XXX. PHOSPHORIC ESTERS IN ALCOHOLIC FERMENTATION.

I. THE SEQUENCE OF THE FORMATION OF PHOSPHORIC ESTERS AND CARBON DIOXIDE IN FERMENTATION BY DRIED YEAST.

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After Harden and Young [1906] had shown the importance of phosphates in fermentation, discovered hexosediphosphate and advanced the equations

$$(1)\quad 2C_6H_{12}O_6 + 2R_2HPO_4 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O,$$

$$(2)\quad C_6H_{10}O_4(PO_4R_2)_2 + 2H_2O = C_6H_{12}O_6 + 2R_2HPO_4,$$

Harden and Robison [1914] isolated hexosemonophosphoric ester from fermenting yeast juice. Realising that this would complicate the original equation, Harden and Henley [1927] investigated the relationship between the carbon dioxide and the hexose mono- and di-phosphates formed when inorganic phosphate is added to fermenting zymin and yeast juice. They found the ratio of the two esters to be very variable, but the amount of carbon dioxide evolved was always somewhat less than the equivalent of the total phosphorus esterified. On the average of the results obtained with zymin (acetone yeast), the carbon dioxide was slightly more than equivalent to the hexosediphosphoric ester formed. These results can be taken to support the view of Meyerhof and Lohman [1927] who suggested that hexosemonophosphate is first formed without liberation of any carbon dioxide, but later reacts to liberate carbon dioxide and produce hexosediphosphate. With some yeast juices the amount of carbon dioxide is much greater than the amount of diphosphoric ester, though it is never more than equivalent to the total phosphorus esterified.

Recently, Robison and Morgan [1928] have isolated a disaccharidemonophosphate, trehalosemonophosphoric ester, from the products of fermentation of sugar by dried yeast. Up to the present trehalosemonophosphoric ester has only been isolated from dried yeast though there have been indications of its occurrence in the products of fermentation by zymin.

In the present paper the sequence of ester production and carbon dioxide evolution and the ratios of the gas to the esters formed by dried yeast are examined.

Experimental method.

The method used in the first experiments was almost identical with that described by Harden and Henley [1927]. The precipitation of the barium salt of the diphosphoric ester was modified by adding 10 % alcohol in order to obtain more complete precipitation. Later investigation indicated, however, that this addition of alcohol caused a slight precipitation of the monophosphate, so that it did not give a perfectly clean separation. The free and combined phosphate in the solutions were estimated by the colorimetric method developed by Martland and Robison [1926].

The carbon dioxide was measured by the method of Harden, Thompson and Young [1910], and was corrected for the basic rate of fermentation and for the gas evolved owing to the production of the relatively acid esters from the free phosphate. For this purpose in all cases the gas evolved on addition of the trichloroacetic acid was measured. The difference in the amounts of gas evolved by this acid at various stages of the fermentation indicates the extent of this acid production and the correction is applied to the volume of gas liberated by the reaction of the phosphate.

The influence of the concentration of phosphoric esters on the basic rate of fermentation.

The volume was also corrected for the amount of gas which would have been formed by the normal fermentation without addition of phosphate. This last is the basic rate of fermentation caused by the steady hydrolysis of the hexosediphosphate (see equation 2). It was possible to show that in the case of many dried yeasts this basic rate of fermentation varied considerably with the concentration of phosphoric esters present. It was therefore necessary to investigate the relationship between the basic rate of fermentation and the concentration of phosphoric esters.

Several lots of 4 g. dried yeast + 25 cc. of 10 % fructose solution + 0·2 cc. of toluene were incubated at 30° and the gas evolved was measured. When equilibrium was attained (after at least an hour) various amounts of 0·6 *M* dipotassium hydrogen phosphate solution were added to the different preparations. After all the free phosphate had reacted and equilibrium was again attained the basic rate of fermentation was carefully measured and the fermentation then stopped in the usual way with trichloroacetic acid, and the phosphoric esters were estimated in the trichloroacetic acid filtrate. The results of such an experiment are to be found in Table I and in Fig. 1.

Table I. *The relation between phosphoric ester concentration and the basic rate of fermentation.* K_2HPO_4 *added.*

Exp. 44. Dried yeast of 9. iii. 28.

Molecular concentrations			Basic or hydrolysis rate cc. per 5 min.	Ratios		
Total P	P as diphosphate	P as monophosphate		P as diphosphate / P as monophosphate	Basic rate / P as monophosphate	Basic rate / P as diphosphate
0·072	0·061	0·011	2·5	5·5	227	41
0·098	0·084	0·014	3·0	6·0	215	36
0·107	0·091	0·016	4·3	5·7	268	47
0·152	0·136	0·017	4·9	8·0	290	36
0·188	0·171	0·017	5·0	10·0	294	29
0·228	0·211	0·017	5·0	12·4	296	24

Except that no large amount of carbon dioxide was evolved and that equilibrium was quickly attained, the addition of hexosediphosphate instead of potassium phosphate gave almost identical results as regards the equilibrium attained and the change in basic rate of fermentation. Such an experiment is shown in Table II and in Fig. 2.

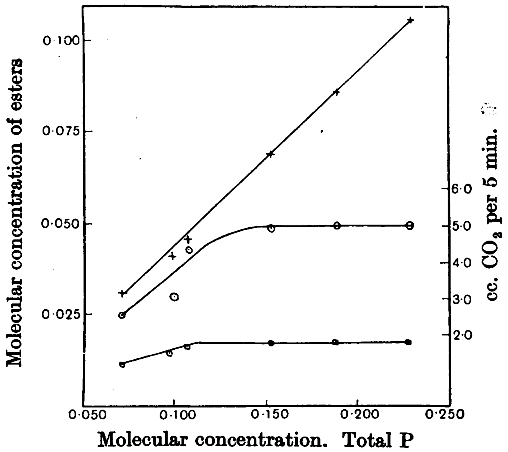

Fig. 1.

—✗—✗—✗— Diphosphoric ester.

—□—□—□— Monophosphoric esters.

—○—○—○— Hydrolysis or basic rate (cc. CO_2 per 5 min.).

Table II. *The relation between the phosphoric ester concentration and the basic rate of fermentation after the addition of hexosediphosphate.*

Exp. 45. Dried yeast of 20. ii. 28.

Molecular concentrations			Hydrolysis rate cc. per 5 min.	Ratios		
Total P	P as diphosphate	P as monophosphate		P as diphosphate / P as monophosphate	Basic rate / P as monophosphate	Basic rate / P as diphosphate
0·071	0·061	0·010	3·9	6·1	390	64
0·080	0·068	0·012	4·3	5·7	358	63
0·087	0·074	0·013	4·7	5·7	362	64
0·102	0·088	0·014	5·3	6·3	382	60
0·119	0·105	0·014	6·1	7·5	434	58
0·131	0·116	0·015	5·8	7·7	387	50
0·163	0·149	0·014	6·0	10·6	428	40

These results are in agreement with the earlier work of Harden and Young [1910], who found that the addition of phosphate to a fermenting mixture of yeast juice and sugar containing very little phosphate, caused a greatly increased amount of fermentation. The increase was much greater than the carbon dioxide equivalent of the added phosphate, and it was probably due in part to the increased basic rate caused by the added phosphate. It is therefore probable that there is a necessary phosphoric ester concentration for the maximum basic rate of fermentation of yeast juice as well as of dried yeast. Yeast juice seems to require less of this phosphate than does dried yeast.

In calculating the amount of carbon dioxide evolved by the reaction of inorganic phosphate, the basal fermentation is therefore assumed to proceed at a rate varying with the amount of esterification that has occurred. Thus up to a certain concentration (varying slightly with the preparation) the basic rate of fermentation is assumed to be proportional to the concentration of esterified phosphate. At the beginning of the reaction the rate is assumed to be the same as that observed before the addition of phosphate. After the time of complete esterification it is taken as being the same as that of the final basic rate. At a point when half the phosphate is esterified it is assumed to be half-way between these two rates, and so on. If the basic rate is assumed to be that which occurred before the addition of phosphate, then the calculated amount of carbon dioxide liberated by the phosphate will be too high. If it is taken as the final basic rate then the correction will be too large and the final figure for the carbon dioxide will be too low. The different methods of calculating the basic rate may cause differences of as much as 10 % in the calculation of the amount of gas evolved. It is deduced from the previous experiments that the assumption of the basic rate being proportional to the esterified phosphorus gives the most accurate value for the carbon dioxide.

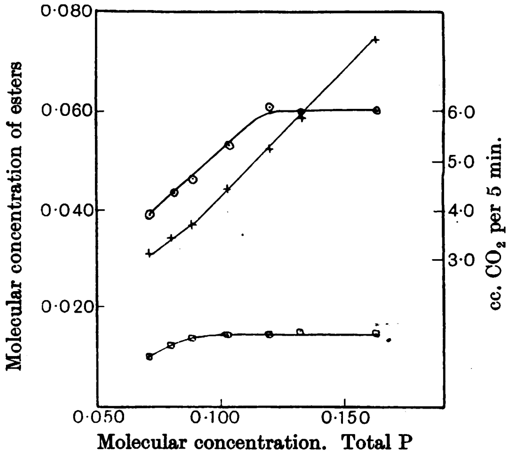

Fig. 2.

—×——×——×— Diphosphoric ester.

—▫——▫——▫— Monophosphoric esters.

—⊘——⊘——⊘— Hydrolysis or basic rate (cc. CO_2 per 5 min.).

The equilibrium of the phosphoric esters.

These results also show that up to a certain concentration of phosphate, there is proportionality between the concentrations of hexosediphosphate, monophosphate and the hydrolysis rate. Above this critical concentration, the amount of monophosphate and the hydrolysis rate remain constant. The significance of the fact that the basic rate and the monophosphoric ester concentration have the same critical concentration is not quite clear, although it possibly indicates that the slowest reaction in the system is the formation of the monophosphoric ester. The rate of the formation of this ester increases up to a certain point with increase in the concentration of hexosediphosphate, but beyond the critical point the enzyme acting on the diphosphate becomes saturated with this substrate. The limiting factor after this point is not the amount of ester present, but rather the amount of enzyme.

The monophosphoric ester isolated from dried yeast fermentations in equilibrium, on analysis and determination of the optical rotation and reducing power, seems to contain 70 % of its phosphorus bound as trehalosemonophosphate.

Hexosediphosphate added to dried yeast fermenting fructose is in part converted into trehalosemonophosphate, thus maintaining the equilibrium ratio between the two esters. If this equilibrium is true, it should also be obtained when trehalosemonophosphate is added to the fermenting mixture. In order to test this, a solution of the potassium salt of trehalosemonophosphate was added to fermentations which were stopped in the usual way during the reaction. The changes in the esters are shown in Table III. The change appeared to be accompanied by a slight increase in carbon dioxide evolution.

Table III. *Phosphoric ester equilibrium after the addition of trehalosemonophosphate to dried yeast and fructose.*

Exp. 81. Dried yeast of 9. iii. 28.

Time after addition	Molecular concentrations expressed as P: Diphosphate	Monophosphate	P as diphosphate / P as monophosphate
No addition	0·078	0·013	6·0
0	0·078	0·020	3·9
5	0·080	0·018	4·5
10	0·082	0·016	5·1
15	0·083	0·015	5·6
30	0·084	0·014	6·0

Thus trehalosemonophosphate appears to be rapidly hydrolysed by fermenting dried yeast with excess sugar and the usual equilibrium ratio between the two esters is soon reached.

The equilibrium is reached in about the same time if hexosediphosphate is added to the fermenting mixture, in place of trehalosemonophosphate, as is shown in Table IV. In this experiment a solution of the potassium salt of hexosediphosphate was added to the fermenting preparations which were stopped at various intervals after the additions. The carbon dioxide evolution was measured, but the large change in the basic rate and the period required for the completion of the reaction made it impossible to calculate the extra carbon dioxide evolved, if indeed any was evolved.

Table IV. *The rate of change in phosphoric esters after the addition of hexosediphosphate to dried yeast and fructose.*

Exp. 83. Dried yeast of 9. iii. 28.

Time after addition	Molecular concentration expressed as P		P as diphosphate / P as monophosphate
	Diphosphate	Monophosphate	
No addition	0·101	0·016	6·3
0	0·140	0·016	8·8
10	0·136	0·018	7·5
20	0·131	0·020	6·6
30	0·130	0·020	6·5
40	0·133	0·020	6·6

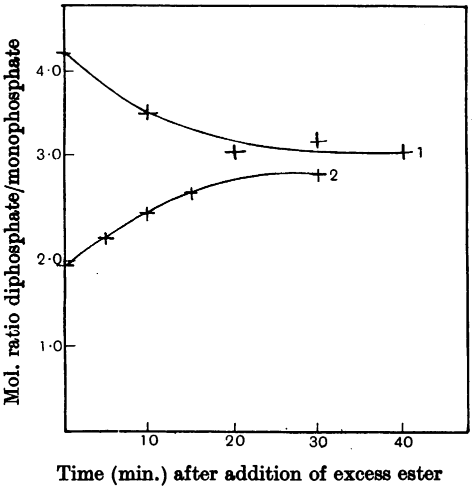

Fig. 3. In curve 1 hexosediphosphate was added; in curve 2 trehalosemonophosphate was added to dried yeast fermenting fructose.

These last two experiments are shown graphically in Fig. 3, where it can be seen that the equilibrium of the two esters is a true equilibrium, which is attained from either side at almost the same rate. The occurrence of this equilibrium ratio of the esters probably indicates that one of these esters is derived from the other.

THE LAG BETWEEN ESTERIFICATION AND CARBON DIOXIDE EVOLUTION.

In order to study the relations of the formation of the various products of the reaction of phosphate with dried yeast, the same amount of phosphate was added to several preparations which were stopped at various times during the reaction. The carbon dioxide evolved was measured and the free phosphate and phosphoric esters in solution were estimated. The carbon dioxide was corrected for the amount evolved by the formation of the relatively acid phosphoric esters and for the basic hydrolysis rate.

The fully corrected results of typical experiments of this kind are shown in Tables V, VI and VII and in Fig. 4. In the tables the ratios of the P in the form of the two esters and of the amount of carbon dioxide formed to the amount of P as diphosphoric ester, and to the amount of P esterified plus the amount of P as monophosphate (P as diphosphate plus twice the P as monophosphate) are given.

Table V. *The time relations of the products of the reaction of phosphate with dried yeast.*

Exp. 42. 2·5 cc. 0·6 *M* K_2HPO_4 added to 4 g. dried yeast + 25 cc. 10 % fructose solution. Basic rate before addition 2·5 cc.; after, 3·0 cc. per 5 min. The mono- and di-phosphate are expressed in terms of P.

	Mol. conc.		Mol. conc. of substances formed		Ratios		
Time min.	Mono-phosphate	Di-phosphate	Di-phosphate	CO_2	$\frac{\text{Diphosphate}}{\text{Monophosphate}}$	$\frac{CO_2}{\text{P esterified}}$	$\frac{CO_2}{\text{P esterified} + \text{P as monophosphate}}$
0	0·026	0·078	—	—	3·0	—	—
10	0·028	0·101	0·023	0·024	3·6	0·96	0·89
20	0·032	0·105	0·027	0·046	3·3	1·40	1·18
30	0·033	0·107	0·029	0·046	3·2	1·28	1·07
40	0·031	0·109	0·031	0·046	3·5	1·28	1·12
50	0·033	0·107	0·029	0·046	3·2	1·28	1·07

Table VI.

Exp. 32 (cf. Fig. 3). 5 cc. 0·6 *M* K_2HPO_4 added to 4 g. dried yeast + 25 cc. 10 % fructose solution. Basic rate before addition 4·4 cc.; after, 6·5 cc. per 5 min. The mono- and di-phosphate are expressed in terms of P.

	Mol. conc.		Mol. conc. of substances formed		Ratios		
Time min.	Mono-phosphate	Di-phosphate	Di-phosphate	CO_2	$\frac{\text{Diphosphate}}{\text{Monophosphate}}$	$\frac{CO_2}{\text{P esterified}}$	$\frac{CO_2}{\text{P esterified} + \text{P as monophosphate}}$
0	0·011	0·073	—	—	6·6	—	—
15	0·009	0·147	0·076	0·061	15·2	0·85	0·88
30	0·010	0·170	0·097	0·098	17·0	1·01	1·02
45	0·023	0·156	0·083	0·113	6·8	1·18	1·05
60	0·025	0·154	0·081	0·113	6·2	1·18	1·03
75	0·021	0·158	0·085	0·113	7·5	1·18	1·07
90	0·025	0·154	0·081	0·113	6·2	1·18	1·03

Table VII.

Exp. 62. 10 cc. 0·6 *M* K_2HPO_4 added to 4 g. dried yeast + 25 cc. 10 % fructose solution. Basic rate before addition 4·4 cc.; after, 6·6 cc. per 5 min. The mono- and di-phosphate are expressed in terms of P.

	Mol. conc.		Mol. conc. of substances formed		Ratios		
Time min.	Mono-phosphate	Di-phosphate	Di-phosphate	CO_2	$\frac{\text{Diphosphate}}{\text{Monophosphate}}$	$\frac{CO_2}{\text{P esterified}}$	$\frac{CO_2}{\text{P esterified} + \text{P as monophosphate}}$
0	0·016	0·064	—	—	4·0	—	—
5	0·016	0·078	0·014	0·009	4·9	0·65	0·65
10	0·019	0·097	0·033	0·018	5·1	0·50	0·46
15	0·023	0·111	0·047	0·036	5·1	0·68	0·61
40	0·034	0·196	0·132	0·149	5·7	1·00	0·89
50	0·034	0·204	0·140	0·167	6·0	1·06	0·96
75	0·034	0·204	0·138	0·168	6·0	1·05	0·97

This type of experiment was carried out many times, with different samples of dried yeast and with different amounts of added phosphate, and similar results were always obtained. Examination of these observations shows that the hexosediphosphoric ester appears as the first product of the reaction and that there is a considerable lag between esterification and the evolution of carbon dioxide. This is not due to physical causes such as supersaturation, because such would be accounted for in the correction applied for the production of acid esters from inorganic phosphate. In the presence of free phosphate, therefore, the esterification proceeds at a higher rate than the reaction, whatever it may be, which results in the formation of alcohol and carbon dioxide. At the end of the reaction the carbon dioxide production overtakes the ester formation and finally somewhat more gas is evolved than is equivalent to the reacting phosphate.

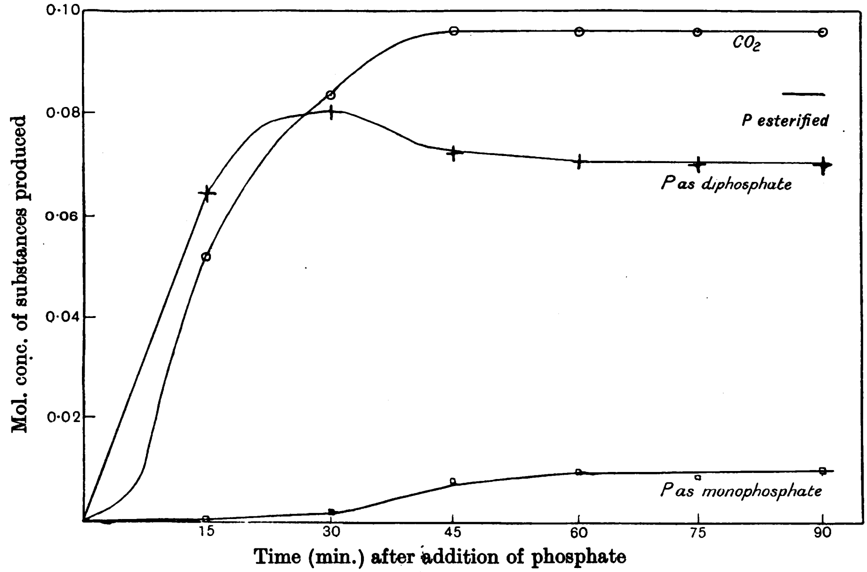

Fig. 4.

A similar delay in carbon dioxide evolution after the addition of phosphate has been indicated by Euler and Johansson [1913]. They considered that the activation of the sugar, the formation of hexosediphosphate and then of a triosemonophosphate were stages in the reaction of phosphate which all occurred before the evolution of carbon dioxide in the reaction of phosphate with dried yeast in the presence of dextrose.

The ratio of carbon dioxide to phosphoric esters.

In all cases in which the gas evolved from the complete reaction of phosphate was measured, it was found to be somewhat more than equivalent to the phosphate. On the other hand maceration extract, yeast juice and zymin, so far as at present known, tend to give less than the equivalent amount of carbon dioxide from added phosphate [Harden and Henley, 1927; Kluyver and Struik, 1928]. Coupled with this is the fact that dried yeast alone gives a monophosphate fraction consisting mainly of trehalosemonophosphate. In all the experiments, the ratios of carbon dioxide to the total P esterified, and to the total P esterified plus the P as monophosphate were calculated. The results of over 50 such determinations, the averages of which are shown in Table VIII, point to equivalence between the carbon dioxide and the total P esterified plus the P as monophosphate, *i.e.* to the amount of P as diphosphate plus twice the amount of P as monophosphate. Thus the phosphorus finally bound as monophosphate appears to react twice. This may possibly be explained by a change of the diphosphoric ester into the monophosphoric ester and free phosphate at a relatively high rate, the latter then reacting again to liberate carbon dioxide and form hexosediphosphate.

The ratio of carbon dioxide to esterified phosphorus appears to vary with the amount of reacting phosphate, being lower with large amounts of phosphate; this may be due to two causes. Firstly, the addition of large amounts of phosphate causes an inhibition in the carbon dioxide production, probably due to a lowering of the basic rate of fermentation so that the observed corrected amount of carbon dioxide will be too low. Smaller amounts of phosphate produce no such inhibition and there is consequently no error of this kind. Secondly, with small amounts of phosphate there is a relatively larger amount of monophosphate formed, so that, if the ratio of carbon dioxide to phosphorus esterified plus the amount of P as monophosphate remains at unity, then the ratio of carbon dioxide to the phosphorus esterified will necessarily be high.

Table VIII. *The ratio of carbon dioxide evolved to phosphoric esters produced on adding potassium phosphate to dried yeast fermenting fructose.*

			Average ratios	
Yeast	cc. 0·6 M K_2HPO_4 added to 4 g. dried yeast	No. of experiments	$\frac{\text{Carbon dioxide}}{\text{P esterified}}$	$\frac{\text{Carbon dioxide}}{\text{P esterified + P as monophosphate}}$
20. ii. 28	2·5	7	1·16	0·98
	5·0	14	1·14	1·02
	7·5	2	1·07	1·00
	10·0	1	1·06	1·00
9. iii. 28	2·5	8	1·22	1·01
	5·0	8	1·04	1·00
	7·5	2	1·05	1·02
	10·0	7	1·05	0·99
11. vi. 28	5·0	3	1·16	1·01
Scotch bottom yeast	5·0	2	1·08	1·00
	Total	54	Average 1·12	1·00

The maximum error in the determination of the ratio of carbon dioxide to esterified phosphate is probably about 5 % in each single experiment. The amount of phosphate can be measured easily to 1 % but the carbon dioxide cannot be determined to nearer than 2 or 3 %. The determination of the monophosphate may have a 10 % error, but, as it forms only one-seventh of the total phosphate, the error will only influence the final ratio by 2 %. The average of 50 determinations of the ratio of carbon dioxide to the P esterified plus the amount of P as monophosphate is 1·00, and this is probably accurate to within 2 %.

DISCUSSION.

The ratio of the various enzymes in dried yeast is such that under equilibrium conditions (*i.e.* when a steady rate of carbon dioxide evolution is attained) the esterified phosphate consists mainly of hexosediphosphate together with a little monophosphoric ester, which is largely the ester of the disaccharide trehalose. The equilibrium attained after the addition of phosphate (below a certain critical concentration) gives the same ratio of the phosphoric esters. There appears to be true equilibrium between the esters, depending upon the amounts of the various enzymes present in the preparation. If relatively large amounts of phosphate are added (*i.e.* above the critical concentration) the ratio of hexosediphosphate to monophosphate is increased. This can be explained by the saturation of the enzyme acting on the diphosphate, so that increases in its concentration beyond a limiting value do not affect the rate of its decomposition.

In presence of dried yeast the monophosphoric esters are produced late in the reaction with phosphate—after much hexosediphosphoric ester has been formed—and the phosphorus bound in this form appears to liberate two equivalents of carbon dioxide.

The significance of trehalosemonophosphoric ester in the process of fermentation is not yet clear but it is hoped that it will be further elucidated in a later communication.

SUMMARY.

Experiments on the action of phosphates and observations on the esters occurring in fermenting dried yeast preparations have given the following results.

(1) Up to a limiting concentration of phosphoric esters, the basic rate of fermentation varies with the concentration of phosphorus present as phosphoric esters. Up to this same concentration the ratio of diphosphoric ester to monophosphoric ester remains constant, but above the limiting concentration the relative amount of diphosphoric ester increases.

(2) The addition of inorganic phosphate to dried yeast fermenting fructose is followed by esterification and liberation of carbon dioxide, but there is a considerable time lag between esterification and the evolution of carbon dioxide.

(3) The carbon dioxide liberated by phosphate is somewhat more than equivalent to the added phosphate, but is approximately equivalent to the phosphorus esterified plus the amount of P as monophosphate formed (*i.e.* the P as diphosphate plus twice the P as monophosphate).

(4) In fermentations by dried yeast in presence of phosphate the monophosphoric esters are formed late in the reaction. Under the conditions described in this paper these esters consist mainly of trehalosemonophosphate.

Throughout this work my indebtedness to Prof. A. Harden and to Dr R. Robison will be obvious, and I wish to thank them for the constant help they have given me during the progress of the research.

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