Biochemistry. — *The so-called co-enzyme of alcoholic fermentation.* By A. J. KLUYVER and A. P. STRUYK.

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I. In 1905 it was shown by HARDEN and YOUNG [1]) that it is possible to divide zymase containing liquids by ultrafiltration into two parts each of which misses the power to ferment glucose, whereas by reuniting both this power is again restored. In the same year it was proved by BUCHNER and ANTONI [2]) that a similar separation could be effected also by dialysis of yeast-juice. It soon appeared from further experiments that contrary to what counts for the remaining mass in the ultrafilter or in the dialysator, the filtrate and the dialysate could be heated to 100° C. without causing the least change in the observed effect. The possibility that the inactivity of the residue should have to be reduced exclusively to the removal of the soluble alkaliphosphates proved indispensable to fermentation, was excluded by further experiments of HARDEN. Hence this investigator concluded that for the fermentation of the hexoses not only the presence of the zymase proper — and of alkaliphosphates — was required, but also the presence of an ultrafiltrating, dialysable, thermostable substance, which he gave the name of "co-enzyme" [3]).

The interest for this co-enzyme has risen considerably in the last years, since namely O. MEYERHOF proved in 1917 that the occurrence of this principle was by no means confined to the yeast-cell; on the contrary the co-enzyme was also found in various animal tissues. Moreover MEYERHOF made also acceptable that the co-enzyme was not only acting in fermentation but also in respiration processes.

Notwithstanding the fact that the co-enzyme has been the subject of many investigations in recent years, one has not yet succeeded in elucidating the nature of this so mysterious substance. So there are to be found assembled in recent summaries of our knowledge in this domain much varying and to a certain extent hardly combinable opinions about the nature of the co-enzyme [4]).

Characteristic however for the mode of view of all investigators is that they have not the least objections to accept the necessity of the co-operation of an unknown factor for the realisation of the alcoholic fermentation of the

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[1]) Compare: A. HARDEN, Alcoholic Fermentation, 3rd Ed., 1923, p. 61.

[2]) E BUCHNER und W. ANTONI, Zeitschr. f. physiol. Chemie, Bd. 46, p. 136, (1905).

[3]) In literature the names co-enzyme, coferment, cozymase and codiastase are mixed up.

[4]) For the sake of brevity this assertion will not be further documented here; compare the wellknown summaries of FUCHS, NEUBERG, HARDEN, VON EULER and SCHOEN.

hexoses. This cannot wonder when one realizes that biochemists are still generally used to reduce the chemical conversions, caused by the living cell, to a chain of reactions each of which passes under the influence of its own specific catalysator. Under these circumstances there is little objection to the acceptance of one single mysterious agent — the co-enzyme — more.

However this becomes different when one puts himself on the standpoint taken in by DONKER and one of us (KLUYVER) [1]) according to which the biochemical conversions — apart from some hydrolyses and esterifications, which are for the greater part of a preparatory nature — are to be reduced to a chain of reactions which may all be considered as a catalytic transference of hydrogen and which pass in each cell under the influence of one and the same agent.

Under these circumstances biochemistry is reduced for the greater part to a series of heterogeneous catalytic processes the model of which is to be found in organic chemistry in the coupled dehydrogenations and hydrogenations under the influence of finely divided nickel.

The simplification of insight brought along with this view renders the acceptance of the necessity of the collaboration of a co-enzyme for the fermentation process much more difficult and it seemed therefore desirable to us to consider in how far the necessity of an agent, besides the zymase proper, was to be derived from our special scheme for alcoholic fermentation based on the general theory referred to above.

II. For the scheme mentioned, we think we may refer to the last cited paper of KLUYVER and DONKER, and also to a former paper of ours [2]). In view of the fact that an esterification namely the formation of a phosphoric ester occurs as the preliminary reaction, it seemed in the first place advisable to consider whether the co-enzyme was the agent, the phosphatese (resp. phosphatase) catalysing this reaction, resp. the hydrolysis of the triose phosphoric ester. Meanwhile this idea has been rejected at once on account of the fact that it is quite unacceptable to attribute the properties of easy dialysability and especially large thermostability to an enzyme such as the phosphatase [3]).

However, a further consideration of the fermentation scheme leads to the conclusion that still another factor than only the zymase in its restricted sense and the phosphatase is required for the setting in of fermentation. As known the dehydrogenation of the methylglyoxal hydrate occurs in a normal way with acetaldehyde as acceptor whereby meanwhile the acetaldehyde is formed by the reaction ensuing therefrom (the decarboxylation of the pyruvic acid). So this situation implies the presence of a substance necessary for the commencement of fermentation, which

[1]) A. J. KLUYVER and H. J. L. DONKER, These Proceedings **28**, 297. (1925); **28**, 605, (1925) and Chemie der Zelle und Gewebe, Bd. **13**, p. 134, (1926).

[2]) These Proceedings **29**, p. 322, (1926).

[3]) As stated before it was already early shown by HARDEN that the effect of the co-enzyme does not rest exclusively upon the removal of the alkaliphosphates.

substance takes over the function of the at first missing acetaldehyde. This consideration made it possible that the co-enzyme should only act as a hydrogen acceptor and looking at this hypothesis we decided to approach experimentally the co-enzyme problem.

III. Before giving a short review of the result of our own experiments we must point out that some experiments were already made by NEUBERG [1]) as well as by HARDEN [2]) the results of which could be interpreted at least partly in the described way. We will not discuss these results any further here, but will only remark that on the one side HARDEN ends his summary given in 1923 [3]) with the words: "It seems highly probable that the co-enzyme fulfils a similar function and it will be found like aldehyde, to be a substance capable of reduction in presence of the enzymes of yeast" whilst on the other side MEYERHOF, on the ground of his own experiments firmly denies the possibility of the substitution of the co-enzyme by aldehydes.

When making our own experiments we decided first of all to use the technic originally followed by HARDEN, with the difference, however, that maceration extract was used instead of BUCHNER's yeast-juice. Small quantities thereof were divided into two parts in an ultrafiltration apparatus according to BECHHOLD, after which the material remaining on the filter was washed with a little water. Further it was investigated how far the fermenting power of the inactive residue, which caused strong fermentation after addition of the ultrafiltrate, was also restored by adding small quantities of acetaldehyde accompanied by suitable quantities of alkaliphosphates.

The results of a series of investigations in this direction have been varying; sometimes it has been possible indeed to lead an otherwise wholly negative remaining mixture to strong fermentation by the addition of acetaldehyde and sometimes this addition remained without any effect, although, even then activation by ultrafiltrate occurred. The nature of the yeast used for the preparation of the maceration extract as well as the duration of the filtration and the intensity of washing of the residue seemed to influence the result.

In the meantime it could be said with certainty that the function of the co-enzyme was not only that of an introductory hydrogen acceptor as otherwise substitution by acetaldehyde should have been possible in all cases.

IV. With this series of experiments we had purposely not yet taken into account a possible complication to which MEYERHOF [4]) drew the attention by his important investigations about the co-enzyme. Starting

[1]) C. NEUBERG und E. SCHWENK, Biochem. Zeitschr., Bd. **71**, p. 135, (1915).

[2]) A. HARDEN, Biochemical Journal, Vol. **11**, p. 64, (1917).

[3]) A. HARDEN, Alcoholic Fermentation, 3rd Ed. London, 1923, p. 72.

[4]) O. MEYERHOF, Zeitschr. f. physiol. Chemie, Bd. **101**, p. 165, (1918), Ibid. **102**, p. 1, (1918), Ibid, **102**, p. 185, (1918).

from the phenomenon of induction shown by certain maceration extracts, discovered by LEBEDEFF [1]), MEYERHOF points to the part which this phenomenon can play with experiments about the co-enzyme made with normal maceration extract.

We can summarise MEYERHOF's theories on this point by saying that he shows the necessity of making difference between the apparent inactivity of a zymase preparation — which is a consequence of the induction — and a real inactivity caused by the absence of the co-enzyme.

It is possible to distinguish between these two phenomena because of MEYERHOF's important discovery that the induction contrary to inactivity caused by lack of co-enzyme can be eliminated by addition of a small amount of hexosephosphate. So the result is that no zymase preparation, which does not remain inactive after addition of hexosephosphate, may be considered free from co-enzyme and MEYERHOF points out that to obtain this effect continued washing on an ultrafilter is indispensable.

Entirely in accordance with this it appeared to us from a new series of experiments — whereby we used like MEYERHOF the more simple ultrafiltrating apparatus of ZSIGMONDY — that the zymase preparations inactivated by too little washing, were only apparently inactive, in so far, that the addition of hexosephosphate [2]) was sufficient to bring about a strong fermenting power. Further we found also that continued washing to a 200 fold dilution of the materials accompanying the zymase — led indeed to a zymase preparation which could be activated by the usual co-enzyme solutions, such as boiled maceration extract, but not by hexosephosphate.

Thus it had now become necessary to repeat the experiments concerning the possibility of activation by acetaldehyde of a zymase preparation really freed from co-enzyme. These experiments, however, gave without exception a negative result, so that the conclusion had to be drawn that except the hexosephosphate, still another substance — the co-enzyme proper — is required to make the zymase capable of producing sugar fermentation.

V. Herewith the way to a closer understanding of the function of the co-enzyme seemed to be blocked up for the present. After ample deliberation it became however alluring to connect the problem with phenomena in a somewhat further off area which — although full attention was given to it by the first zymase investigators — had been left later on with a single exception, without any consideration. We allude to the fact that it was already proved by HAEHN in 1898 that zymase preparations always possess a strong proteolytic power and in connection herewith are also subject to a strong autolysis by which the zymase itself is also destroyed. Hereto were added the later observations of BUCHNER and his

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1) A. LEBEDEFF, Annales de l'Institut Pasteur, T. **26**, p. 8, (1912).

2) When in this paper is spoken of hexosephosphate always the with natriumoxalate conversed "Candiolin BAYER" or the corresponding "hexosediphosphorsaures Natrium", from the same firm is meant. We want to express here also our best thanks to the named firm, which was so kind to put the mentioned preparations at our disposal.

collaborators, including a.o. HAEHN, who proved that boiled maceration extract possesses an undeniable antiproteolytic action [1]). The possibility lying at hand that the co-enzyme function of boiled zymase preparations should have to be reduced to the supposed presence of a so-called "anti-protease", in other words to the zymase protecting action of these juices was meanwhile rejected by the named investigators, a.o. after observation that by continued boiling the co-enzyme function can be destroyed, whilst the "antiprotease" function remains then for the greater part preserved. The same conclusion was drawn by HAEHN and SCHIFFERDECKER [2]) who published in 1923 a detailed investigation on this point.

Now we specially noted the fact that whilst on the one side MEYERHOF in his former cited investigations does not pay any attention to the studies of BUCHNER and collaborators about the proteolytic function of the zymase preparations, on the other side HAEHN and SCHIFFERDECKER seem to have remained ignorant of the 6 years earlier by MEYERHOF published results and thus had not taken into consideration at all the according to MEYERHOF secondary influence of a possible want of hexosephosphate. This situation seemed to us to imply the possibility that the principle considered by BUCHNER and SCHIFFERDECKER as "co-enzyme" might not be anything else than hexosephosphate which is destroyed in small concentrations by continued boiling whereas the by MEYERHOF as "co-enzyme" (Coferment) indicated principle should have to be identified with the antiproteolytic factor present in the boiled juices.

Numerous observations in the series of experiments of the different investigators proved to be in perfect accordance with this hypothesis, but for shortness' sake we will not go further into this here.

An experimental test of the hypothesis given above led to a satisfactory result. It appeared to be possible indeed according to HAEHN and SCHIFFERDECKER to free a solution of co-enzyme by seven hours boiling entirely of its original activating power and to regenerate this by simple addition of some hexosephosphate. On the other hand a zymase preparation remaining inactive with hexosephosphate, could be activated by simple addition of the meant "Schutzsaft", so that there stood nothing in the way of identifying MEYERHOF's co-enzyme with BUCHNER's "antiprotease".

In the meantime as long as the nature of the antiproteolytically acting substances was not further ascertained, the above mentioned experiments did not exclude all doubts regarding the correctness of this last contention. It might be possible that besides these substances still another substance, the co-enzyme proper, had also endured without difficulty the long process of boiling, although this seemed improbable since it follows also from

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[1]) E. BUCHNER und H. HAEHN, Biochem. Zeitschr., Bd. **19**, p. 198, (1909); Ibid. Bd. **26**, p. 171, (1910).

[2]) H. HAEHN und H. SCHIFFERDECKER, Biochem. Zeitschr., Bd. **138**, p. 209, (1923).

THOLIN's [1]) experiments that the co-enzyme is practically entirely destroyed by heating to 100° C. during seven hours.

Meanwhile we did our best to consolidate our view concerning the nature of the co-enzyme of MEYERHOF. Therefore it was necessary to form a more precise idea regarding the nature of the antiproteolytic action. It seemed probable to ascribe this action to proteins which might act as substrates of the endotryptase and in this way function to a certain extent as "lightning conductor" for the zymase. However great difficulties were in the way of an experimental test of this hypothesis, as protein extracts from much varying vegetable and animal organs were just tested by MEYERHOF on their activating function with positive result, where from this investigator concluded to the very general occurrence of the "co-enzyme".

Thus it was of importance for us to find a protein containing substance which was beyond any suspection of containing MEYERHOF's co-enzyme. Such a material was only to be found, in the from co-enzyme, according to MEYERHOF's prescriptions, freed zymase. It seemed probable that a zymase preparation free of co-enzyme and subjected to a moderated autolysis after boiling up, in other words after destroying the zymase and the endotryptase should contain smaller complexes of proteins which in a second portion of zymase preparation free of co-enzyme, would protect the zymase against the endotryptase action.

In fact it now appeared that a similar effect could be observed so that our theory regarding the nature of MEYERHOF's co-enzyme was thus further confirmed.

VI. We may now presume from the above mentioned experiments that the preparations, used by the various investigators of which the activating function on inactivated zymase is indicated by them as "co-enzyme" action, contain, dependent on the different applicated methods of inactivation, different activating principles.

On the one hand inactivation might be a consequence of withdrawal of the introductory hydrogen acceptor, on the other hand of hexosephosphate or in the end also of the antiproteolytic acting substances. Not seldom these factors will act simultaneously and this in dependence of the followed mode of operation, the nature of the used zymase preparation etc. in varying degrees. We are of the opinion that this is very much the case with the extensive investigations of H. VON EULER and his collaborators, not discussed here, but we will not go further into this now.

Thus from all the foregoing the conclusion could be drawn that it would be advisable to distinguish henceforth more co-enzymes, which might be indicated as the co-enzyme of HARDEN (introductory hydrogen acceptor) that of BUCHNER and HAEHN (hexosephosphate) and that of MEYERHOF (antiprotease).

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[1]) TH. THOLIN, Zeitschr. f. physiol. Chemie, Bd. **115**, p. 235, (1921).

We should, however, prefer to strike out the whole conception of co-enzyme from the biochemistry of alcoholic fermentation and replace it by the insight that for fermentation of the hexoses by the usual zymase preparations a certain number of factors must be realized, namely: except the presence of alkaliphosphates, that of an introductory hydrogen acceptor and of hexosephosphate [1]), whilst further there must be reckoned with the autolytic properties of the named preparations.

For a further documentation of this preliminary communication we refer to a thesis to be shortly forthcoming from one of us (Str.) as also to an extensive paper to be published elsewhere.

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Delft, April 1927.

[1]) In the meantime we have strong evidence that hexosephosphate on the condition that it is present in a somewhat higher concentration, can itself fulfil the function of introductory hydrogen acceptor.