Biochemistry. — *The conditions of activation of washed zymin, III.* By A. A. STHEEMAN. (Communicated by Prof. A. J. KLUYVER.)

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I.

In previous communications [1]), dealing with the conditions of reactivation of washed zymin it has been described how by washing the zymin with water several stages of inactivation of the zymase system may be obtained. Further it was stated that these stages of inactivation can be distinguished from each other sharply by investigating which of the known reactivating principles of alcoholic fermentation are able to give rise to a reactivating effect.

In the second communication it has been demonstrated that the occurrence in the fermenting medium of a suitable hydrogen-accepting substance along with hexosebiphosphate and VON EULER's principle, always sufficed to substitute the boiled washing as reactivating principle [2]). It was also made clear that in this mixture of substances the reactivating effect of hexosebiphosphate may be replaced by that of a large amount of VON EULER's principle, but that the reversal is not at all the case. Therefore it is also possible to substitute the boiled washings by large amounts of E.P. along with a suitable hydrogen acceptor.

In a recent communication E. AUHAGEN [3]) describes the results of experiments with dried yeast (Trockenhefe), which was washed (with 0.2 mol. phosphatebuffer ph. 7, 7—7, 8) to such an extent, that the addition of anorganic phosphate along with hexosebiphosphate, a magnesium salt and VON EULER's principle did no more suffice to reactivate the inactivated zymase system. When however besides this mixture of substances "Kochsaft" was also added, reactivation of the washed zymase-system could be observed.

From these observations AUHAGEN concludes that the reactivating effect of this addition of "Kochsaft" has to be explained by the occurrence in the "Kochsaft" of another unknown reactivating principle which is designated by him as "Co-zymase II".

There would be no objection to accept the existence of another until now unknown activating principle of alcoholic fermentation, if AUHAGEN

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[1]) Proc. Kon. Akad. v. Wetensch. Amsterdam, **32,** 426 (1929); **33,** 889 (1930).

[2]) In all these experiments a sufficient amount of magnesium always remains in the washed zymin.

[3]) E. AUHAGEN, Naturwissenschaften, **19,** 916 (1931).

had only shown, that the addition of one or more of the already known activators did not suffice to reactivate his zymase-system.

More especially one would have expected, that AUHAGEN should have given attention to the fact, already demonstrated many years ago by HARDEN and by NEUBERG and on which much stress has been laid in my previous communications, that incidentally inactivated zymase systems could be reactivated by the addition of a hydrogen acceptor like acetaldehyde or pyruvates (along with potassium ions). However AUHAGEN does not seem to have done any experiments in this direction.

This is the more remarkable, since in my second communication on the subject mention was made of the socalled third stage of inactivation of the zymin, for which it was proved, that the simple addition of hexosebiphosphate did not reactivate the system, whereas reactivation did occur, when an extract of zymin guaranteed to be free from EULER's principle was added as well [1]). For the activator present in this extract of zymin I was able to show, that it could be substituted by hydrogen acceptors like acetaldehyde or methylene blue. In this way the indispensability of a suitable hydrogen accepting substance in a medium in which alcoholic fermentation occurs — as already assumed by HARDEN (1917) — was clearly demonstrated.

Under these circumstances it was highly probable that in the experiments reported by AUHAGEN the inactivation of his zymasesystem had to be ascribed to the absence of a suitable hydrogen acceptor in the medium.

Now indeed it proved to be possible to reactivate zymin, washed with phosphate buffer (pH 7.70) till it would no longer ferment glucose, even after addition of potassium phosphate together with hexosebiphosphate and VON EULER's principle and magnesium chloride by simply adding acetaldehyde or methylene blue or the boiled washings or "Kochsaft", or the protein solution made from washed zymin as mentioned above.

II.

Experimental.

To inactivate the zymin, 6 grams of zymin, obtained from baker yeast, ("koningsgist") were washed one time with 50 cc. water for 10 minutes (to remove the acid phosphates) and twice with 50 cc. of AUHAGEN's phosphate buffer (0,2 mol. pH 7.70). Although with zymin the washing with water is as effective, in these experiments AUHAGEN's method of washing was adhered to.

For the fermentation experiments the quantitative apparatus according to VAN ITERSON—KLUYVER was used again.

The phosphate solutions used in the experiments was a mixture of 5/12

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[1]) This guarantee was obtained in this way that the extract was prepared from washed zymin. which was proved to be in its *fourth* stage of inactivation.

mol. K_2HPO_4 and 1/12 mol. KH_2PO_4 made up to 1 litre with water. The protein solution was made from the zymin washed till it contained no more VON EULER's principle (fourth stage of inactivation) according to the method described earlier. The "Kochsaft" was obtained by boiling 10 grams of baker yeast with 5 cc. water for two minutes; and removing the residue by centrifuge. The two last mentioned solutions were saturated with carbon dioxyde at the temperature of the experiment (26° C.).

All mixtures used in the experiments were saturated with toluene.

A.

The first set of experiments shows conclusively that a zymin washed until glucose is no more fermented, even when hexosebiphosphate and VON EULER's principle together with magnesiumchloride and potassiumphosphate also occur in the fermenting medium, may be reactivated by the simple addition of acetaldehyde.

6 grams of zymin were once washed with water for 10 minutes and twice with phosphate buffer (pH 7.70) for 15 minutes. After having been centrifuged the final zymin residue was suspended in water and made up to 24 cc. In each experiment 1,5 cc. of this suspension (0.375 gram of zymin) were added to the solutions mentioned below and the mixture made up with water to 5,1 cc. One cc. of each of these mixtures was introduced into the fermentation apparatus.

One cc. of the solution of VON EULER's principle (E.P.) contained 2 mgrs. of this substance.

The hexosebiphosphate solution used contained 5 mgrs. of organic P. per cc.

Besides 0.25 gr. of glucose, 2 mgrs. of $MgCl_2 . 6 H_2O$ and 1 cc. 0.5 M potassium phosphate solution, the mixtures contained in:

Exp. 1. 0.5 cc. Na-hexosebiphosphate + 0.5 cc. acetaldehyde (1 %).
Exp. 2. 0.5 cc. Na-hexosebiphosphate + 0.5 cc. acetaldehyde + 0.5 cc. E.P.
Exp. 3. 0.5 cc. acetaldehyde + 0.5 cc. E.P.
Exp. 4. 0.5 cc. Na-hexosebiphosphate + 0.5 cc. E.P.
Exp. 5. 0.5 cc. E.P.

TABLE A.

Time in hours	Evolution of CO_2 in cc. at atmospheric pressure. Number of experiment.				
	1	2	3	4	5
1	—	0.4	—	—	—
4		3.6	—	—	—
18	—	9.7	—	—	—
20	—	10.0	—	—	—
48	—	11.0	—	—	—

Experiments 2 and 4 prove clearly that a zymase-system washed until the stage of inactivation is reached, which was also attained in AUHAGEN's experiments, may be reactivated by acetaldehyde.

B.

The following set of experiments demonstrates that in the stage of inactivation circumscribed above reactivation does not only occur by the addition of "Kochsaft", but as well by that of the boiled washings, of acetaldehyde, of methylenblue or of the proteinsolution mentioned above.

The arrangement of this set of experiments was performed in quite the same manner as described under A.

Besides 0.25 gr. of glucose, 2 mgrs. of $MgCl_2 . 6 H_2O$ and 0.5 cc. of the Na-hexosebiphosphate solution, the solution contained in:

Exp. 1. 1 cc. K phosphate 0.5 M + 0.5 cc. acetaldehyde (1 %).
Exp. 2. 1 cc. K phosphate 0.5 M + 0.5 cc. acetaldehyde + 0.5 cc. E.P.
Exp. 3. 1 cc. K phosphate 0.5 M + 0.5 cc. E.P.
Exp. 4. 1 cc. K phosphate 0.5 M + 1 cc. methylene blue (sol. sat.) + 0.5 cc. E.P.
Exp. 5. 0.25 cc. K phosphate 0.5 M + 1 cc. proteinsol. + 0.5 cc. E.P.
Exp. 6. 0.25 cc. K phosphate 0.5 M + 2 cc. boiled washings (conc. 10 times) + 0.5 cc. E.P.
Exp. 7. 0.25 cc. K phosphate 0.5 M + 2 cc. „Kochsaft" + 0.5 cc. E.P.
Exp. 8. 1 cc. Na phosphate 0.5 M + 0.5 cc. acetaldehyde (1 %) + 0.5 cc. E.P.
Exp. 9. 0.5 cc. K phosphate 0.5 M + 3 cc. „Kochsaft". [1])

TABLE B.

	Evolution of CO_2 in cc. at atmospheric pressure								
	Number of experiment								
	1	2	3	4	5	6	7	8	9
After 20 hours.	—	9.7	—	6.4	8.5	11.6	11.2	—	8.9

SUMMARY.

These experiments show once more that VON EULER's principle is unable to reactivate an inactivated zymase system unless at the same time a suitable hydrogen accepting substance occurs in the medium. It was proved that either methylene blue, or acetaldehyde, or "Kochsaft", or the boiled washings, or a protein solution free from VON EULER's principle, can act as such.

Since in these experiments the same method of inactivation was used as described by AUHAGEN, it may be concluded, that the supposedly new activator, named by this author "Co-Zymase II", is nothing but a hydrogen acceptor naturally present in yeast, the occurrence of which was already proved long ago by several investigators.

[1]) In exp. 9, besides glucose only anorganic phosphate was added.