XCII. NOTE ON THE REACTIVATION OF REDUCTASE IN WASHED YEAST PREPARATIONS.

BY ARTHUR HARDEN AND MARJORIE GIFFEN MACFARLANE.

From the Biochemical Department, Lister Institute, London.

(Received April 28th, 1931.)

It was shown by Harden and Norris [1914, 1915] that after being washed with water dried (Lebedev) yeast did not reduce methylene blue; this property however was restored on the addition of boiled washings and also of definite chemical substances such as salicylaldehyde, benzaldehyde and lactate. It was suggested by Lebedev and Griaznov [1912] that a co-reductase was necessary for the action of reductase, in a manner analogous to that of co-enzyme; this theory, though later withdrawn by Lebedev [1927] is upheld by Euler and his colleagues [Euler and Nilsson, 1925] who regard the co-reductase as being identical with co-mutase and the co-enzyme of alcoholic fermentation. In considering the evidence for this view the possibility must be borne in mind that boiled yeast extract may restore the activity of the reductase only by reason of hydrogen donors contained therein rather than by the presence of a true co-enzyme. Euler and Nilsson [1927] however showed that neither a co-enzyme preparation which had been considerably purified nor a hexosediphosphate diminished the time required for the reduction of methylene blue by washed dried yeast when added separately, whereas when both were present together decoloration took place rapidly. If however the addition of co-enzyme together with hexosediphosphate, aldehyde or other substance induces alcoholic fermentation in a washed preparation, the chemical changes involved may provide an easily oxidisable substance which will allow the reduction of methylene blue to proceed, without any necessity for postulating a co-reductase. Consequently, it is unjustifiable to draw any conclusions as to the necessity for a co-reductase from experiments on the reactivation of reductase in washed yeast by the addition of any substance, if the possibility exists that such a reactivation is a secondary effect of alcoholic fermentation induced by the substance.

The earlier experiments of Harden and Norris were made at a time when failure to ferment sugar in presence of inorganic phosphate was accepted as evidence of the complete absence of co-enzyme from the washed yeast preparation. Meyerhof [1918] showed that the presence of a small amount of hexosediphosphate rendered this test more delicate, since its fermentation required a smaller concentration of co-enzyme. Later Kluyver and Struyk

[1927, 1928] showed that the reactivation of apozymase by acetaldehyde described by Harden [1917] did not take place in maceration residue after adequate washing; while Stheeman [1930] has made it clear that four distinct stages of inactivation of the fermenting complex of zymin can be attained by various degrees of washing. In the first stage the washed zymin is reactivated by a hydrogen acceptor, such as methylene blue or acetaldehyde; in the second by hexosediphosphate; in the third by hexosediphosphate and a hydrogen acceptor, and in the last by hexosediphosphate and co-enzyme. It has been confirmed in this laboratory that the adequate criterion for absence of co-enzyme in a washed preparation is a failure to ferment in presence of hexosediphosphate, and that the reactivation by acetaldehyde previously described [Harden, 1917] does not take place in the complete absence of co-enzyme.

It is obvious that a preparation in any of the first three stages of inactivation of zymase may be reactivated by aldehydes and hexosediphosphate with regard to reductase, if the fermentation so induced does in fact provide a suitable hydrogen acceptor. It seemed probable that the results obtained by Harden and Norris [1914] might require revision in the light of the later evidence as to the stages of inactivation by washing. It was therefore decided to repeat their experiments on the reactivation of reductase by chemical substances, using a washed preparation whose state of inactivation was checked by simultaneous fermentation experiments. Since it was impossible to use the same type of yeast as before, dried baker's yeast was chiefly used, as this is easily freed from co-enzyme. The results given below were obtained from various washed preparations of dried yeast.

Preparation 1. Dried baker's yeast washed three times by centrifuging with 8 parts of water, once on the filter with 5 parts, and dried with acetone and ether.

Preparation 2. Same dried yeast, washed four times with 8 parts of water by centrifuging, once on the filter and suspended in water (5 parts).

Preparation 3. Same dried yeast, washed five times with 8 parts of water, once on the filter with 5 parts and suspended in water (5 parts).

Preparation 4. Dried Copenhagen yeast, washed five times with 8 parts of water by centrifuging, once on the filter and suspended in water (5 parts).

Fermentation experiments. 0.2 g. of the washed preparation, 0.5 cc. of 20 % fructose and 0.1 cc. 0.6 M K_2HPO_4 were placed in each of three micro-fermentation flasks to which were added:

Flask (a) 0.5 cc. 0.1 M hexosediphosphate;

(b) 1.0 cc. yeast extract (prepared from ale yeast zymin);

(c) 0.5 cc. hexosediphosphate + 1.0 cc. yeast extract;

together with water to make a total volume of 2.5 cc. The flasks were placed in a thermostat at 30°, the CO_2 evolved being measured (Table I).

Reductase experiments. The reducing action of the preparations towards

methylene blue was then tested in the presence of various substances. A measured quantity of the washed suspension with 0.1 cc. of 1/1000 methylene blue and 0.1 cc. toluene was incubated at 26° in a total volume of 4 cc. with the addition of the desired substance. After constant observation for some hours, the mixtures were allowed to stand overnight, the minimum reduction time being given for such as were decolorised in the morning (Table II).

Table I. *Fermentation by washed yeast preparations. cc. CO₂.*

Time in hrs.	Washed preparation							
	1		2		3		4	
	2	20	2	20	2	20	2	20
Flask (a)	0	>5	0	>5	0	0.15	0	0.25
(b)	0.06	>5	0	>5	0	0	0.06	1.45
(c)	1.49	>5	3.16	>5	2.6	>5	0.24	2.20

Table II. *Reduction time of methylene blue by washed preparations.*

Additions	Preparation			
	1	2	3	4
(a) —	>5 hrs.	>4 hrs.	>4 hrs.	>3 hrs.
(b) 0.5 cc. hexosediphosphate	>5 hrs.	3½ hrs.	3 hrs.	>3 hrs.
(c) 1 cc. yeast extract	>5 hrs.	15 min.	—	5 min.
(d) 1 cc. yeast extract + 0.5 cc. hexosediphosphate	20 min.	15 min.	—	5 min.
(e) 0.01 cc. salicylaldehyde	>5 hrs.	>4 hrs.	>4 hrs.	>3 hrs.
(f) 0.01 cc. benzaldehyde	>5 hrs.	>4 hrs.	>4 hrs.	>3 hrs.
(g) 0.1 cc. 0.01 M lactate	1 hr.	30 min.	20 min.	15 min.
(h) 0.1 cc. lactate + 0.5 cc. hexosediphosphate	45 min.	—	20 min.	—
(i) 0.1 cc. 0.01 M succinate	—	3 hrs.	—	—

Another sample of washed yeast did not reduce methylene blue after 20 hours alone, but did so in 30 minutes with yeast extract, 1½ hours with lactate and 2½ hours with methylglyoxal.

The washed yeast preparations varied somewhat in their properties according to the manner of preparation. Thus preparation 1, which had been treated with acetone, required both yeast extract and hexosediphosphate for a rapid reduction, while preparation 2 reduced rapidly with yeast extract alone, although there was no significant difference in their fermenting power. The Copenhagen yeast reduced quickly with yeast extract, but fermented very poorly. The preparations were all consistent in that in the absence of yeast extract and hexosediphosphate, neither fermentation nor reduction took place during the period of constant observation (2–6 hours), though in the majority of cases both fermentation and reduction took place overnight. In all cases the addition of lactate caused a reduction of methylene blue in a time comparable with that taken by an addition of yeast extract + hexosediphosphate. With preparation 3, for instance, where no fermentation occurred in absence of co-enzyme, lactate effected reduction in 20 minutes, and with preparation 5, which did not reduce methylene blue alone, in 1½ hours. Succinate and methylglyoxal were also capable of acting as hydrogen donors,

though less rapidly than lactate but salicylaldehyde and benzaldehyde were ineffective.

During the reduction of methylene blue by washed dried yeast in presence of lactate, the lactate is decomposed with evolution of CO_2 , presumably according to the equations

the pyruvic acid being decomposed by the carboxylase of the yeast. The acetaldehyde produced might conceivably also have acted as an acceptor for the dehydrogenation of lactate. That this was not the case was shown in the following experiments.

0.2 g. washed yeast + 0.25 cc. K_2HPO_4 were placed in each of three micro-fermentation flasks to which were added

1. 1 cc. 1 % lactate + 0.01 g. methylene blue + 1.25 cc. H_2O ;
2. 1 cc. lactate + 0.03 g. methylene blue + 1.25 cc. H_2O ;
3. 1 cc. lactate + 0.1 cc. 10 % acetaldehyde + 1.24 cc. H_2O .

The flasks were placed in a thermostat at 30° and the CO_2 measured. Flask 1 became decolorised in 45 minutes, giving 0.73 cc. CO_2 ; flask 2 in 80 minutes, giving 1.82 cc. CO_2 , the theoretical amounts for these quantities of methylene blue being 0.8 and 2.4 cc. respectively. No evolution of CO_2 took place in flask 3. Rather less than the equivalent amount of CO_2 was evolved for the methylene blue reduced, showing that the acetaldehyde produced in the reaction or added independently did not act as a hydrogen acceptor for lactate. Moreover, the addition of pyruvic acid, which might liberate acetaldehyde in a more reactive state, was also ineffective in inducing a fermentation of lactate.

SUMMARY.

The addition of lactate, succinate or methylglyoxal restored the power of reducing methylene blue to yeast preparations washed to a point at which no fermentation of hexosediphosphate occurred. From these results it is concluded that a co-enzyme is probably not required for the action of the reductase in yeast preparations.

REFERENCES.

- Euler and Nilsson (1925). *Z. physiol. Chem.* **149**, 44.
 ——— (1927). *Z. physiol. Chem.* **162**, 84.
 Harden (1917). *Biochem. J.* **11**, 64.
 ——— and Norris (1914). *Biochem. J.* **8**, 100.
 ——— (1915). *Biochem. J.* **9**, 330.
 Kluver and Struyk (1927). *Proc. K. Akad. Wetensch. Amsterdam*, **30**, 569.
 ——— (1928). *Biochem. Z.* **201**, 212.
 Lebedev (1927). *Z. physiol. Chem.* **172**, 255.
 ——— and Giaznov (1912). *Ber. deutsch. chem. Ges.* **45**, 3256.
 Meyerhof (1918). *Z. physiol. Chem.* **101**, 165.
 Stheeman (1930). *Proc. K. Akad. Wetensch. Amsterdam*, **33**, 889.