CXXXII. STUDIES ON XANTHINE OXIDASE.

III. THE REDUCTION OF NITRATES.

BY MALCOLM DIXON AND SYLVA THURLOW.

From the Biochemical Laboratory, Cambridge University.

(Received July 31st, 1924.)

IN a recently published paper, Haas and Hill [1923] described the property possessed by milk of reducing nitrates in presence of aldehydes as "activators." This observation had previously been made by Bach [1911]. To the substance present in the milk which causes this reduction they gave the name "atite," and they regarded the "atite" as the actual "oxidisable substance," or, in other words, reducing agent, the aldehyde acting as an accelerator or apparently a catalyst.

It is well known that milk contains an enzyme, Schardinger's enzyme, capable of oxidising aldehydes in presence of such substances as methylene blue which themselves become reduced in the process. It occurred to us that the aldehyde in the system described by Haas and Hill might really be the reducing substance and not the catalyst. In this case the Schardinger enzyme would be the catalyst and the nitrate would simply be taking the place of the methylene blue, so that the term "atite" would merely designate the oxidising enzymes present in milk.

Haas and Hill gave reasons for believing that "atite" was not identical with the Schardinger enzyme. They stated that on heating milk to certain temperatures they could destroy the Schardinger enzyme without destroying "atite." They do not, however, state the amount of methylene blue used in this particular experiment, but in other similar experiments where quantities are given they use twenty times the usual amount employed in the methylene blue technique. Moreover, they give no actual reduction times and merely indicate their results as positive or negative. Thus a solution containing a small amount of enzyme would be considered by them to give a negative result, as the reduction time with so large an amount of methylene blue might easily be several days. On the other hand, the Griess-Ilosvay test for nitrites is an extremely delicate one so that very small amounts of enzyme would be detected. It does not therefore seem proved by their results that they actually effected a separation, particularly as they state that when the Schardinger reaction was first observed to be negative, the nitrite reaction was also very weak. We have repeated their experiments using smaller quantities of methylene blue and have been unable to effect a separation of the two activities. Haas and Hill

also stated that the nitrate-reducing agent of the potato was not identical with the Schardinger enzyme, but we have been led to believe that the "atite" of milk is different from the "atite" of the potato, as we have found that the two are "activated" by different substances. This will be explained later on in the paper. Since our evidence shows that the systems in the potato and milk are different we feel that arguments based on the properties of one system are inconclusive when applied to the other.

On the other hand, there are a number of facts which seem to point to the identity of "atite" with the xanthine and aldehyde oxidases of milk, which, as we have shown in the second paper of this series, are probably one and the same. Wherever the oxidase has been found we have found also the power to reduce nitrates in the presence of aldehyde. In the first paper of this series we gave a method for the preparation of the milk oxidases, i.e. the xanthine and aldehyde oxidase. This active caseinogen preparation was found to reduce nitrates as well as milk did in the presence of aldehyde. On clotting milk with rennin, both the oxidase and "atite" were found in the whey. Filter-paper removes a large proportion of the oxidase as shown in the former papers of this series, and the "atite" is similarly removed by this procedure.

If the hypothesis that the aldehyde is merely the reducing substance is true, it would be expected that hypoxanthine and xanthine could replace the aldehyde in the system. This was indeed found to be the case. Both milk and our caseinogen preparation in the presence of hypoxanthine actively reduce nitrate to nitrite. In fact all those substances which in the previous papers were found to be oxidised by the milk oxidase were able to bring about the reduction of nitrate. Furthermore, the amount of nitrite produced in a given time in presence of different substances was exactly parallel to the rate of reduction of methylene blue by these substances.

Since Haas and Hill have shown that milk contains an agent which will oxidise nitrites in the presence of oxygen, these experiments were carried out in vacuum tubes. The procedure employed was the same as that described in the previous papers. After standing in a thermostat for a definite time the tubes were opened and the Griess-Ilosvay reagent added immediately. The following table gives a typical experiment.

Each tube contained 5 cc. enzyme solution + 1 cc. 5 % NaNO_3 or 0.5 cc. methylene blue and 0.5 cc. water + 0.1 cc. of substance given in column 1. The evacuated nitrate tubes were heated $\frac{1}{2}$ hour in a water-bath at 37°.

Substance	Nitrite test	Reduction time
Control	—	∞
Hypoxanthine (2 mg. per cc.)	++++	2'
Xanthine (2 mg. per cc.)	+++	4'
Aldehyde (10 %)	+	58'
Adenine (2 mg. per cc.)	+	50'
Uric acid (20 mg. per cc.)	—	∞
Guanine (2 mg. per cc.)	—	∞
Caffeine (20 mg. per cc.)	—	∞

If the nitrate is merely replacing the methylene blue in this system, it would be expected that the substances that inhibit methylene blue reduction

would also inhibit nitrite formation. The following table shows the parallelism in inhibitory effects between the reaction with methylene blue and that with nitrate.

Each tube contained 5 cc. enzyme solution + 1 cc. 5 % NaNO_3 or 0.5 cc. methylene blue + 0.1 cc. Hx (2 mg. per cc.) + 0.5 cc. of substance in column 1.

Inhibiting substance	Nitrate test	Reduction time
Control	+ + + +	3'
N/10 KCN	+	7'
1 % NaF	+ + +	4'
Guanine (2 mg. per cc.)	+	15'
Caffeine (20 mg. per cc.)	+ + + +	3'
Uric acid (20 mg. per cc.)	+	15'

As has been shown in the previous paper, the oxidase begins to undergo destruction at p_H 9.3, and on adjusting the p_H of the solution to this value the nitrate-reducing power was similarly diminished.

It is of interest that the same inhibition by purines that was observed in the previous paper occurs also with nitrates, since this indicates that this is not a specific property of methylene blue. We have observed the same effect as before with high concentrations of hypoxanthine. The reduction of nitrate proceeds much more rapidly with a small than with a large concentration of hypoxanthine.

This very striking correspondence seems definitely to establish the view expressed above that the nitrate simply serves as an oxidising agent, replacing methylene blue, and that the "atite" of milk is merely another name for the oxidising enzymes of milk. The "atite" of the potato we have found to be activated by formaldehyde and acetaldehyde but not by the purine bases, in this respect contrasting sharply with the milk "atite." We think this is evidence that the two "atites" are of different natures.

It will be seen that the reduction of nitrate to nitrite gives us another technique for the investigation of oxidising enzymes and tissue respiration generally, and can be employed as an alternative to the methylene blue technique of Thunberg [1920] and others, and the dinitrobenzene technique of Lipschitz and Gottschalk [1921]. The method can easily be made accurately quantitative by the use of the colorimetric estimation method with metapenylenediamine; or qualitative results can be quickly obtained by means of the Griess-Ilosvay reagent.

SUMMARY.

1. Milk or the preparation of xanthine oxidase will reduce nitrates to nitrites in presence of hypoxanthine, xanthine, adenine or aldehyde.
2. The nitrate takes the place of the methylene blue in the system described in the previous papers.
3. The rate of reduction of nitrate is proportional to the rate of reduction of methylene blue under various conditions.

4. The inhibition effects by purines previously observed with methylene blue also occur with nitrates.

5. The "atite" of Haas and Hill is identical with the oxidase or oxidases in milk.

We wish to express our indebtedness to Professor F. G. Hopkins for his continual interest during this work and for much helpful advice.

REFERENCES.

- Bach (1911). *Biochem. Z.* **33**, 282.
Haas and Hill (1923). *Biochem. J.* **17**, 671.
Lipschitz and Gottschalk (1921). *Pflüger's Arch.* **191**, 1.
Thunberg (1920). *Skand. Arch. Physiol.* **40**, 1.