XIII. THE ENZYMES OF WASHED ZYMIN AND DRIED YEAST (LEBEDEFF). II. REDUCTASE.

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The reducing powers of yeast have long been known, and it was shown by Hahn that yeast juice and zymin possessed similar properties.

Recently the relation of the reducing ferment of yeast to the enzymes concerned in alcoholic fermentation has become of some interest, owing on the one hand to the theories of Neuberg, Kostytscheff and v. Lebedeff which postulate the reduction of acetaldehyde to ethyl alcohol as an essential step, and on the other hand to the experiments of Palladin [1908] who has found that during the fermentation of glucose by zymin the reducing action of the latter on sodium selenite and methylene blue is greatly diminished, and to those of Lvoff [1913, 1, 2, 3] who finds that during the reduction of methylene blue by dried yeast or maceration extract there is a correspondingly smaller fermentation of glucose, one molecule of methylene blue being equivalent to one molecule of glucose.

The reduction of selenite or methylene blue by living yeast is known [see Grüss, 1908] to be accelerated by the presence of glucose, and hence the conclusions of Palladin do not seem warranted without further examination.

In the following experiments the work of Palladin has been repeated and at the same time the effect of washing dried yeast and zymin upon the reducing power of the preparation has been ascertained.

The reducing powers of yeast, zymin and dried yeast towards sodium selenite.

Reduction of sodium selenite by living yeast. Influence of carbohydrates on the rate of reduction. The following mixtures were made up and incubated at 25°.

A.	2 g. yeast	+	30 cc. 0·5 per cent. sodium selenite solution.				
B.	,,	,,	,,	,,	,,	,,	+1 g. glucose.
C.	,,	,,	,,	,,	,,	,,	+1 g. galactose.
D.	,,	,,	,,	,,	,,	,,	+1 g. lactose.
E.	,,	,,	,,	,,	,,	,,	+1 g. arabinose.

The red colour due to metallic selenium appeared first in B and was quite marked in one hour. At this time there was only a faint reduction in all the other flasks. Two hours later the flasks all showed reduction, but that in B was very much more marked than in the others, which were all equally reduced. Hence the rate of reduction is increased by the presence of a fermentable sugar as found by Grüss [1908]. The other sugars tried had no influence. A control experiment was carried out in which glucose was incubated with sodium selenite solution alone but no reduction took place. A second experiment in which other fermentable sugars, cane sugar and maltose, were employed, gave a similar result.

Reduction of sodium selenite by zymin. Similar experiments to those described above were next carried out, using zymin instead of living yeast. In no case was any acceleration produced by the presence of a fermentable sugar. Palladin [1908] states that the reduction in the case of zymin is greatly hindered and in some cases entirely inhibited under these circumstances. In the authors' experiments, however, such a retardation has only been observed when very high concentrations of sugar have been employed. With low concentrations (5 per cent.) of glucose little effect was produced.

These points are illustrated by the following experiments.

The following mixtures were made up and incubated at 25°.

									Concentration of glucose per 100 cc. in g.
A.	3 g. zymin	+	10 cc. 1 % selenite	+	10 cc. H_2O	...	...		0
B.	,,	,,	,,	,,	+9·4	,,	+ 1 g.	glucose	5
C.	,,	,,	,,	,,	+8·8	,,	+ 2 g.	,,	10
D.	,,	,,	,,	,,	+7	,,	+ 5 g.	,,	25
E.	,,	,,	,,	,,	+4	,,	+10 g.	,,	50

The total volume was thus the same in each case, while the concentration of glucose varied from 5 to 50 g. per 100 cc. After incubation for 60 minutes the reduction in A, B, C and D was well marked and was approximately equal in extent. In E reduction had just started but was much less pronounced than in the other flasks. Hence glucose in concentrations up to 25 per cent. had little influence on the rate of reduction. Palladin's explanation of his results was that in the presence of glucose the reductase was directly concerned in the alcoholic fermentation and was therefore not free to reduce the selenite.

In view of the different results obtained by the authors, with lower concentrations of glucose, it seemed desirable to examine to what extent fermentation was influenced by the presence of selenite, for which purpose the following experiments were carried out:

A. *Living Yeast.*

(1) 2 g. yeast + 2 g. glucose + 20 cc. H_2O.
(2) ,, ,, ,, + 20 cc. 1 % sodium selenite.

The rate of fermentation of these mixtures at 25° was observed with the following results:

Duration of experiment, mins.	Total CO_2 evolved in cc. 1 Yeast + glucose	2 Yeast + glucose + selenite
5	9·1	10·4
10	17·8	20·6
15	28·0	31·2
20	36·6	40·9
30	54·6	60·8

In the above experiment therefore the mixture containing the selenite gave a slightly higher rate of fermentation than the control.

Reduction in (2) was well marked after incubation for 20 minutes. With zymin on the other hand a very different result was obtained.

B. *Zymin.*

(1) 2 g. zymin + 2 g. glucose + 20 cc. H_2O.
(2) ,, ,, ,, + 10 cc. 1 % selenite + 10 cc. H_2O.
(3) ,, ,, ,, + 20 cc. 1 % selenite.

Duration of experiment, mins.	Total CO_2 evolved in cc. 1 No selenite	2 0·5 % selenite	3 1 % selenite
50	11·0	2·4	2·1
65	25·5	2·7	2·3
80	33·8	3·1	2·4
95	40·3	3·4	2·4
155	64·2	4·4	3·1

With zymin therefore the presence of even 0·5 per cent. sodium selenite almost entirely inhibited the fermentation and this result was confirmed by further experiments. Both (2) and (3) showed signs of reduction after 50 mins. and the production of selenium increased steadily during the experiment. As Palladin [1908] used concentrations of selenite varying from 2 to 5 per cent. it is improbable that alcoholic fermentation was proceeding at all in any of his experiments.

C. *Dried Yeast.* (Lebedeff.)

(1) 2 g. dried yeast + 20 cc. H_2O + 2 g. glucose.
(2) ,, ,, + 20 cc. 1 % selenite + 2 g. glucose.
(3) ,, ,, + 10 cc. 1 % selenite + 10 cc. H_2O + 2 g. glucose.

Duration of experiment, mins.	Total CO_2 evolved in cc.		
	1 No selenite	2 1 % selenite	3 0·5 % selenite
35	18·8	0·6	0·9
95	36·6	1·0	1·5
155	55·6	1·5	1·9
380	98·6	3·8	3·8

Here, as with zymin, fermentation was almost entirely inhibited. Reduction was observed in (3) at the end of the experiment but not in (2).

The sample of dried yeast had a very low reducing power towards selenite as shown by the following experiment:

(1) 2 g. dried yeast + 20 cc. 1 % selenite.
(2) ,, ,, + 20 cc. 1 % selenite + 2 g. glucose.

These mixtures were incubated at 25°. After incubation for 4·5 hours no reduction could be detected in either. An hour later however reduction was visible in (2) and still later appeared in (1). These results with zymin and dried yeast cannot be explained on the ground of Lvoff's interpretation of his own experiments, according to which one molecule of glucose gives up two atoms of hydrogen to the reducible substance (in his case methylene blue), so that an amount of glucose equivalent to this escapes fermentation. In these experiments therefore in presence of 0·1 g. sodium selenite there should have been a deficit of about 29 cc. of CO_2, whereas as a matter of fact the deficit in the case of dried yeast was about 95 cc. and in the case of zymin 64 cc., without any sign of fermentation setting in.

It may here be remarked that the conclusions drawn by Lvoff [1913, 2 and 3] from his experiments cannot at present be accepted. In all the experiments in which the fermentation was continued beyond the stage at which the methylene blue was completely reduced, the deficit of carbon dioxide and alcohol increased considerably with the time. This indicates that the inhibition of fermentation even in the earlier stages cannot be solely attributed to the deviation of hydrogen, and indeed makes it doubtful whether any of it can be attributed to this cause. Experiments on the change in the amount of sugar during the process which are being carried out by Lvoff and, it may be suggested, an investigation on the degree of inhibition produced with different concentrations of dried yeast or maceration extract are required before any definite conclusion can be legitimately drawn. Experiments VI

and VIII [Lvoff, 1913, 3, pp. 304–5] suggest that the effect varies considerably with the amount of dried yeast employed.

Inactivation of dried yeast and zymin by washing.

It was observed that when zymin or dried yeast was washed several times with cold water and thus rendered incapable of fermenting sugar, it also lost its power of reducing methylene blue or sodium selenite.

It seemed therefore of interest to ascertain the cause of this loss of reducing power and also whether any substance capable of restoring it would at the same time restore the power of alcoholic fermentation. That the action is enzymic is shown by the fact that when dried yeast is boiled with water, the mixture does not reduce methylene blue. As a result it was found that the addition of certain aldehydes or of bouillon restored the reducing power but not the fermenting power, whilst the boiled washings restored both.

It seems probable therefore that washing removes some substance which acts as an acceptor for the oxygen activated during the reduction process and that the place of this can be taken by certain aldehydes or by some constituent of bouillon. The reducing enzyme of yeast therefore bears a close resemblance to that of potato juice recently investigated by Bach [1913, 1 and 2].

The zymin and dried yeast (Lebedeff) obtained from Schroder were washed in the manner previously described [Harden, 1913].

I. Washed zymin and sodium selenite.

5 g. zymin were washed and made to 60 cc.

1. 20 cc. zymin suspension + 20 cc. H_2O + 50 cc. 0·5 % sodium selenite.
2. ,, ,, ,, + 20 cc. boiled washings + 50 cc. 0·5 % sodium selenite.
3. ,, ,, ,, + 20 cc. H_2O + 50 cc. 0·5 % sodium selenite + 0·1 cc. formalin.
4. ,, boiled washings + 20 cc. H_2O + 50 cc. 0·5 % sodium selenite.

On incubation for 17 hours at 25° the only flask which showed reduction was (2), which contained washed zymin and boiled washings. The reducing power was not restored by the addition of formaldehyde (4).

II. Washed dried yeast and sodium selenite.

20 g. of dried yeast made to 100 cc.

1. 15 cc. yeast suspension + 10 cc. H_2O + 7 cc. 1 % selenite + 1 cc. toluene.
2. ,, ,, ,, + 10 cc. boiled washings + 7 cc. 1 % selenite + 1 cc. toluene.
3. 0 + 15 cc. H_2O + 10 cc. boiled washings + 7 cc. 1 % selenite + 1 cc. toluene.

Reduction was marked in (2) after 3 hours at 25° and did not occur in either (1) or (3) in 17 hours.

III. Washed dried yeast and (*a*) Methylene blue, (*b*) Schardinger's reagent.

10 g. dried yeast washed and made to 100 cc.

1. 20 cc. yeast suspension + 20 cc. H_2O + 1 cc. methylene blue + 1 cc. toluene.
2. ,, ,, ,, + 20 cc. boiled washings + 1 cc. methylene blue + 1 cc. toluene.
3. ,, H_2O + 20 cc. boiled washings + 1 cc. methylene blue + 1 cc. toluene.
4. ,, yeast suspension + 20 cc. H_2O + 1 cc. Schardinger + 1 cc. toluene.
5. ,, ,, ,, + 20 cc. boiled washings + 1 cc. Schardinger + 1 cc. toluene.
6. ,, H_2O + 20 cc. boiled washings + 1 cc. Schardinger + 1 cc. toluene.

The methylene blue was made by diluting 5 cc. of a saturated alcoholic solution to 200 cc.; Schardinger's reagent by mixing 5 cc. of saturated alcoholic methylene blue with 5 cc. formalin and diluting to 200 cc.

After 19 hours reduction had occurred in (2) and (5) but in none of the others.

Hence formaldehyde does not restore the reducing power.

IV. Reducing power of inactivated yeast in presence of various substances.

A number of qualitative experiments were made to ascertain the efficacy of various substances in producing reduction when added to washed dried yeast and methylene blue.

The results may be tabulated as follows:

Reducing power restored	No change
Salicylaldehyde*	Quinol*
Benzaldehyde	*p*-Phenylene diamine
Anisaldehyde	Pyrogallol
Isovaleraldehyde	Pyruvic acid
Bouillon*	Citral
	Acetaldehyde

The substances marked with an asterisk were tested as to their capacity to restore the power of alcoholic fermentation to washed dried yeast in presence of a small concentration of phosphate, in all cases with negative results.

Methylene blue and sodium selenite were also found to be inactive in this respect.

SUMMARY.

1. The presence of a fermentable sugar favours the reduction of selenite by living yeast but has little influence on the reducing power of zymin unless the sugar is present in high concentration, when inhibition occurs.

2. Sodium selenite in concentration of 0·5 g. per 100 cc. almost totally inhibits the fermentation of glucose by zymin and dried yeast (10 g. per 100 cc. of 10 per cent. glucose solution).

3. When dried yeast or zymin is washed with cold water it loses its power of reducing methylene blue and sodium selenite.

4. Such washed preparations reduce methylene blue in presence of many aldehydes and of bouillon, but these do not restore to it the power of producing alcoholic fermentation.

5. Addition of the boiled washings to these washed preparations restores both the power of reducing methylene blue and of producing alcoholic fermentation.

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