XXXIV. THE SALT EFFECT IN ALCOHOLIC FERMENTATION.

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WHEN the necessary hydrogen acceptor (in the form of acetaldehyde) is supplied to a mixture of yeast juice and sugar containing the appropriate concentration of phosphate, the maximum rate of fermentation is attained with practically no delay [Harden and Henley, 1920]. If, however, the acetaldehyde be omitted, the other conditions remaining unchanged, there will be a considerable delay in attaining the maximum rate of fermentation. With increasing concentration of phosphate, the period of delay is lengthened, as the maximum rate of fermentation is in general not attained until the concentration of phosphate has been reduced approximately to the optimum by the formation of hexosephosphate (see Figs. 1 and 2). If the concentration of phosphate be raised still more a point is reached at which the rate of fermentation never reaches the maximum. In presence of added acceptor (acetaldehyde) the maximum rate of fermentation attainable and optimum concentration of phosphate are higher than in its absence [Harden and Henley, 1920].

It appears therefore that the enzymes concerned are extremely sensitive to even a slightly excessive concentration of phosphate.

Meyerhof [1918] has shown that NaCl and other salts exercise a similar depressing effect both on the rate of attainment of the maximum and on the maximum rate attained and he therefore considers that phosphate, in addition to its specific function, exerts a general effect on the process which is shared by other salts. The following experiments were undertaken with the object of comparing the relative effects of different salts and of ascertaining whether the effect is a general one or is specific to any particular stage of the fermentation process.

It was found in the first place, in full confirmation of Meyerhof's results, that the effect of other salts is in general similar to that of excess of phosphate. Using equimolecular solutions the chlorides of Na and K have about the same effect, which is, however, less than that of the corresponding sulphates.

As regards the effect in different stages of the process, direct experiment showed that chlorides and sulphates, in the concentrations employed in these experiments, had no depressing effect on the rate of decomposition of pyruvic acid, whilst phosphates had a slightly stimulating effect, probably due to the

diminution of $[H]$ in their presence. It was next established that the characteristic salt effect was not removed or greatly modified by the addition of acetaldehyde. Finally it was found that the rate of action of the hexose-phosphatase was diminished by the salts employed.

As regards the effect of excess of phosphate it was found that this differs from that of sulphates and chlorides by being relatively greater in the absence of acetaldehyde and less in its presence.

Experimental.

I. EFFECT OF $NaCl$, Na_2SO_4 , KCl AND K_2SO_4 ON THE FERMENTATION OF FRUCTOSE AND GLUCOSE BY ZYMIN.

Expt. 1. The fermenting mixtures had a concentration $0.1 M$ of K_2HPO_4 and $0.25 M$ of the experimental salt. The maxima attained and the times required for their attainment were as follows:

	Maximum rate cc. per 5 mins.	Time mins.
Control	7.7	35
$NaCl$	5.2	48
Na_2SO_4	4.3	57
KCl	5.2	45
K_2SO_4	4.4	52

The results with glucose and fructose were similar. Meyerhof's observations are thus confirmed and it is further to be noticed that the chlorides of Na and K have about the same effect, which is less than that produced by the corresponding sulphates. The greater effect of these latter salts indicates that it is probably the anion of the salt which is efficacious.

II. EFFECT OF SALTS ON PARTICULAR STAGES OF THE FERMENTATION PROCESS.

A. *Effect of K_2HPO_4 , KCl and K_2SO_4 on the action of carboxylase.*

In order to obviate the difficulties introduced by autofermentation, washed zymin was used in presence of a pyruvate and boric acid [see Harden, 1913]. The pyruvate solution was made by neutralising 2.42 g. of pyruvic acid with KHO and making to 25 cc.

Expt. 2. Five flasks were made up each containing 10 cc. of washed zymin suspension (2 g. zymin), and 0.2 cc. toluene with the following additions, the total volume being 15 cc.

No.	Glucose	Pyruvate	Boric acid	$1.2 M K_2HPO_4$	$1.2 M KCl$	Water
1	0.5 g.	0 cc.	0 g.	0.75 cc.	0.5 cc.	13.5 cc.
2	0	6	1.2	0	0	9
3	0	6	1.2	9	0	0
4	0	6	1.2	0	4	5
5	0	6	1.2	0	9	0

After incubation for 10 minutes at 25° , readings of the gas evolution were taken at frequent intervals, with the following results. The absence of fermentation in No. 1 shows that the washing had been efficacious.

No.	Total gas evolved		
	1 hour	2 hours	4 hours
1	0.1 cc.	0.1 cc.	0.1 cc.
2	15.3	18.9	25.6
3	19.0	26.8	33.7
4	13.4	18.6	23.0
5	13.7	19.0	23.2

By comparing the results of Nos. 2, 4 and 5, it is seen that the KCl has had practically no effect, though present in higher concentration than in the experiment previously described (Expt. 1). A similar result was obtained on another occasion with K_2SO_4 .

The K_2HPO_4 has had a slight beneficial effect as the total gas produced in No. 3 is greater than that in No. 2. This effect may perhaps be due to a lowering of the $[H^+]$ by the K_2HPO_4 .

B. Effect of K_2SO_4 and of acetaldehyde on the rate of hydrolysis of hexosephosphate by hexosephosphatase.

In presence of excess of sugar the rate of fermentation produced by either yeast juice or zymin is conditioned by the concentration of free phosphate. As soon as any free phosphate present in the juice has been converted into hexosephosphate the rate of renewal of the supply depends mainly on the rate of action of the hexosephosphatase present. In these circumstances the rate of fermentation is a measure of the rate of action of the hexosephosphatase [see Harden, 1914, p. 119]. By adding a salt solution to a fermenting mixture in which these conditions have been attained, it is possible to determine whether the added salt has any effect on the hexosephosphatase rate.

A study of the effect of phosphate on the rate of action of hexosephosphatase cannot be made in this way because it enters into the reaction and the active masses of the reacting substances are thereby changed.

The effect of K_2SO_4 was tried both in the absence and presence of acetaldehyde for the sake of comparison with later experiments.

Expt. 3. Four flasks were made up each containing 2 g. zymin + 2 g. glucose + 0.2 cc. toluene and the following additions were made: .

No.	1 % acetaldehyde	Water	0.4 M K_2SO_4	
1	0 cc.	20.9 cc.	0 cc.	
2	3	17.9	0	
3	0	13.4	7.5	} Added after the steady rate had been attained.
4	3	10.4	7.5	

After a steady rate had been attained, readings were made every 5 minutes, with the following results:

No.	1	Average rate over 125 minutes	0.71 cc. CO_2 per 5 mins.
2	"	"	0.71
3	"	"	0.45
4	"	"	0.59

The added K_2SO_4 has caused a marked reduction in the rate but this is not so great in the presence of acetaldehyde.

C. *Salt effect in presence and absence of added acceptor (acetaldehyde).**Comparison of effects produced by K_2SO_4 and K_2HPO_4 .*

Expt. 4. Six flasks were made up each containing 20 cc. of yeast-juice + 2 g. fructose + 0.2 cc. toluene, and the following additions were made:

No.	1 % acetaldehyde	Water		0.4 M K_2SO_4	0.6 M K_2HPO_4	Water
1	0 cc.	5 cc.		0 cc.	10 cc.	7.5 cc.
2	5	0	} And after incubation at 25° for 25 mins.	0	10	7.5
3	0	5		7.5	10	0
4	5	0		7.5	10	0
5	0	5		0	15	2.5
6	5	0		0	15	2.5

Readings of the gas evolution were taken at intervals of 5 minutes.

As the concentration of phosphate in the fermenting liquid is progressively changing, whereas the concentration of K_2SO_4 presumably remains unchanged, it is useless to make a direct comparison of the rates of fermentation observed in the several experiments at equal intervals of time measured from the moment at which the various additions were made. Since a simple relation exists between the K_2HPO_4 added and the CO_2 evolved in excess of that which would have been produced had no K_2HPO_4 been added, it is possible to calculate from the total gas evolution, measured from the time at which additions were made, the amount of free phosphate remaining at any moment during the course of the experiment. When this calculation has been made at a series of intervals during each experiment, comparison of the various experiments can be made at equal concentrations of free phosphate. In making the calculations it is assumed that 1 cc. of 0.6 M K_2HPO_4 is equivalent to 14.8 cc. of CO_2 under the conditions of the experiment, and that 0.9 cc. CO_2 must be allowed as the normal rate of fermentation in absence of added phosphate. The latter assumption is not strictly accurate for all the experiments, since the normal rate of fermentation, *i.e.* the hexosephosphatase rate, is affected by the addition of phosphate or sulphate as shown in section B above. The errors introduced from this cause are probably not large, and are to some extent unavoidable, as it is very difficult to estimate the hexosephosphatase rate exactly after the addition of large amounts of phosphate. The curves shown in Figs. 1 and 2 have been prepared in this way by calculation from the observed gas evolution in experiment 4 (Nos. 1-6). In the cases (Nos. 5 and 6) in which the effect of 5 cc. of extra phosphate is to be determined, it is necessary to assume the available free phosphate as equivalent to only 10 cc. of the total of 15 cc. which are actually present, and only to utilise the observations made while the extra 5 cc. of phosphate remained intact. Hence the comparison can only be carried as far as the point at which the concentration of phosphate in the experiments with 10 cc. of phosphate alone, or in presence of K_2SO_4 (Nos. 1, 2, 3, 4) is such as to produce the maximum rate. After this point the rate in these experiments falls off for lack of phosphate, whereas in the experiments with the extra 5 cc. of phosphate (Nos. 5 and 6) this does not occur so soon, and the rate maintains a higher level at the expense of some of the extra phosphate.

In order to compare the depressing effects of the added salts the ratio of the rates of fermentation, at points on the curves where the available phosphate concentration is equal, are employed. These ratios may legitimately be calculated at any points of equal phosphate concentration. But it should be noted that the results at the beginning and end of the experiments are not so trustworthy as the others. In the former case, because the rates are changing rapidly, in the latter because with diminishing phosphate and rate of fermentation the relative errors of calculation are greatly increased.

The results of the observations in absence of added acetaldehyde are shown in Fig. 1, and in presence of acetaldehyde in Fig. 2.

When the curves 1 and 3 (K_2SO_4 in absence of acetaldehyde) and 2 and 4 (K_2SO_4 in presence of acetaldehyde) are compared it is found that the ratios are fairly constant over a considerable range of free phosphate concentration, the values being given in the following table.

Depressing effect of K_2SO_4 .

Available phosphate in cc. of CO_2	Without acetaldehyde		With acetaldehyde	
	Ratio	$\frac{\text{Rate from 3}}{\text{Rate from 1}}$	Ratio	$\frac{\text{Rate from 4}}{\text{Rate from 2}}$
135		0.78		0.77
130		0.78		0.78
120		0.77		0.77
110		0.76		0.77
100		0.74		0.76
90		0.71		0.75
Average ...		0.76		0.77
% Depression ...		24		23

Hence the depressing effect of K_2SO_4 is the same in presence or absence of acetaldehyde.

When, however, the ratios are calculated from 1 and 5 (extra phosphate in absence of acetaldehyde) and from 2 and 6 (extra phosphate in presence of acetaldehyde) it is found that they are very different in the two cases.

Depressing effect of phosphate.

Available phosphate in cc. of CO_2	Without acetaldehyde		With acetaldehyde	
	Ratio	$\frac{\text{Rate from 5}}{\text{Rate from 1}}$	Ratio	$\frac{\text{Rate from 6}}{\text{Rate from 2}}$
130		0.58		0.85
120		0.60		0.88
110		0.61		0.89
100		0.60		0.89
90		0.58		—
Average ...		0.59		0.88
% Depression ...		41		12

It is seen from these figures that the depression produced by extra phosphate is greatly diminished by the presence of acetaldehyde and it would hence appear that a large part of the depressing effect of phosphate is exercised on the production (or possibly the reduction) of the acceptor which is necessary for the attainment of a high phosphate rate [see Harden and Henley, 1920, 1921].

Fig. 1.

Fig. 2.

The sulphate on the other hand does not appear to have this specific action, since the effect is not altered by the addition of acceptor. The effect which it does exercise, and which may be regarded as the general salt effect of Meyerhof, appears to be a depression of the rate of one or more of the reactions in an early stage of the fermentation process, since, as we have seen, this salt is without any marked action on the decomposition of pyruvic acid by carboxylase. Whether the residual effect of phosphate, which is exercised in presence of acetaldehyde, is of the same nature or not cannot at present be stated [see Harden, 1914, p. 72]. It is interesting to note that whilst the total effect of phosphate in absence of acetaldehyde is much greater than that of sulphate, the residual effect in presence of acetaldehyde is much less. It is also worthy of note in this connexion that the normal function of phosphate is probably connected with one of these early stages.

Parallel experiments with glucose and yeast juice in which the concentrations of phosphate and sulphate were only half as great as in the experiments with fructose gave results in good agreement with those described above. The percentage depressions produced by K_2SO_4 were: in presence of acetaldehyde, 23 %; in absence of acetaldehyde, 26 %. The percentage depressions produced by K_2HPO_4 were: in presence of acetaldehyde, 17 %; in absence of acetaldehyde, 41 %. Since the ratio $\frac{\text{concentration of } K_2HPO_4}{\text{concentration of } K_2SO_4}$ is the same in both series of experiments and since the percentage depressions are approximately equal, it appears to follow that these salts affect the fermentation of glucose by yeast juice in a similar manner to that of fructose.

The salt effect seems to be correlated with the phosphate rate; and just as the maximum phosphate rate and tolerance of phosphate are greater with fructose than with glucose the tolerance of salt is also greater in the case of fructose.

SUMMARY.

1. As stated by Meyerhof, the chlorides and sulphates of sodium and potassium exert a depressing effect on the maximum rate obtainable and the rate of attainment of this maximum in the fermentation of glucose and fructose by yeast juice or zymine in the presence of phosphate. The effect of the sulphates is greater than that of the chlorides.
2. Salts diminish the rate of action of hexosephosphatase, but are without effect on that of carboxylase.
3. The depressing effects produced by potassium sulphate and by excess of phosphate differ in character, the latter being greatly diminished by addition of acetaldehyde, whereas the former is practically unaffected.

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