XXXVI. THE FERMENTATION OF GLUCOSE AND FRUCTOSE BY DRIED YEAST IN THE SIMULTANEOUS PRESENCE OF PHOSPHATE AND SULPHITE.

By FUMIWO HEMMI.

From the Biochemical Department, Lister Institute.

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NEUBERG has conclusively demonstrated in a long series of brilliant researches that when sugar is fermented by yeast, or by yeast preparations such as dried yeast or maceration extract, acetaldehyde is formed as an intermediate product and is then converted by reduction into alcohol. When the acetaldehyde is "fixed" by the addition of a sulphite, another intermediate product is reduced with the formation of glycerol,

and the fermentation is thus enabled to continue.

Now it has been shown by Harden and Young [1910] that in the normal fermentation of sugar by yeast preparations hexosediphosphate is formed according to the equation:

This is regarded by these authors as also representing the course of fermentation in the yeast cell, whereas Neuberg regards it as a pathological phenomenon which does not occur normally in the yeast cell, but only when the cell has been injured in some way, by drying, grinding, action of toluene, etc. Under these circumstances it was thought to be of interest to examine the effect of the presence of phosphate on the production of aldehyde by yeast preparations in the presence of sulphite, in order to ascertain whether either of these two changes in any way interfered with the progress of the other.

The results show that the addition of phosphate in no way interferes with the production of aldehyde. On the contrary the percentage of aldehyde, as calculated on the sugar actually fermented is usually slightly greater in the presence than in the absence of added phosphate. The fermentation of potassium hexosephosphate itself also yields aldehyde in presence of sulphite.

Further, the presence of sulphite does not prevent esterification of the phosphoric acid (see Exps. 5 and 6) so that the two processes in no way interfere. This is consistent with the view of Harden and Young that the phosphate is concerned in the early stages of the decomposition of the sugar molecule.

EXPERIMENTAL.

In order to obtain a series of comparable results dried yeast was employed in presence of toluene. Three different samples of dried yeast (Nos. 1, 2, 3) were used at different times. They were all prepared by pressing out fresh brewer's yeast at about $\frac{1}{2}$ ton per sq. inch and then drying at 37° for 48 hours.

The experiments were carried out by placing 5 g. of the dried yeast + 25 cc. H_2O + 0.2 cc. toluene + 1 (or sometimes 2) g. of the sugar in a flask and incubating at 30° in a thermostat, the CO_2 evolved being measured. After an interval, usually of 1 hour, additions were made in the form of 0.6M K_2HPO_4 , a solution of 20 g. $Na_2SO_3 \cdot 7H_2O$ per 100 cc. and if necessary water, all previously saturated at 30° with CO_2 . No estimations of p_H were made.

After the requisite period of incubation, during which any CO_2 evolved was measured, the contents of the flask were cooled in ice and made up to 100 cc. One portion of 50 cc. was filtered and 20 cc. taken for the estimation of inorganic phosphate by means of magnesium citrate solution and ammonia. The results are expressed as mgm. of K_2HPO_4 . In the remainder the acetaldehyde was estimated by the method described by Neuberg [1918]. Excess of barium chloride solution was added, the mixture allowed to stand in the cold, filtered and a portion of the filtrate distilled with $CaCO_3$ and the aldehyde estimated in the distillate.

Another portion (10 cc.) of the filtrate was taken for the estimation of reducing sugar by Bertrand's method, after precipitating the excess of barium by sodium sulphate.

The amount of sugar thus obtained does not however represent the total sugar which has escaped fermentation to CO_2 and either alcohol or aldehyde. In the first place any hexosediphosphate present at the close of fermentation would be almost completely precipitated by the barium chloride and in the second place any hexosemonophosphoric acid [Robison, 1922] would still be present, but would have a reducing power considerably less than that of free glucose or fructose. Further information can be obtained from a consideration of the amount of inorganic phosphate in the liquid, the difference between this and the total of the amounts added and originally present in the yeast representing phosphate combined either as hexosediphosphate or hexosemonophosphate.

In order to obtain an approximate idea of the amount of sugar fermented it has been assumed that the combined sugar is all present as hexosediphosphate.

In the first place it was found, in agreement with Neuberg's results [1919], that the addition of sulphite must be made after a vigorous sugar fermentation had commenced. When the sulphite was added along with the sugar at the commencement of the experiment, fermentation did not ensue, and even when the addition was made at the end of 30 minutes very little action occurred. When an hour was allowed to elapse before the addition was made,

satisfactory results were obtained. This is clearly shown in the following experiment.

Exp. 1. 5 g. of dried yeast (No. 2) + 1 g. glucose + 25 cc. H_2O + 0.2 cc. toluene, incubated at 30° .

To four series of flasks the following additions were made after intervals of 5, 30 and 60 minutes from the commencement of incubation and the mixtures were incubated for a further period of six hours.

No.	0.6M K_2HPO_4 cc.	20 % $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ cc.	H_2O cc.
1	5	2.5	2.5
2	0	2.5	7.5
3	5	5	0
4	0	5	5

Results.

No.	Time of addition mins.	Glucose mgm.		Free phosphate as K_2HPO_4 mgm.		Acetaldehyde mgm.
		Initial	Final	Initial	Final	
1	5	1000	1014	900	826	0
	30	916	923	880	791	5.1
	60	864	422	820	668	10.9
2	5	1000	1005	380	366	0
	30	916	970	360	383	4.5
	60	864	0	300	337	32
3	5	1000	1005	900	820	0
	30	916	920	880	781	8.3
	60	864	630	820	708	14.7
4	5	1000	1005	380	374	0
	30	916	978	360	381	7.7
	60	864	702	300	358	12.8

The initial figures are calculated from the amount of CO_2 evolved during the preliminary incubation.

In all subsequent experiments therefore the sulphite was added at least one hour after the fermentation had commenced.

It was also found that both phosphate and sulphite tended to stop the fermentation so that it was necessary to ascertain the best amounts to add. It was not found possible to use more than 5 cc. of 20 % sulphite (= 0.5 g. Na_2SO_3) + 10 cc. of 0.6M K_2HPO_4 (= 1.045 g. K_2HPO_4) with 5 g. dried yeast in a total volume of about 40 cc., and even this was excessive and resulted in a very small amount of fermentation. This only amounts to a concentration of sulphite of 1.25 g. Na_2SO_3 per 100 cc., whereas Neuberg (with living yeast) used 12.6 g. per 100 cc. This amount of sulphite is theoretically capable of combining with 174 milligrams of aldehyde corresponding with 0.73 g. of sugar fermented according to Neuberg's equation. In many experiments only 2.5 cc. of 20 % sulphite and 5 cc. of 0.6M K_2HPO_4 were used and in some only 1.25 cc. of sulphite.

The following is a typical experiment in which 5 cc. of sodium sulphite and 10 cc. of K_2HPO_4 were used.

Exp. 2. 5 g. dried yeast (No. 1) + 1 g. glucose + 25 cc. H_2O + 0.2 cc. toluene.

After 75 mins. incubation the following additions were made to two series of flasks.

No.	0.6M K_2HPO_4 cc.	20 % $Na_2SO_3 \cdot 7H_2O$ cc.	H_2O cc.
1	10	5	0
2	10	0	5
3	0	5	10
4	0	0	15

After the addition the flasks of Series 1 were incubated for three hours and those of Series 2 for six hours.

Results.

No.	Incubation period	Residual glucose mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
			Total	Free	Combined	
1	3	476	1405	1105	300	10.1
	6	450	1405	1271	134	16.8
2	3	50	1405	1193	212	0
	6	0	1405	1304	101	0
3	3	512	360	310	50	9.1
	6	492	360	360	0	11.2
4	3	100	360	202	158	0
	6	0	360	228	132	0

The sugar present (free and combined) at the moment when the additions were made was 0.65 g. and the phosphate content of the dried yeast was 360 mgm. (K_2HPO_4).

In this case the amount of fermentation was greatly reduced by the addition of the sulphite, compare 1 and 2, 3 and 4. The amount of phosphate is also somewhat excessive.

Other experiments in which less sulphite was employed are the following:

Exp. 3. 5 g. dried yeast (No. 2) + 1 g. glucose + 25 cc. H_2O + 0.2 cc. toluene at 30°.

After one hour's incubation, the following additions were made and incubation continued for six hours.

No.	0.6M K_2HPO_4 cc.	20 % $Na_2SO_3 \cdot 7H_2O$ cc.	H_2O cc.
1	5	2.5	2.5
2	2.5	2.5	5
3	0	2.5	7.5
4	5	1.25	3.75
5	0	1.25	8.75
6	0	0	10

After one hour the total remaining glucose (free and combined) was 747 mgm. The analyses gave the following results:

No.	Residual glucose mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
		Total	Free	Combined	
1	438	902	669	233	10.9
2	180	641	389	252	13.5
3	0	380	344	36	31.7
4	0	902	770	132	35.0
5	0	380	340	40	28.2
6	0	380	210	170	0

Here in presence of 5 cc. phosphate, 2.5 cc. of Na_2SO_3 solution has greatly diminished the fermentation, whereas in absence of the phosphate the sugar has been practically all fermented away as has also been the case in the presence of 1.25 cc. of Na_2SO_3 even in the presence of 5 cc. of phosphate.

On the assumption that the whole of the combined phosphorus is present as hexosediphosphate, the percentages work out as follows:

No.	Sugar used	Sugar as phosphoric ester	Sugar fermented	Acetaldehyde	Acetaldehyde % of sugar fermented
1	309	121	188	10.9	5.8
2	567	130	437	13.5	3.1
3	747	19	728	31.7	4.3
4	747	68	679	35.0	5.2
5	747	21	726	28.2	3.9

It will be observed that both in presence of 2.5 and 1.25 cc. of sulphite the higher percentage is produced in presence of added phosphate. It must be remembered at the same time that the sulphite in 4 and 5 was only capable of combining with about one-third of the aldehyde theoretically obtainable from the sugar fermented.

Exp. 4. 5 g. dried yeast (No. 2) and 1.933 g. fructose (2 g. of 96.6 %) + 25 cc. H_2O + .2 cc. toluene.

After one hour's incubation, the following additions were made and incubation continued for six hours.

No.	0.6 M K_2HPO_4 cc.	20 % $\text{Na}_2\text{SO}_3 \cdot 7\text{H}_2\text{O}$ cc.	H_2O cc.
1	5	2.5	2.5
2	2.5	2.5	5
3	0	2.5	7.5
4	5	1.25	3.75
5	0	1.25	8.75

After one hour the total remaining fructose (free and combined) was 1680 mgm.

Results.

No.	Residual fructose mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
		Total	Free	Combined	
1	293	902	209	693	56.3
2	173	641	59	582	42.7
3	348	380	0	380	46.7
4	108	902	484	418	47.4
5	422	380	0	380	33.9

Treating these figures as those in Exp. 3, the following percentages are obtained.

No.	Fructose used	Fructose as phosphoric ester	Fructose fermented	Acetaldehyde	
				mgm.	% of fructose fermented
1	1387	359	1028	56.3	5.5
2	1507	301	1206	42.7	3.5
3	1332	197	1135	46.7	4.1
4	1572	216	1356	47.4	3.5
5	1258	197	1061	33.9	3.2

Exp. 5. 5 g. dried yeast (No. 2) + 1.933 g. fructose (2 g. of 96.6 %) + 25 cc. H_2O + .2 cc. toluene at 30°.

After one hour's incubation the following additions were made and incubation continued for nine hours.

No.	0.6M K_2HPO_4 cc.	20 % $Na_2SO_3 \cdot 7H_2O$ cc.	H_2O cc.
1	5	2	3
2	5	2.5	2.5
3	0	2.5	7.5
4	5	3	2
5	0	3	7

After one hour the total remaining fructose (free and combined) was 1680 mgm.

Results.

No.	Residual fructose mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
		Total	Free	Combined	
1	72	902	675	227	62.7
2	87	902	458	444	74.2
3	231	380	153	227	66.0
4	216	902	311	591	65.3
5	159	380	15	365	51.1

Treating these figures as those in Exp. 3, the following percentages are obtained.

No.	Fructose used	Fructose as phosphoric ester	Fructose fermented	Acetaldehyde	
				mgm.	% of fructose fermented
1	1608	117	1491	62.7	4.2
2	1593	230	1363	74.2	5.4
3	1449	117	1332	66.0	5.0
4	1464	306	1158	65.3	5.6
5	1521	189	1332	51.1	3.8

In No. 1 the amount of aldehyde is not far from the maximum capable of being combined with the sulphite present.

Experiments were also made to ascertain whether fructose, which reacts more readily than glucose with phosphate, would yield aldehyde more or less readily than glucose (Exp. 6) and whether hexosephosphate was also fermented with production of aldehyde (Exp. 7).

Exp. 6. 5 g. dried yeast (No. 3) + 25 cc. water + 0.2 cc. toluene and in (1) and (2) 2 g. glucose and in (3) and (4) 1.97 fructose.

After one hour's incubation the following additions were made and incubation continued for nine hours.

No.	0.6M K_2HPO_4 cc.	20 % $Na_2SO_3 \cdot 7H_2O$ cc.	H_2O cc.
1	5	2.5	0
2	0	2.5	5
3	5	2.5	0
4	0	2.5	5

Results.

No.	Residual sugar mgm.	Phosphate as K_2HPO_4 mgm.			Acetaldehyde mgm.
		Total	Free	Combined	
1	0	902	773	129	75.8
2	0	380	363	17	50.9
3	0	902	794	108	74.9
4	0	380	371	9	53.8

Treating these figures as before:

No.	Sugar used	Sugar as phosphoric ester	Sugar fermented	Acetaldehyde	
				mgm.	% of sugar fermented
1	1681	67	1614	75.8	4.7
2	1699	9	1690	50.9	3.0
3	1636	56	1580	74.9	4.7
4	1629	5	1624	53.8	3.3

Glucose and fructose appeared to act exactly alike. In both cases the percentage production in presence of added phosphate is appreciably greater than in its absence.

Exp. 7. In this experiment, in order to ascertain whether the decomposition product of hexosediphosphate had any special significance for the production of aldehyde, fructose and phosphate were added in such proportion that after the preliminary incubation the whole of the residual sugar should be present in the combined form.

The results obtained (summarised below) were of the same order as when free glucose or fructose was fermented.

No.	Time of incubation hours	Sulphite cc.	Sugar fermented calculated from hexosephosphate decomposed mgm.	Acetaldehyde	
				mgm.	% of sugar fermented
1 (a)	6	2	106	10.2	9.7
(b)	6	2.5	108	10.9	10.5
2 (a)	24	2	216	11.5	5.3
(b)	24	2.5	222	12.2	5.5

SUMMARY.

When the fixation method of Neuberg is applied to the fermentation by dried yeast of glucose or fructose, acetaldehyde is produced to about the same extent in the presence or absence of added phosphate.

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