# VIII. THE CONDITIONS OF ACTIVATION OF WASHED ZYMIN AND THE SPECIFIC FUNCTION OF CERTAIN CATIONS IN ALCOHOLIC FERMENTATION.

By ARTHUR HARDEN.

*From the Biochemical Department, Lister Institute.*

(*Received March 12th, 1917.*)

It was observed by Oppenheimer [1915] that the addition of a small amount of a pyruvate (1 per mille) or of pyruvic acid (1 %) to a mixture of Lebedev's maceration juice (prepared from dried yeast) and glucose caused an increase in the rate of fermentation of the glucose, and that a similar, but much smaller, acceleration was produced by the addition of acetaldehyde (1 : 200,000). Neuberg [1915, p. 75] somewhat later made a similar observation and extended it to the fermentation of fructose, mannose, cane sugar and maltose and also found [1915, p. 83] that other $\alpha$-ketonic acids produced an analogous effect. He further attempted [Neuberg and Schwenk, 1915] to activate yeast-juice, which had been freed from coenzyme by dialysis, and zymin (acetone-yeast), rendered inactive by washing, by adding to them salts of individual $\alpha$-ketonic acids and a phosphate. In this, however, he was unsuccessful, but by adding a mixture of salts of a large number of $\alpha$-ketonic acids (pyruvic, $\alpha$-ketobutyric, $\alpha$-ketoisovaleric, $\alpha$-ketocapronic, phenylglyoxylic, phenylpyruvic, $p$-hydroxyphenylpyruvic, hydroxypyruvic, oxalacetic and $\alpha$-ketoglutaric) along with dipotassium hydrogen phosphate, he succeeded in obtaining a small amount of fermentation (of the order of 5 cc. of $CO_2$ from excess of sugar by the action of 20 cc. of inactivated maceration juice or 2 g. of inactivated zymin), and concluded that this mixture could in part replace the coenzyme.

On repeating these experiments it was found that zymin, which had been prepared from top-yeast and rendered inactive by thorough washing, was readily activated by potassium pyruvate in presence of a suitable concentration of a phosphate. The experiments were then extended to acetaldehyde, since this substance is the immediate product of the decomposition

of salts of pyruvic acid by the carboxylase of the zymin, which, as has been previously shown [Harden, 1913; Neuberg and Rosenthal, 1913] is not inactivated by washing. The first experiments were carried out in the presence of sodium phosphate and were unsuccessful, but it was subsequently found that, when the sodium phosphate was replaced by the potassium or ammonium salt, acetaldehyde activated the zymin in the same manner as the pyruvate.

It thus appears that potassium and ammonium ions play some special part in the process of fermentation in which they cannot be replaced by sodium ions. The presence of sodium ions is not in itself inhibitory, since activation occurs in their presence, provided only that potassium or ammonium ions are also present. This observation complements in an interesting manner the discovery by Adolf Mayer [1874] that potassium phosphate cannot be replaced by the sodium salt in a synthetic culture medium for yeast.

The fact that washed zymin can be activated by acetaldehyde in presence of a phosphate is of considerable interest from several points of view. In the first place it is consistent with, and may even be regarded as strong evidence in favour of, the theory now held by many investigators [for literature see Harden, 1914] that acetaldehyde is an intermediate product in alcoholic fermentation and is reduced in that process to alcohol by hydrogen liberated at a previous stage of the decomposition. According to this view the acetaldehyde would fulfil the function of an activator by serving as an acceptor for the hydrogen and would thus enable the reaction to start. It is possible however that other reducible substances would act in the same way and this would to some extent lessen the weight of the evidence in support of the acetaldehyde theory although it would be in agreement with the view that a hydrogen acceptor is a factor necessary for fermentation. This question will form the subject of further experiments as soon as circumstances permit.

These considerations lead directly to the question whether acetaldehyde can be regarded as constituting the coenzyme of yeast-juice. There is much in favour of this idea, but in view of the possibility that some other reducible substance may play the same part as acetaldehyde it is impossible to come to a definite conclusion without further investigation respecting the actual presence of acetaldehyde in yeast-juice and the activation of yeast-juice freed from coenzyme by dialysis, ultrafiltration and continued fermentation. As regards washed zymin, acetaldehyde certainly acts in all respects as a coenzyme, provided that phosphate and potassium or ammonium ions are present in suitable concentrations.

A second consideration is that the possibility of reactivating washed zymin by means of acetaldehyde and a phosphate affords a means of examining the effect of various cations and anions on the initiation and course of the fermentation and this opens a field of enquiry which may be expected to yield interesting results.

The stimulating effect of pyruvate and acetaldehyde on fermentation by yeast-juice observed by Oppenheimer and by Neuberg, also becomes of great interest in the light of these results and will form the subject of a further communication.

## Experimental.

The zymin used in these experiments was prepared from a top-fermentation yeast employed in an English brewery by the usual method of treatment with acetone. It was washed five times with 6 parts of water in the manner previously described [Harden and Young, 1911] each washing lasting $\frac{3}{4}$ hour, and was then made up to a given volume with water. The same sample of zymin was used throughout the experiments quoted so that the results of experiments done with different batches are roughly comparable. All the fermentations were carried out in presence of toluene at 25°, the solutions being previously saturated with $CO_2$ at this temperature and incubated for 10—15 minutes before readings were made. The carbon dioxide evolved was collected and measured in the apparatus previously described [Harden, Thompson and Young, 1910] and the figures given are cc. of gas at atmospheric pressure and temperature.

### *Experiments with zymin.*

I. The activation of washed zymin by potassium pyruvate and potassium phosphate and the effect of the replacement of these salts by the corresponding sodium salts.

14 g. of zymin were washed and made up to 80 cc.

In each experiment 10 cc. of this suspension (1·75 g. zymin) were used along with 1 g. of glucose and water to a total volume of 20·6 cc. To five quantities were added:

*Exp.* 1. 2 cc. 0·5 M $K_2HPO_4$.
„ 2. 2 cc. 0·5 M $K_2HPO_4$ + 3 cc. 1 % pyruvic acid (as K salt).
„ 3. 3·3 cc. 0·3 M $Na_2HPO_4$ + 3 cc. 1 % pyruvic acid (as Na salt).
„ 4. 1·6 cc. 0·3 M $Na_2HPO_4$ + 3 cc. 1 % pyruvic acid (as Na salt).
„ 5. 10 cc. boiled washings.

| Time | | Evolution of $CO_2$ in cc. | | | | |
|---|---|---|---|---|---|---|
| h. | m. | 1 | 2 | 3 | 4 | 5 |
| 1 | 5 | 0·5 | 12·6 | 1·9 | 2·1 | 11·1 |
| 2 | 5 | 0·5 | 20·4 | 2·1 | 2·8 | 17·6 |
| 20 | 20 | 2·9 | 55·8 | 2·5 | 6·6 | 69 |

In presence of the sodium salts (Nos. 3 and 4) little more $CO_2$ was evolved than in the control (No. 1) in the absence of coenzyme, whereas in the presence of the potassium salts (No. 2) the fermentation is almost as vigorous as in the presence of boiled washings (No. 5).

II. The activation of zymin by acetaldehyde and potassium phosphate or ammonium phosphate and the effect of the replacement of these salts by sodium phosphate.

A. *Exp.* 6. This was carried out along with the foregoing series. To the mixture of zymin glucose and toluene were added 1 cc. acetaldehyde solution (0·7 %) + 2 cc. 0·5 M $K_2HPO_4$.

| Time | | Evolution of $CO_2$ in cc. | |
|---|---|---|---|
| | | Control | |
| h. | m. | No. 1 (above) | 6 |
| 1 | 5 | 0·5 | 13·3 |
| 2 | 5 | 0·5 | 19·8 |
| 20 | 20 | 2·9 | 52·4 |

B. 10 cc. of washed zymin suspension and water to 20·6 cc. To six quantities were added:

*Exp.* 7. 10 cc. boiled washings + 1 g. glucose.
„ 8. 3 cc. 0·5 M $K_2HPO_4$ + 1 g. glucose.
„ 9. 3 cc. 0·5 M $K_2HPO_4$.
„ 10. 3 cc. 0·5 M $K_2HPO_4$ + 1 g. glucose + 1 cc. acetaldehyde.
„ 11. 2 cc. 0·5 M $K_2HPO_4$ + 1 g. glucose + 1 cc. acetaldehyde.
„ 12. 1·6 cc. 0·3 M $Na_2HPO_4$ + 1 g. glucose + 1 cc. acetaldehyde.

| Time | | Evolution of $CO_2$ in cc. | | | | | |
|---|---|---|---|---|---|---|---|
| h. | m. | 7 | 8 | 9 | 10 | 11 | 12 |
| | 55 | 14·1 | 0·2 | 0·5 | 14·2 | 11·7 | 0·9 |
| 1 | 55 | 25·1 | 0·6 | 1·1 | 29·6 | 19·5 | 1·6 |
| 3 | 0 | 33·2 | 0·7 | 1·3 | 37·7 | 23·6 | 2·3 |
| 18 | 10 | 89 | 0·8 | 1·3 | 80·1 | 51·6 | 2·3 |

The amount of sodium phosphate added was only half the equivalent of the K salt because a previous experiment had shown that even in the presence of a small concentration of K ions, no fermentation occurred in presence of the full equivalent.

C. 10 g. zymin to 60 cc. 10 cc. of zymin suspension + 1 g. glucose were taken. Total vol. = 20·6 cc. To three lots were added:

*Exp.* 13. 10 cc. boiled washings.
„ 14. 2 cc. 0·5 M $K_2HPO_4$.
„ 15. 2 cc. 0·5 M $(NH_4)_2HPO_4$ + 1 cc. acetaldehyde.

| Time | | Evolution of $CO_2$ in cc. | | |
|---|---|---|---|---|
| h. | m. | 13 | 14 | 15 |
| 1 | 10 | 8·1 | 1·4 | 6·5 |
| 3 | 25 | 16·1 | 1·5 | 15·6 |
| 20 | 35 | 39·8 | 1·5 | 34·6 |

Here again acetaldehyde and potassium phosphate (Nos. 10, 11) or ammonium phosphate (No. 15) produce a good fermentation whereas acetaldehyde and sodium phosphate (No. 12) are without action.

III. Negative effect of potassium ions and acetaldehyde in absence of mineral phosphate.

Six quantities of 10 cc. of zymin suspension + 1 g. glucose + water to 20·6 cc. were taken and to these were added:

*Exp.* 16. 10 cc. boiled washings.
„ 17. 3 cc. 0·5 M $K_2HPO_4$ + 1 cc. acetaldehyde.
„ 18. 3 cc. M $KHCO_3$ + 1 cc. acetaldehyde.
„ 19. 3 cc. M $NaHCO_3$ + 1 cc. acetaldehyde.
„ 20. 1·6 cc. 0·3 M $Na_2HPO_4$ + 1 cc. acetaldehyde.
„ 21. 0·8 cc. 0·3 M $Na_2HPO_4$ + 1 cc. acetaldehyde.

| Time | | Evolution of $CO_2$ in cc. | | | | | |
|---|---|---|---|---|---|---|---|
| h. | m. | 16 | 17 | 18 | 19 | 20 | 21 |
| 1 | 10 | 11·7 | 14·4 | 2·1 | 1·3 | 0·8 | 1·5 |
| 2 | 10 | 18 | 28·3 | 2·3 | 1·7 | 1·4 | 1·9 |
| 18 | 10 | 69 | 74·9 | 3·3 | 3·8 | 1·5 | 2 |

This shows that K ions and acetaldehyde in the absence of phosphate (No. 18) are incapable of producing fermentation and further that the same is true of Na ions (No. 19) and that the reduction of the concentration of sodium phosphate does not render it efficacious as an activator (No. 21).

*Experiments with dried yeast.*

Since dried yeast can be inactivated by washing [Euler and Bäckström, 1912] it can readily be used for experiments on activation. It is however not so satisfactory as zymin since, probably owing to the presence of a certain proportion of living cells, some samples yield irregular results. The yeast was dried in the air at 37° and then ground in a coffee mill. It was washed in a similar manner to the zymin.

IV. Effect of the pyruvates and phosphates of potassium and sodium.

20 g. yeast, washed four times and made to 100. 15 cc. yeast suspension + 1 g. glucose + 1 cc. toluene; water to 30·6 cc.; five quantities were taken.

*Exp.* 22. 5 cc. 1 % pyruvic (as K salt).
„ 23. 5 cc. 1 % „ „ + 1·5 cc. 0·5 M $K_2HPO_4$.
„ 24. 5 cc. 1 % „ „ + 2·5 cc. 0·3 M $Na_2HPO_4$.
„ 25. 5 cc. 1 % „ (as Na salt) + 1·5 cc. 0·5 M $K_2HPO_4$.
„ 26. 5 cc. 1 % „ „ + 2·5 cc. 0·3 M $Na_2HPO_4$.

| Time | | Evolution of $CO_2$ in cc. | | | | |
|---|---|---|---|---|---|---|
| h. | m. | 22 | 23 | 24 | 25 | 26 |
| | 20 | 2·6 | 9·7 | 15·6 | 13·1 | 1·4 |
| 1 | 10 | 5·6 | 16·2 | 22·8 | 20·2 | 2·7 |
| 2 | 5 | 6·7 | 24·3 | — | — | 3·9 |
| 3 | 5 | 7·1 | 34·1 | — | — | 5·2 |
| 19 | 20 | 9·9 | 71·1 | — | — | 8·6 |

Here the substitution of sodium for potassium salts (No. 26), in equivalent amounts has resulted in the complete absence of any evolution of $CO_2$ beyond

that in the control in the absence of phosphate (No. 22). That the presence of sodium ions is not seriously harmful is shown by a comparison of Nos. 24 and 25 with No. 23.

V. Effect of substituting sodium phosphate for potassium phosphate in presence of acetaldehyde.

The dried yeast was treated as in IV. 15 cc. yeast suspension + 1 g. glucose + 1 cc. toluene; water to 30·6 cc.

*Exp.* 27. 1·5 cc. 0·5 M $K_2HPO_4$.
„ 28. 1·5 cc. 0·5 M $K_2HPO_4$ + 1·4 cc. 1 % acetaldehyde.
„ 29. 2·5 cc. 0·3 M $Na_2HPO_4$ + 1·4 cc. 1 % acetaldehyde.

| Time | | Evolution of $CO_2$ in cc. | | |
|---|---|---|---|---|
| h. | m. | 27 | 28 | 29 |
| 1 | | 1·5 | 25·5 | 2·9 |
| 3 | | 1·5 | 41·8 | 3·2 |
| 5 | 30 | 1·7 | 58·3 | 3·8 |
| 21 | 45 | 4·4 | 86·3 | 6·1 |

The contrast here between the action of equivalent quantities of sodium and potassium phosphate is very marked.

VI. Effect of the addition of various chlorides in presence of sodium phosphate and acetaldehyde.

Dried yeast, treated as above. 15 cc. of yeast suspension + 1 g. glucose + 1 cc. toluene + 0·5 cc. 0·3 M $Na_2HPO_4$ + 5 cc. 0·1 % acetaldehyde; water to 30·6 cc. Four quantities.

*Exp.* 30. 1·5 cc. M KCl.
„ 31. 1·5 cc. M $NH_4Cl$.
„ 32. 1·5 cc. M NaCl.
„ 33. Control.

| Time | | Evolution of $CO_2$ in cc. | | | |
|---|---|---|---|---|---|
| h. | m. | 30 | 31 | 32 | 33 |
| 1 | 15 | 7·7 | 7·8 | 0·3 | 1·9 |
| 2 | 15 | 10·6 | 11·1 | 2·5 | 2·8 |
| 18 | | 11·6 | 13·4 | 3·3 | 3·3 |

This shows that whereas the addition of KCl (No. 30) or $NH_4Cl$ (No. 31) to a mixture containing inactivated dried yeast, acetaldehyde and sodium phosphate causes a definite, though small, degree of fermentation, that of NaCl (No. 32) has no effect, the amount of $CO_2$ evolved being the same as in the control (No. 33).

SUMMARY.

1. In the presence of potassium phosphate, zymin and dried yeast, which have been inactivated by washing, can be activated by the addition of a pyruvate or acetaldehyde.

2. A specific difference in relation to alcoholic fermentation exists between the ions of sodium on the one hand and of potassium and ammonium on the other.

REFERENCES.

Euler and Bäckström (1912). *Zeitsch. physiol. Chem.*, **77**, 394.
Harden (1913). *Biochem. J.*, **7**, 214.
—— (1914). *Alcoholic Fermentation* (London, Longmans & Co.).
—— and Young (1911). *Proc. Roy. Soc.* B, **83**, 451.
—— Thompson and Young (1910). *Biochem. J.*, **5**, 230.
Mayer (1874). *Lehrbuch der Gährungs-Chemie* (Heidelberg).
Neuberg (1915). *Biochem. Zeitsch.*, **71**, 1.
—— and Rosenthal (1913). *Biochem. Zeitsch.*, **51**, 128.
—— and Schwenk (1915). *Biochem. Zeitsch.*, **71**, 135.
Oppenheimer (1915). *Zeitsch. physiol. Chem.*, **93**, 235.