XXXIII. THE REDUCING ENZYMES OF DRIED YEAST (LEBEDEFF) AND OF RABBIT MUSCLE.

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In a previous communication [Harden and Norris, 1914] it was shown that the power of reducing methylene blue is restored to dried yeast (Lebedeff), which has been washed until it has been rendered inactive in this respect, by a number of substances (e.g. salicylaldehyde) and the suggestion was made that these substances were capable of acting as acceptors for the oxygen activated during the reduction process. On the other hand many easily oxidisable substances (e.g. quinol) are without effect.

Further qualitative experiments carried out in a similar manner to those previously described have shown that comparatively few substances are able to act as acceptors in this way. The following list gives the results so far obtained, including those previously mentioned.

Positive.

Salicylaldehyde	Dihydroxyacetone	Bouillon
Benzaldehyde	Glycollic acid	Boiled yeast extract
Anisaldehyde	Citric acid	Normal horse serum
Isovaleraldehyde	Glyceric acid	Sterile milk
Phloroglucinol	Succinic acid	

Negative.

Quinol	Glucose	Creatine
<i>p</i> -Phenylene diamine	Laevulose	Asparagine
Pyrogallol	Glycocoll	Guanidine-acetic acid
Resorcinol	Alanine	Guanidine nitrate
Citral	Tyrosine	Glycol
Glycerol	Malic acid	Witte peptone
Propylene glycol	Tartaric acid	Acetone
2-3-Butylene glycol	Mandelic acid	Pyruvic acid
Formic acid	Yeast nucleic acid	Methylglyoxal
Acetaldehyde		

Of the hydroxy acids examined, lactic acid, either as free acid or sodium salt, was very active, whilst glycollic, glyceric and citric acid produced a very slow reduction. Sodium succinate also gave a very slow reduction.

Owing to their special interest the cases of acetaldehyde (negative) and lactic acid (positive) were more closely investigated.

Acetaldehyde. As acetaldehyde might be expected to exert some inhibitory action on the enzyme concerned in the reduction, special experiments were first made to ascertain how far this was the case.

Exp. 1. Four flasks were set up, each containing 5 cc. of a 10 per cent. suspension of the washed yeast (Lebedeff). To these were added the following solutions, and the mixtures incubated for two hours at 37°.

	Water cc.	Methylene Blue cc.	Boiled Yeast Washings cc.	1 per cent. Acetaldehyde cc.	Result
1.	6	1	0	0	—
2.	1	1	5	0	Reduced in 49 min.
3.	5	1	0	1	—
4.	0	1	5	1	Reduced in 78 min.

In this experiment therefore the acetaldehyde has a pronounced inhibitory effect. When the concentration is halved however this inhibition ceases to be perceptible.

Exp. 2. A similar experiment was made with half the concentration of acetaldehyde.

	Water cc.	Methylene Blue cc.	Boiled Yeast Washings cc.	1 per cent. Acetaldehyde cc.	Result
1.	6.5	0.5	0	0	—
2.	1.5	0.5	5	0	Reduced in 21 min.
3.	6	0.5	0	0.5	—
4.	1	0.5	5	0.5	Reduced in 21 min

That the amount of aldehyde here employed is sufficient to reduce the methylene blue is proved by the fact that in presence of washed muscle (p. 335) half this amount produced complete reduction.

A further experiment with varying amounts of acetaldehyde also gave completely negative results.

Exp. 3. 10 cc. of a 10 per cent. suspension of washed yeast (Lebedeff) were used, together with the following additions.

	Saline cc.	Methylene Blue cc.	1 per cent. Acetaldehyde cc.	Result
1.	5	0.5	0	—
2.	3	0.5	2	—
3.	4	0.5	1	—
4.	4.5	0.5	0.5	—
5.	4.75	0.5	0.25	—
6.	4.9	0.5	0.1	—

Lactic Acid. Both free lactic acid and sodium lactate were found to have a positive action, and attempts were made to establish some quantitative relation between the amounts of methylene blue reduced and the amount of lactic acid oxidised and also to ascertain the nature of the products formed from the lactic acid.

1. No completely satisfactory solution of the former of these two problems was attained.

Any oxygen present in the various solutions interferes with this estimation by reoxidising the leuco-methylene blue and thus increasing the amount of lactic acid required for the reduction of a given weight of the colouring matter. Complete removal of the oxygen was very difficult to effect, especially from the yeast suspension, but the partial removal attained by boiling out the water and other solutions which were used and working in an atmosphere of nitrogen caused a great acceleration of reduction and a fall in the ratio of lactic acid to methylene blue.

Exp. 4. A solution of methylene blue was prepared containing 0.4 mg. per cc., i.e. 0.001 molar.

10 cc. of a 10 per cent. suspension of the washed yeast were taken in each case, along with 1 cc. N/100 lactic acid and 1 cc. of the methylene blue solution. The yeast suspension was made with water saturated with nitrogen.

(a) The solutions of methylene blue and lactic acid were boiled up and cooled in nitrogen, and the yeast suspension then added. Reduction occurred in 7 min.

(b) The solutions were boiled up and cooled in air and the yeast suspension then added. Reduction occurred in 21 min.

Exp. 5. Working with air free solutions in an atmosphere of nitrogen, the following results were obtained.

10 cc. of a 10 per cent. yeast suspension and 1 cc. 0.01 N lactic acid were used, the total volume being made to 16 cc. with water. Temperature 37°.

Methylene Blue cc.	Time for Reduction min.
2	19
3	43.5
4	68
5	90

The greatest amount of methylene blue reduced by 1 cc. of 0.01 N lactic acid was 5 cc., the molecular equivalent being 10 cc.

The interesting observation was made that the reduction in certain cases was greatly accelerated by exposure to light. This only occurred with solutions containing dissolved oxygen, and was ultimately traced to the accelerating influence of light on the reoxidation of leuco-methylene blue by oxygen, a reaction which is being further investigated.

2. *Products of oxidation of lactic acid.* The distillate from a mixture of the washed dried yeast, methylene blue and lactate gave a pronounced reaction for acetaldehyde. This suggested that the lactic acid was first converted by oxidation into pyruvic acid. The latter would then be decomposed by the carboxylase of the washed yeast into carbon dioxide and acetaldehyde, which, being incapable of further oxidation under the conditions of the experiment, could be detected in the liquid. Quantitative experiments showed that a considerable amount of acetaldehyde was produced. This amount however was considerably less than the equivalent of the lactic acid destroyed, and it is to be supposed either that a considerable proportion of the acetaldehyde condenses to alcohol or disappears in some other way, or that only a part of the lactic acid oxidised is converted into pyruvic acid. A special experiment showed that none of the aldehyde was converted into alcohol, there being somewhat more found in the absence than in the presence of methylene blue.

Pyruvic acid itself could not be detected.

Exp. 6. 20 g. of dried yeast were washed and made to 50 cc. and two mixtures made:

A. 25 cc. yeast suspension + 25 cc. sodium lactate solution (approx. N/5) + 0.4 g. methylene blue.

B. 25 cc. yeast suspension + 25 cc. sodium lactate solution.

These were incubated at 37° for 3.75 hours, at the end of which the methylene blue in A was completely reduced. The two mixtures were separately

Hence in the reduction of 1.6 g. of methylene blue, 0.8 g. lactic acid has disappeared and at least 0.118 of this has been converted into acetaldehyde.

Preliminary experiments with rabbit muscle.

When the minced muscle of the rabbit is washed several times with saline, it behaves in a similar manner to dried yeast, and loses the power, possessed by the normal muscle, of reducing methylene blue.

The experiments with muscle are not however as satisfactory as those with yeast. When the muscle is very finely divided, the enzyme is removed as well as the acceptor. On the other hand, when the division is not so fine certain portions retain the acceptor and local reduction occurs. In any case the removal of the acceptor is not so complete as with dried yeast and reduction occurs in the controls in about 2-3 hours.

The chief interest in the results so far obtained with muscle lies in the fact that specific differences exist between the activating action of certain substances on the enzyme in muscle and on that in yeast. Thus acetaldehyde reactivates washed muscle, whereas, as shown above, it has no effect on washed yeast. Hitherto only qualitative experiments have been made with muscle and the positive results obtained are given below. The experiments are being continued.

Exp. 8. The muscle was minced and then washed with normal saline three times. Portions of 5 g. were then weighed out, a given volume of the substance to be tested and of the methylene blue solution, and if necessary of saline, added and the whole well shaken and incubated at 37°.

Substance tested	Vol. cc.	Methylene		Saline cc.	Time required for reduction at 37°, minutes		
		Blue cc.					
Boiled muscle washings	5	0.5	—	—	14		
Boiled yeast washings	5	0.5	—	—	12.5		
Sodium lactate, 5 per cent.	1	1.0	8		28		
Acetaldehyde, 1 per cent.	1	0.5	9				41
" "	0.25	0.5	9.75				46
Control	—	—	—		- in 50	- in 120	- in 60

None of the substances used reduced the methylene blue in the absence of the muscle.

SUMMARY.

1. The power of reducing methylene blue is restored to washed dried yeast (Lebedeff) by a number of oxidisable substances but not by others.
2. Lactic acid restores the power of reduction and is itself oxidised in the reaction, yielding acetaldehyde; the amount found however is not equivalent to the lactic acid lost. The lactic acid is probably first converted into pyruvic acid, which is then decomposed by the carboxylase of the yeast into acetaldehyde and carbon dioxide.
3. Rabbit muscle, like dried yeast, when washed loses the power of reducing methylene blue.
4. This power is restored to washed muscle by various substances. The enzyme concerned seems to be specifically different from that present in yeast, since acetaldehyde restores the power of reduction to washed muscle but not to washed dried yeast.

REFERENCE.

Harden and Norris (1914), *Biochem. J.* 8, 190.