and of fructose by dried yeast in presence of phosphates and was found in amounts representing up to 5 % of the total esterified phosphorus. The dried yeast used for the

fermentations was shown to contain neither trehalosemonophosphoric ester nor trehalose in quantities sufficient to account for the amount of the ester found in the fermentation products, but the experimental data did not justify any conclusion as to the significance of this disaccharide phosphoric ester in the fermentation process. The ester, however, was shown to be readily fermented by dried yeast, yeast juice and zymin.

A more general account of these experiments is given in the present paper.

MATERIAL AND METHODS.

Yeast preparations.

(a) Yeast juice: prepared from English mild ale top yeast by the method of Buchner modified by Harden and Henley [1927].

(b) Dried yeast: prepared from English mild ale top yeast (in one experiment from a bottom yeast).

(c) Zymin: prepared from English mild ale top yeast.

Fermentation.

Method A. Glucose or fructose (30 g.) was added to 300 cc. yeast juice, or to 50 g. dried yeast or zymin mixed with 180 cc. water. A further 70 g. of the sugar was added in two or three portions during the fermentation. To the fermenting mixture, maintained at 28°–30°, a 20 % solution (0.56 *M*) of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ was added in six equal portions as indicated by the evolution of carbon dioxide in a guide experiment. Except when otherwise stated, the additions were made when the rate of evolution of gas had fallen nearly to the minimum (hexosephosphatase rate). The total amount of phosphate added was equivalent to about 7 g. P. Dried yeast or zymin was allowed to remain in the sugar solution for 1 hour before the first addition of phosphate.

Method B (used in the later experiments). 30 g. sugar was added to the yeast and, after the preliminary period, a solution containing both phosphate (20 %) and sugar (20 %) was allowed to run in continuously at a controlled rate throughout the fermentation. The fermentation was carried out in a large bottle immersed in a water-bath and connected by two rubber tubes with (a) a 100 cc. burette containing the phosphate-sugar solution, (b) a nitrometer, capacity 1 litre, in which the carbon dioxide evolved was collected over brine. At intervals of 5 minutes the bottle was vigorously shaken and from the volume of gas evolved the rate of addition of the phosphate sugar solution was regulated, in accordance with the plan of the particular experiment. The fermentation was stopped or portions of the fermenting mixture were removed at definite intervals of time after the last addition of phosphate.

Precipitation of proteins.

The precipitation of yeast proteins by heat coagulation, though employed in the first experiments which led to the isolation of fermentation hexosemonophosphate, was abandoned owing to the risk that a hexosemonophosphate might thereby be formed by partial hydrolysis of the hexosediphosphate in the solution. The method then adopted [Robison, 1922], namely precipitation of the protein along with the barium salts by alcohol, avoided this risk, but the successive extraction of the barium hexosephosphates from the mixture of salts and coagulated protein was laborious and difficult to carry out in a quantitative manner. Neuberg and Leibowitz [1927] have described a more rapid method for the preparation of hexosemonophosphate, in which the proteins are precipitated by heat coagulation after the solution has been neutralised to phenolphthalein. The protein-free filtrate, after the addition of calcium or barium acetate, is once more heated to boiling in a brine-bath and filtered hot, by which means the phosphate and nearly all the hexosediphosphate are removed as insoluble salts.

We have made some experiments to determine the extent to which hydrolysis of hexosediphosphate might occur under such conditions.

Exp. 1. Solutions of sodium hexosediphosphate (0.03 *M*), adjusted to different hydrogen ion concentrations, were heated for different periods of time at different temperatures. The degree of hydrolysis was measured by the increase in inorganic phosphate.

Exp. 2. 10 cc. of a saturated solution of barium hexosediphosphate, at p_H 8.4 ("neutral to phenolphthalein"), was slowly heated to 100° in a water-bath and maintained at the boiling point for a further 5 minutes. The inorganic phosphate liberated was then determined. The results are shown in Table I.

Table I.

Exp.	p_H	Time of heating min.	% P hydrolysed			
			70°	80°	90°	100°
1	6.9	30	0.8	2.4	6.5	16.0
1	7.8	30	1.0	2.6	8.7	18.0
1	8.4	5	0.2	0.6	2.1	5.5
2	8.4	15 min. up to 100° 5 min. at 100°	—	—	—	9.7

Since the phosphoric acid group in position 1 of hexosediphosphoric ester is much more readily hydrolysed than the remaining group, it is probable that in the above experiments a monophosphate would be formed in amount equivalent to the greater part of the inorganic phosphate set free. The use of high temperatures during the separation of the products must therefore result in some contamination of the fermentation monophosphate with the ester produced by partial hydrolysis of the diphosphate. For some purposes this might not be of serious importance, but it would be very undesirable in these

experiments in which one of the main objects was to separate and identify the components of the monophosphoric ester formed during fermentation.

For the last six years trichloroacetic acid has been used in this laboratory for the precipitation of the yeast proteins and has been found satisfactory for this purpose. The acid is added to the fermentation mixture in amount equal to 4 % of the total volume; the mixture is thoroughly shaken and allowed to stand, preferably at 0°, for 2 hours before being filtered. The latter operation is much accelerated by the addition of a few drops of capryl alcohol. The possibility of the hydrolysis of hexosediphosphate occurring during this stage owing to the acidity of the solution (p_H about 4) was not overlooked, but repeated tests failed to indicate any increase in the amount of inorganic phosphate under experimental conditions.

General separation of the barium salts.

The acid protein-free filtrate was treated with barium acetate equal in weight to the crystalline sodium phosphate added during the fermentation, and was then neutralised to phenolphthalein with baryta. The greater part of the alkali was added in concentrated, hot solution in order to avoid unnecessary dilution, but the final adjustment to p_H 8.4 was made with cold saturated baryta solution. The precipitate was filtered off, washed with small quantities of water, pressed out as thoroughly as possible, washed with absolute alcohol and dried.

"10 % A" fraction¹. The filtrate from the sparingly soluble salts was treated with one-tenth its volume of absolute alcohol and the precipitate filtered off, washed with a little 10 % alcohol, then with absolute alcohol and dried in a desiccator. In certain experiments no precipitate whatever was given at this stage. The reason for these anomalous results will be discussed later.

"Soluble A" fraction. The filtrate from the 10 % A precipitate was treated with basic lead acetate, avoiding any large excess of this reagent; the precipitate was decomposed with hydrogen sulphide, the lead sulphide being filtered off, washed with water and again treated with hydrogen sulphide. The combined filtrates and washings, after thorough aeration, were treated with baryta solution until pink to phenolphthalein and poured into twice the volume of alcohol. It is important that these precipitates should be thoroughly washed with absolute alcohol and dried rapidly in a vacuum desiccator.

"10 % B" and "soluble B" fractions. The soluble A fraction was thoroughly ground with ten times its weight of water, to which one-tenth the volume of alcohol was finally added before filtration. The insoluble portion formed the 10 % B fraction; on pouring the filtrate into twice its volume of alcohol the soluble B fraction was obtained.

Other fractions. The organic phosphate in the filtrate from the 10 % A

¹ These somewhat inaccurate terms used in the laboratory have been retained for convenience.

fraction was not entirely precipitated by basic lead acetate, the amount remaining in solution representing about 1 % of the total phosphorus esterified during the fermentation. The greater part of this residual phosphorus was brought down by basic lead acetate and ammonia, but the barium salts recovered from this fraction ("ω") were highly contaminated with phosphorus-free compounds and could not readily be purified. The alcoholic filtrates from the soluble A and B fractions also contained small amounts of phosphoric esters which were not recovered.

Micro-analytical methods.

Total and inorganic P were estimated by the modified Briggs method [Martland and Robison, 1926].

Reducing power was estimated by the Hagedorn and Jensen method, omitting the zinc hydroxide precipitation. Inconsistent results were at first obtained by this method when applied to the hexosephosphates. The reducing power, calculated as per cent. glucose, varied with the amount of substance used for the estimation and was markedly increased by traces of alkali. It was found that these differences could be nearly eliminated by adding 0.5 cc. of a 0.5*N* solution of sodium hydroxide to the solution before heating for 15 minutes in the water-bath. The reducing power, calculated from the glucose table, was increased by as much as 30 % and was nearly independent of the amount of substance taken. This modification was adopted in all subsequent estimations, the same amount of alkali being added to the blank. The value of the blank titration is not, however, altered by this addition nor is the reducing power of glucose itself increased.

Aldose was estimated by the iodimetric method [Macleod and Robison, 1929]. Caution was necessary in interpreting the values so obtained for the crude fractions, owing to the possibility that some of the contaminating substances derived from the yeast and precipitated with the phosphoric esters might react with iodine. It is probable, however, that errors due to this cause were usually small except in the case of the very impure ω fractions.

Selivanoff's reaction. A roughly quantitative method was employed for the estimation of the fructose equivalent by the Selivanoff reaction. A suitable quantity of the solution diluted to 5 cc. was mixed with 5 cc. of a 0.05 % solution of resorcinol in 5*N* HCl. A standard solution containing 0.2 mg. fructose was similarly treated. Both tubes were heated for 6 minutes in a boiling water-bath and, after cooling, the intensities of the colours were compared in a colorimeter. The results, though not very consistent, were useful in supplementing the aldose determinations, more especially on the purified fractions.

The values obtained for highly purified specimens of the four phosphoric esters by the above methods together with the specific rotations are shown in Table II.

Table II.

	P %	Reducing power as glucose		"Fructose" Selivanoff %	[α] _{D481}
		H. and J. %	Iodine %		
Barium hexosediphosphate	10.16	12	2	10	+ 3.5°
" aldosemonophosphate ¹	7.85	36	41	0	+ 19.5°
" fructosemonophosphate (Neuberg)	7.85	36	2	22	+ 0.7°
" trehalosemonophosphate	5.57	0	0	0	+ 132°

COMPOSITION OF THE FRACTIONS.

Using these values and assuming the four esters to be the only compounds present having reducing properties or optical activity, the probable composition of the various fractions was deduced arithmetically. This assumption was not necessarily correct, but such an analysis might furnish evidence of the presence of other phosphoric esters. The application of this method in the early stages of the investigation, when only three esters were known, led to the isolation of the fourth, trehalosemonophosphate.

Table III.

Experiment	Fraction	P in fraction as % of total P recovered	Percentage composition of fraction (barium salts)					
			Hexose- diphos- phate	Fructose- monophos- phate	Aldose- monophos- phate	Trehalose- monophos- phate	Inorganic phosphate	P-free impurities
5 Yeast juice-fructose	Sp. soluble	38.6	73	0	20	0	2	5
	10 % A	15.8	31	22	28	2	5	12
	10 % B	7.9	15	7	40	0	20	18
	Soluble B	37.3	6	16	68	1.5	1.5	7
6 Yeast juice-fructose	Sp. soluble	18.6	89	1	3	0	3	4
	10 % A	18.3	43	11	28	0	4	14
	10 % B	16.6	28	6	39	1.5	11	14.5
	Soluble B	44.8	2	30	58	2.5	1	6.5
7 Zymin-fructose	Sp. soluble	80.1	90	0	0	0	5	5
	10 % A	7.3	77	0	10	0	1	12
	10 % B	3.4	50	7	12	0	22	9
	Soluble B	7.5	10	12	50	14	0	14
13 Dried yeast-fructose	Sp. soluble	85.3	72	0	0	0	20	8
	10 % A	No precipitate						
	10 % B	6.0	35	4	23	3	4	31
	Soluble B	8.4	10	11	50	17	0	12
15 Dried yeast-fructose	Sp. soluble	85.1	91	0	0	0	0.5	8.5
	10 % A	4.3	81	0	4	2	3	10
	10 % B	2.9	20	3	16	20	3	38
	Soluble B	7.0	6	8	19	54	0	13
20 <i>b</i> Dried yeast-fructose	Sp. soluble	52.3	83	0	0	0	17	0
	10 % A	11.3	93	0	0	0	1.7	5.3
	10 % B	21.4	78	0	10	0	9.5	2.5
	Soluble B	14.3	14	8	37	28	0	13

For most fractions the values of reducing power, etc., calculated from the percentage composition so found, were in good agreement with those obtained experimentally, except that the Selivanoff and the iodimetric figures were in

¹ Results obtained with the most highly purified specimen of the aldose component of hexosemonophosphoric ester. This component is probably glucosemonophosphate (theoretical iodimetric value 45.6). Evidence of the identity of the second component with Neuberg's fructosemonophosphate has been obtained [Robison and King, 1929, and further unpublished work].

some cases mutually inconsistent. Both these values may have been affected by the impurities in the crude fractions and the figures showing the partition of the hexosemonophosphoric ester into aldose and fructose components are therefore of limited significance. This criticism does not apply in the case of the more highly purified fractions obtained in later stages of the separation. The percentage compositions deduced for the four chief fractions of experiments with yeast juice, dried yeast and zymin are shown in Table III.

The fact that the reducing power and other properties of these fractions could be quantitatively accounted for in this way does not prove that no other phosphoric esters were present, since mixtures containing one or more unknown esters might give equally satisfactory values. The evidence on this point will be considered in greater detail, taking each of the main fractions in turn.

Sparingly soluble fraction.

In the experiments with dried yeast and zymin, in which the three monophosphoric esters together represent less than 20 % of the organic phosphorus, the sparingly soluble fraction consisted almost wholly of hexosediphosphate together with most of the inorganic phosphate. A small amount of nucleic acid was present but was not separately estimated. The proportion of P-free impurities was usually small. In the yeast juice experiments, when the monophosphoric esters formed a much larger proportion of the total products, a certain amount of these soluble barium hexosemonophosphates was always carried down with the hexosediphosphate and in one experiment formed over 20 % of the fraction. This was confirmed by the separation of the hexosemonophosphate in nearly pure condition.

It was noticed that when the crude fraction contained much inorganic phosphate the whole of the diphosphate could not be extracted with 200 parts of water nor even by grinding the residue with further quantities of water. The amount of ester in the residue was roughly proportional to the inorganic phosphate and this suggested that an insoluble double salt had been precipitated. Further evidence in the same direction was found in the absence of any 10 % A precipitate in such experiments, indicating that the filtrate from the sparingly soluble fraction was unsaturated with respect to barium hexosediphosphate (see also experiments shown in Table IV).

10 % A and 10 % B fractions.

In dried yeast and zymin experiments the 10 % A precipitate also consisted very largely of hexosediphosphate, but the 10 % B fraction contained up to 30 % of the two hexosemonophosphates. In yeast juice experiments the percentage of hexosemonophosphate was high in both 10 % fractions, often exceeding that of the diphosphate. The occurrence of so large a proportion of the very soluble barium hexosemonophosphates in these fractions

did not seem altogether reasonable and an alternative solution was considered, namely the presence of an ester having a sparingly soluble barium salt and intermediate reducing power.

The precipitates were ground with 10 parts of water and the filtered extract was treated with (a) one-tenth its volume of alcohol and, after filtration, (b) twice its volume of alcohol. The residue was again ground with water and brought into solution with the minimum amount of hydrochloric acid. The p_H of this solution was readjusted to 8.4 by the addition of baryta, and the precipitate filtered off. The filtrate was treated with alcohol as described above. These operations were repeated on the several fractions, the progress of the separation being followed by analyses. Certain of the fractions were then converted into brucine salts which were separated by fractional crystallisation from dilute alcohol and finally reconverted to the barium salts which were analysed. In this way the 10 % A and B precipitates were separated into hexosediphosphate and hexosemonophosphates of sufficient purity for identification, together with fractions of less certain nature. Provided that the latter were included in the computation, the amounts of mono- and diphosphate thus recovered agreed moderately well with those calculated from the analyses of the crude precipitates. The reducing power and other properties of these indefinite fractions were, however, not easy to reconcile with those of any probable mixture of the known phosphoric esters and suggested rather that they contained a new ester, possibly a disaccharide diphosphate having one free aldose group. In spite of much work on these fractions the evidence is still very conflicting, and no conclusion can yet be reached. Experience has shown that great caution is necessary before inferring the homogeneity of brucine salts of these phosphoric esters from their definite crystalline form.

Whatever may be the nature of these fractions, it is certain that the hexosemonophosphates are carried down along with inorganic phosphate and hexosediphosphate and may form a considerable percentage of the crude 10 % precipitates. Since pure barium hexosemonophosphate is not precipitated from its 10 % aqueous solution by the addition of one-tenth the volume of alcohol, its occurrence in the sparingly soluble and 10 % A and B precipitates is probably due to the formation of less soluble double salts with inorganic phosphate and hexosediphosphate. The following experiments support this view. Solutions of barium hexosediphosphate (saturated) and of barium hexosemonophosphate (2.5 %) were mixed with various quantities of dilute phosphoric acid (3.4 %) and were then made pink to phenolphthalein by the addition of baryta, the requisite amount of water being added before neutralisation to make the final volume of all the solutions equal. After filtration the amounts of total and organic phosphate remaining in solution were estimated and from these the amounts in the precipitate were calculated. In some experiments barium acetate (5 %) was added to the hexosephosphate solutions, in order to approach more closely to the conditions under which the 10 % A precipitate was formed. The results are shown in Table IV.

Table IV. *Precipitation of hexosephosphates from aqueous solution with inorganic phosphate.*

Phosphoric ester	Barium acetate %	P in solution before neutralisation mg. per 100 cc.		P in precipitate after neutralisation	
		Organic	Inorganic	mg. per 100 cc. Organic	Organic Inorganic
Hexosediphosphate	0	60	12	22.7	1.90
	"	"	24	30.6	1.28
	"	"	36	35.9	1.00
	"	"	48	38.7	0.81
	"	"	60	42.1	0.70
Hexosediphosphate	5	18.8	12	8.0	0.67
	"	"	24	13.0	0.54
	"	"	36	15.5	0.43
	"	"	48	15.8	0.33
	"	"	60	16.1	0.27
Hexosemonophosphate	5	167	9.5	6.0	0.63
	"	"	28.5	14.0	0.49
	"	"	47.5	19.0	0.40

It is seen that although no alcohol was added to these solutions, very considerable proportions of the hexosephosphates were carried down with the barium phosphate, probably as double salts. The analogous double salt of hexosemonophosphate-hexosediphosphate is probably more soluble in water and is only precipitated in presence of high concentrations of the monophosphate or on addition of alcohol as in the 10 % precipitates.

Soluble B fractions.

The investigation of the soluble fractions from dried yeast and zymine experiments and the isolation of trehalosemonophosphate have already been described. The examination of the fractions from yeast juice experiments will be dealt with more particularly in a further communication, but certain points of interest may be mentioned. In order to satisfy the analytical values it was necessary to postulate the presence of small amounts of trehalosemonophosphate in these fractions, although this ester has not yet been isolated from yeast juice preparations, nor has any purified fraction been obtained with properties definitely indicative of its presence. On the other hand, certain fractions have shown the same general intermediate characters as those mentioned above, which were isolated from the 10 % precipitates. Whether these contain a hitherto unknown ester or represent merely residual mixed salts of hexosediphosphate, hexosemonophosphates and trehalosemonophosphate, as would be required by the analytical figures, remains to be proved. It is not improbable that the trehalose ester is formed during fermentations with yeast juice but that its presence is obscured and its isolation rendered difficult by the large proportion of the monophosphates. Similar but less extreme cases are shown by the zymine fermentations. The high rotation of the soluble B fractions ($[\alpha]_{5461} = +28^\circ, +30^\circ$) pointed to the presence of trehalosephosphoric ester to the extent of 14 and 16 % respectively. Isolation of the

ester in pure condition proved very difficult and was only achieved in the case of one preparation. It is hoped that the isolation may be facilitated by means of the sparingly soluble crystalline barium salt described later in the paper.

The soluble B fractions of the dried yeast and zymin experiments contained by calculation an unexpectedly large proportion of barium hexosediphosphate, an average of 10 %. Unless the solubility of this salt is greatly increased in presence of the monophosphates, this may be a further indication of the occurrence of an unknown ester not included in the calculations.

ω fractions.

These very small fractions were so highly contaminated with phosphorus-free substances that no certain inference could be drawn with regard to the phosphoric esters except that the presence of trehalosephosphate was shown by the specific rotation whenever this ester formed a significant part of the fermentation products.

Table V.

Exp.	Method of fermentation	Sugar	Percentage distribution of esterified phosphorus							
			Duration of fermentation (minutes)		Phosphorus esterified %	Probable partition of hexosemonophosphate				
						Hexose-diphosphate	Hexose-monophosphate	Fructose-monophosphate		Aldose-monophosphate
			t_1	t_2				Fructose-monophosphate	Aldose-monophosphate	
Yeast juice:										
1	A	Glucose	155	40	97.3	67.0	32.5	2.5	30.0	0.5
2	"	Fructose	155	40	97.7	47.5	52.2	13.0	39.2	0.3
3	"	"	160	45	97.6	41.5	58.0	17.0	41.0	0.5
4	"	Glucose	155	40	99.0	61.2	38.1	13.6	24.5	0.7
5	"	Fructose	140	25	99.5	39.3	61.0	12.0	49.0	0.7
6	"	"	145	25	98.8	35.0	64.0	19.0	45.0	1.0
Zymin:										
7	A	Fructose	225	35	95.2	86.5	11.0	2.0	9.0	2.5
8	"	Glucose	230	35	96.1	88.0	9.0	1.5	7.5	3.0
Dried yeast a:										
9	A	Fructose	270	40	96.6	90.0	5.0	0.0	5.0	5.0
10	"	"	270	40	97.3	84.5	10.0	1.5	8.5	5.5
11	"	Glucose	270	40	99.4	85.5	8.5	2.0	6.5	6.0
Dried yeast e:										
12	A	Fructose	270	40	94.4	92.0	3.0	0.0	3.0	5.0
Dried yeast b:										
13	A	Fructose	70	20	82.3	83.0	14.5	2.5	12.0	2.5
14	"	"	140	25	95.4	80.5	12.5	1.5	11.0	7.0
15	"	"	260	75	99.4	89.0	4.5	1.0	3.5	6.5
Dried yeast c:										
16	A	Fructose	65	10	61.6	78.5	20.0	3.0	17.0	1.5
17	"	"	90	10	76.9	90.5	5.0	0.5	4.5	4.5
18	"	"	225	45	96.1	89.5	3.5	0.5	3.0	7.0
19 a	B	Fructose	110	5	89.0	82.5	9.0	1.5	7.5	8.5
19 b	"	"	110	305	96.0	80.0	8.0	1.5	6.5	12.0
20 a	"	"	80	5	80.0	79.0	15.5	3.0	12.5	5.5
20 b	"	"	80	35	94.0	81.0	13.5	2.0	11.5	5.5
Dried yeast d:										
21 a	B	Fructose	80	5	89.0	66.6	30.4	8.5	21.9	3.0
21 b	"	"	80	19 hrs.	98.0	84.6	15.4	3.9	11.5	0.0

t_1 is the time interval between the first and last additions of phosphate.

t_2 is the time interval between the last addition of phosphate and the addition of trichloroacetic acid.

In the experiments with dried yeast and zymin, but not with yeast juice, there was a preliminary period of 1 to 1½ hrs. before the first addition of phosphate.

DISTRIBUTION OF PHOSPHORIC ESTERS IN THE PRODUCTS OF FERMENTATION.

From the foregoing discussion it is clear that the quantitative estimation of the phosphoric esters in fermentation products, by division into fractions according to the solubility of the barium salts, is likely to give erroneous results unless the composition of the fraction is further investigated by analysis. The proportion of the soluble hexosemonophosphates precipitated with the sparingly soluble hexosediphosphate and the amount of the latter remaining in the filtrate will vary according to the relative proportions of these esters and of inorganic phosphate in the fermentation products. Even the analytical methods described in this paper do not furnish entirely dependable results. Table V shows the distribution of the total esterified phosphorus based on the estimated composition of the fractions. The main division into hexosediphosphate, hexosemonophosphate and trehalosemonophosphate may be accepted as approximately correct, since other esters, if present, cannot form more than a very small fraction of the whole. The partition of the hexosemonophosphate into its aldose and fructose components is given with reserve as these figures are based, not on the theoretical iodimetric value for an aldosemonophosphate (45.6 %), but on the value (41 %) experimentally found for the purest specimen of this component yet obtained. Although there are grounds for believing this to be pure glucosemonophosphate, the disparity in the iodine reduction values is difficult to explain. In addition, both the Selivanoff and iodine values are liable to be affected by the impurities present in the crude fraction.

In Exps. 1 to 12 (method A, p. 120) each addition of sodium phosphate was made after the rate of evolution of carbon dioxide had fallen to the minimum and the fermentation was stopped at a similar interval after the last addition.

In the fermentations with yeast juice (1-6) the proportion of hexosemonophosphate was high and reached 64 % of the total esterified phosphorus in one experiment. Still higher percentages of hexosemonophosphate have been recorded by Neuberg and Leibowitz [1927] and by Harden and Henley [1929]. The presence of a very small proportion (1 % or less) of trehalosemonophosphate was deduced by calculation but this ester was not isolated from the products.

For the dried yeast fermentations five different preparations were used, *a*, *b*, *c*, *d*, being made from an English brewer's top yeast, while *e* was a bottom yeast. In Exps. 9-12 hexosediphosphate represented over 84 % of the organic phosphorus, while some 5 or 6 % was present as trehalosemonophosphate, this ester being isolated by the methods previously described.

The products of the zymyn fermentations were similar to those obtained with dried yeast, but the proportion of trehalosephosphate was smaller. The ester was isolated from the products of Exp. 8.

Exps. 13, 14 and 15 (dried yeast *b*) were made in order to study the effect

of varying the intervals between phosphate additions on the distribution of phosphoric esters in the fermentation products. The specific rotation of the three soluble B fractions, $[\alpha]_{5461} = +32^\circ$, $+58^\circ$, $+75^\circ$, clearly indicated the very different proportions of trehalosemonophosphate to hexosemonophosphate. The percentage of the latter ester was highest (Exp. 13) when the fermentation was carried out rapidly and the concentration of inorganic phosphate was maintained at a high level. With longer intervals between phosphate additions (Exp. 14) the proportion of hexosemonophosphate decreased while that of trehalosemonophosphate increased. With still longer intervals (Exp. 15) hexosediphosphate increased, chiefly at the expense of hexosemonophosphate. In Exps. 16, 17 and 18 (dried yeast *c*) the variations were of the same general nature, having regard to the fact that a different yeast preparation was used and that the time intervals between phosphate additions were shorter than in the corresponding experiments of the previous series. In Exp. 16 the total period of fermentation was only $1\frac{1}{2}$ hours and no more than 61.6 % of the phosphorus was esterified, of which 20 % was in the form of hexosemonophosphate and only 1.5 % in the form of trehalosemonophosphate. Exps. 19, 20 and 21 were made by method B (p. 120). The phosphate-sugar solution was added continuously so that a high rate of evolution of carbon dioxide was maintained. In each experiment one half of the fermenting mixture was removed and treated with trichloroacetic acid 5 minutes after the phosphate addition was completed; the remaining half was allowed to ferment for further periods of 5, $\frac{1}{2}$ and 19 hours respectively, further additions of sugar being made in Exps. 19 and 21. The results in each case show a decrease in the amount of hexosemonophosphate during the second period, but this is not so marked as in Exp. 15. In Exp. 19 *b*, 5 hours after the last phosphate addition, the production of trehalosemonophosphate reached the maximum value hitherto observed (12 %). In Exp. 21 *b*, and in other experiments not recorded, trehalosemonophosphate entirely disappeared during 19 hours' fermentation. The divergent results that may be obtained when otherwise similar experiments are carried out with different preparations of dried yeast are illustrated by Exps. 20 *a* and 21 *a*. This difficulty is intensified when the products of fermentation by yeast juice are investigated.

So far as this limited number of experiments can justify any general conclusion, it may be said that the production of hexosemonophosphate is maximum when inorganic phosphate is present in considerable excess and a high rate of fermentation is maintained; this hexosemonophosphate tends to decrease as trehalosemonophosphate accumulates when fermentation is continued with a low concentration of inorganic phosphate, but during very prolonged fermentation trehalosephosphate disappears while hexosediphosphate increases.

No significant differences have been observed between the results obtained with glucose and with fructose.

Similar conclusions with regard to the production of hexosemonophosphate

have been stated by Neuberg and Leibowitz [1927], who used juice prepared from a bottom yeast. The recent work of Boyland [1929] on the sequence of the formation of phosphoric esters by dried yeast led him to conclude that trehalosemonophosphate is formed late in the reaction with phosphate.

Experiments shortly to be published (Harden and Robison) indicate that when the hexosemonophosphates are fermented by zymase a high rate of fermentation, comparable with the phosphate rate in the fermentation of fructose, may be produced. This, however, only occurs in the presence of inorganic phosphate and the phenomenon exactly resembles that which takes place when sugar is fermented in presence of phosphate. If excess of monophosphate be added, the rate eventually falls and a second addition of monophosphate does not cause any further increase.

PREPARATION OF THE PHOSPHORIC ESTERS.

For the preparation of the individual phosphoric esters it is important to choose a type of yeast and experimental conditions which will give a maximum yield of the desired ester. For the preparation of the hexosemonophosphates rapid fermentation with an active yeast juice would be chosen. The monophosphates can be isolated and purified by conversion of the soluble B fractions into brucine salts and recrystallisation from dilute alcohol and other solvents. For the preparation of hexosediphosphate and trehalosemonophosphate a moderately prolonged fermentation with dried yeast may be expected to give the best results. The diphosphate can be readily obtained in pure condition by extracting the sparingly soluble fractions with 200 parts of water at p_H 8.6. The clear filtrate, which should be free from inorganic phosphate, is treated with alcohol until a slight cloud forms and is then heated in 200 cc. portions to 70° in a water-bath and filtered hot. The diphosphate remaining in solution can be recovered in a somewhat less pure condition by adding more alcohol and again heating to 70°. Purification by means of the lead salt is of little advantage and often leads to hydrolysis during the decomposition by sulphuretted hydrogen. The inorganic phosphate always present in the 10 % B fraction must have arisen in this way. Barium hexosediphosphate should be dried at low temperatures. Hydrolysis to the extent of 20 % has been observed after heating *in vacuo* over phosphorus pentoxide at 100° for 6 hours.

A CRYSTALLINE FORM OF BARIUM TREHALOSEMONOPHOSPHATE.

Trehalosemonophosphate may be isolated from the soluble B fraction by means of its crystalline, sparingly soluble brucine salt. The final purification is much simplified by the aid of the crystalline barium salt which has been obtained during the last few months. The amorphous salt is very soluble in water, but if the solution is treated with alcohol (15–20 %) until the first signs of precipitation are observed and then left at room temperature, transparent needles separate out in the course of a few hours and continue to

increase in quantity during some days. The concentration of alcohol may be gradually increased, but with 20 % alcohol almost the whole of the ester ultimately separates in crystalline form. The crystals are very sparingly soluble in cold water, but if brought into solution with the aid of heat or of dilute acid they can be reconverted into the amorphous soluble form by rapid precipitation with alcohol. They appear to contain water of crystallisation (5 mols), but otherwise to have the same composition as the amorphous salt. It is suggested that this crystalline form may consist of two or more molecules joined together through the barium atoms, that is of a double salt similar in type to those discussed earlier in this paper.

SUMMARY.

1. Methods are described for the separation and investigation of the phosphoric esters produced during fermentation of sugars by yeast preparations.
2. Sources of error in the conclusions drawn from results obtained with the aid of these and other methods are discussed.
3. The distribution of total esterified phosphorus between the four known esters, estimated by the application of these methods, is given for a number of fermentation experiments with yeast juice, zymin and dried yeast.
4. Some indication has been obtained of the presence of a fifth ester, but this can at most form only a very small proportion of the fermentation products. The evidence is not yet decisive.
5. The effect of varying the rate of addition of phosphate on the relative proportions of the different esters has been studied. Hexosemonophosphate was obtained in maximum yield when the fermentation was rapid and inorganic phosphate was in considerable excess. Trehalosemonophosphate, on the other hand, increased in amount when the fermentation was continued for some time after the addition of phosphate, but again decreased if the fermentation was prolonged.
6. A sparingly soluble crystalline form of barium trehalosemonophosphate is described.

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