LXXX. FURTHER OBSERVATIONS ON CERTAIN REDUCING AND OXIDISING REACTIONS IN MILK.

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In a recent paper dealing with certain reducing and oxidising reactions in milk [Haas and Hill, 1923] it was suggested that the substance there described as itate might possibly be identical with milk peroxidase. Subsequent experiments have shown, however, that this is not the case, and that the two substances are distinct.

With the object of obtaining some further information regarding the nature of itate, experiments were made upon the nitrite oxidising power of whole milk and of whey. It was then found, as the following experiment shows, that when milk is coagulated by rennet itate remains in the whey[1].

Exp. 1. Into a series of wide mouthed bottles fitted with corks and containing 75 cc. of milk and 1 cc. of 10 % acetic aldehyde, were placed 4, 5, 6, 7, 8, 9, 9·5, 10, 10·5, 11, 11·5, 12 and 13 cc. respectively of 0·182 % sodium nitrite; an exactly similar series of bottles was set up containing whey[2] in place of milk. These bottles were rotated in a thermostat at 45°, and the time in minutes required for the oxidation of the nitrite in each case was determined by testing at short intervals with Griess-Ilosvay reagent. The results so obtained are incorporated in the curves of Fig. 1.

It should be pointed out that employing the above method of experiment it is only possible to obtain approximately, to within five minutes, the time taken to oxidise a given quantity of nitrite. For this reason all the points do not fall onto a smooth curve. A comparison of the two curves, however, reveals the fact that the rate of oxidation of nitrite is slower in whey than in milk, and also that the total amount oxidised by the whey is less than that oxidised by the milk.

[1] One of us (P.H.) is indebted to Dr S. S. Zilva of the Lister Institute for having drawn his attention to this fact. The statement made in the earlier paper to the effect that "itate is precipitated with the proteins when milk is coagulated" referred to precipitation by saturation with ammonium sulphate, not rennet.

[2] The whey was prepared by coagulating milk with Benger's Essence of Rennet at 37° using 2 cc. of rennet for every 100 cc. of milk, the curds being filtered off through butter muslin. This rennet was shown by experiment to be free from nitrite and to be incapable of oxidising nitrite in the presence of acetic aldehyde.

The non-identity of itate and peroxidase.

It has been known for some time that when milk is coagulated by rennet, the peroxidase remains in the whey and the fact above established, that itate likewise remains in the whey, appeared to lend additional support to the possibility of the two substances being identical. On further fractionating the whey however, by half-saturation with ammonium sulphate, it was found that the precipitate (1), so obtained, when redissolved in water, was able to oxidise nitrite in the presence of acetic aldehyde, but on completely saturating the filtrate from precipitate (1), the new precipitate (2) when redissolved in

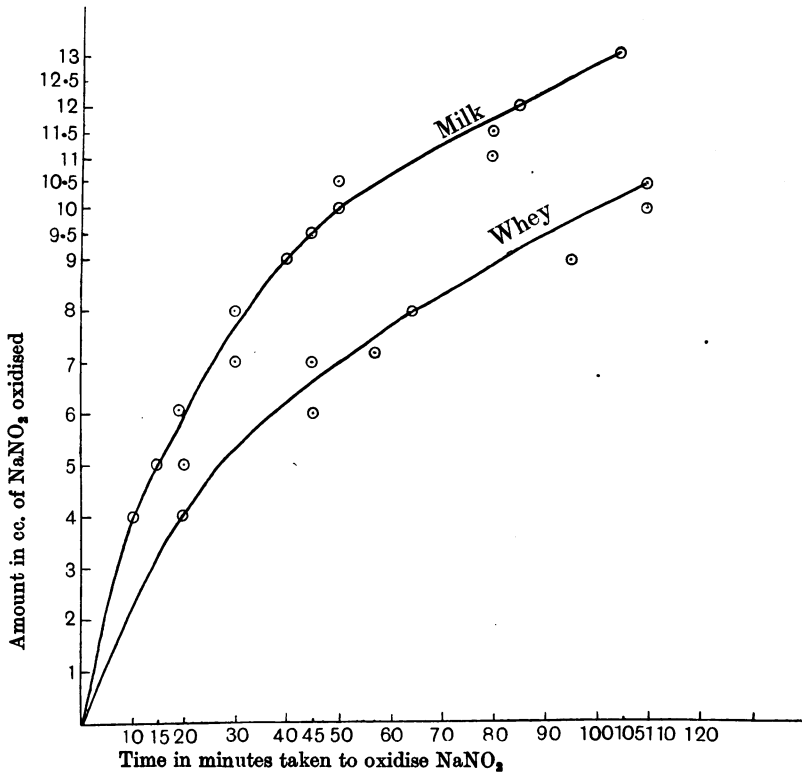

Fig. 1.

water was unable to do so. The solutions of both precipitates (1) and (2) in water gave a peroxidase reaction with guaiacum[1] and hydrogen peroxide, but the reaction was stronger in the case of the latter showing that it contained most peroxidase. Since, however, only fraction (1) was able to oxidise nitrite, it follows that itate and peroxidase are not identical, and that they

[1] Made by dissolving 0·5 g. of guaiacum resin in 5 cc. of absolute alcohol, filtering and diluting with an equal volume of water. The solutions of this reagent used throughout this work were always freshly prepared and ascertained to be free from peroxide by testing against fresh milk.

can be separated by making use of their different solubilities in half-saturated ammonium sulphate. These facts are illustrated by the following experiment:

Exp. 2. 900 cc. of whey were half-saturated with ammonium sulphate and the precipitate, fraction 1, so obtained, was filtered off on a Buchner funnel and redissolved in 200 cc. of water. The filtrate was completely saturated with ammonium sulphate and the second precipitate, fraction 2, filtered off and also dissolved in 200 cc. of water. To 50 cc. of the solutions of each fraction, containing 1 cc. of 10 % acetic aldehyde, were added respectively 1, 2, 3 and 4 cc. of sodium nitrite[1] and the eight bottles were then rotated in the thermostat at 45°. After 20 minutes fraction 1 had oxidised 2 cc. of nitrite and after 60 minutes almost completed the oxidation of 3 cc. but was not able to effect the oxidation of 4 cc. even after 180 minutes. Fraction 2 on the other hand, after 90 minutes, had not appreciably diminished the amount of nitrite in any of the bottles. It is therefore clear that itate activity is confined to the precipitate obtained by half-saturating whey with ammonium sulphate.

It will be noted from the above experiment that the activity of the itate is considerably reduced by the process of precipitation with ammonium sulphate and re-solution in water when compared with its activity in whey. Thus, whereas 75 cc. of whey were able to oxidise 10·5 cc. of nitrite, the solution of fraction 1, obtained from 225 cc. of whey, in 50 cc. of water, could only oxidise between 2 and 3 cc., in other words approximately one tenth of that oxidised by the same volume of whey.

The inactivation of itate and peroxidase.

It was pointed out in the earlier paper [1923, p. 678] that the termination of itate activity coincides with the disappearance of the peroxidase reaction, and that when once a given quantity of nitrite has been oxidised further added nitrite is no longer attacked, even though the amount of milk employed contains sufficient itate to oxidise a much larger quantity of nitrite.

It is now found, however, that the oxidation of a further quantity of nitrite can be effected, provided it is added before the complete oxidation of that already present. These facts suggested that during the course of the reaction some product might be formed which had a destructive action upon both the itate and the peroxidase, rendering them inactive as soon as all the nitrite is oxidised. If this were so it followed that the peroxidase would not be inactivated so long as the nitrite were kept in excess. That this assumption is correct was shown by the following experiment:

Exp. 3. 75 cc. of milk and 1 cc. of 10 % acetic aldehyde were rotated with 16 cc. of sodium nitrite, a quantity known to be in excess of the amount which the itate present could normally oxidise[2]; it was found that, after

[1] The concentration of the sodium nitrite used throughout these experiments was 0·182 %.

[2] The largest volume of nitrite we have observed to be oxidised, using 1 cc. of acetic aldehyde, is 15 cc. of sodium nitrite, although on earlier occasions the maximum was 10 cc. in 210 minutes. We have however, using 2 cc. of acetic aldehyde, oxidised 17 cc. of nitrite in 240 minutes, but we were not able to increase this quantity by the addition of more aldehyde. No great significance must be attached to the absolute values since it is reasonable to expect a certain amount of variation in different specimens of milk.

5½ hours' rotation, the mixture still contained unchanged nitrite but gave a positive reaction with guaiacum and hydrogen peroxide, indicating the presence of unchanged peroxidase, whereas a quarter of an hour's rotation of milk with aldehyde alone was sufficient to inactivate all the peroxidase.

The fact that in this case peroxidase and nitrite could exist together without mutually interacting even after the addition of more aldehyde, suggested for the first time that the peroxidase alone was not responsible for the oxidation of nitrite.

The function of the aldehyde in the reaction.

In attempting to explain the function of the aldehyde in assisting in the oxidation of the nitrite, it was clear that it could not be that of a hydrogen acceptor in a "hydrolytic oxidation-reduction," leaving the oxygen from the water to oxidise the nitrite, since, in that case, no function could be assigned to the atmospheric oxygen which had already been shown to be essential to the reaction [Haas and Hill, 1923].

In view of the known tendency of benzaldehyde to form, with atmospheric oxygen, a peroxide, the possibility of a similar peroxide formation from acetic aldehyde was indicated.

To test this matter further, 75 cc. of water were rotated at 45° with 1 cc. of 10 % acetic aldehyde. No trace of peroxide formation could be detected after four hours' rotation, on addition of a few drops of guaiacum solution and a little fresh milk to supply the peroxidase. When however the 75 cc. of water were replaced by an equal volume of milk, it was found that, after rotating for a quarter of an hour, the mixture no longer gave any reaction for peroxidase but gave a strong positive reaction for peroxide when tested as above with guaiacum and milk.

Since milk alone when rotated in the thermostat produces no peroxide, it would appear that the production of a peroxide depends upon the interaction of acetic aldehyde with some constituent of the milk. Furthermore the essential part played by atmospheric oxygen in the reaction was shown by the fact that no peroxide was produced when milk and aldehyde were rotated in a bottle in which the air was replaced by nitrogen.

Subsequently it was found that a peroxide was also formed when acetic aldehyde was replaced by benzaldehyde but not by formaldehyde.

With regard to the particular constituent of milk concerned in the peroxide formation, two possibilities presented themselves, namely, itate and peroxidase. By rotating solutions of fractions (1) and (2), respectively, with acetic aldehyde, it was shown that the former, which contains itate and peroxidase, was alone able to produce peroxide, while the latter, which contains peroxidase only, gave no peroxide. This shows that it is the itate and not the peroxidase which is concerned in the formation of a peroxide.

THE MECHANISM OF THE OXIDATION OF NITRITE TO NITRATE.

In view of the above facts the conclusion appears justified that when milk is rotated in a thermostat, with acetic aldehyde and sodium nitrite, in a bottle containing air, the first reaction to take place is the formation of a peroxide from the aldehyde and atmospheric oxygen, through the intervention of the itate; the peroxide so formed then interacting with the peroxidase effects the oxidation of the nitrite.

The following equations are put forward tentatively in possible explanation of the above changes:

$$CH_3CHO + O_2 = CH_3CO \cdot O_2 \cdot H$$

$$CH_3CO \cdot O_2\ H + NaNO_2 = CH_3COOH + NaNO_3$$

but it must be expressly stated that neither acetyl peroxide nor acetic acid has actually been isolated; in view however of the minute quantities of material involved the difficulty of doing so will be appreciated.

In support of the above theory of oxidation of the nitrite, by the interaction of peroxide with peroxidase, the following experiment may be quoted:

Exp. 4. Two bottles each containing 75 cc. of milk, 6 cc. 0·3 % hydrogen peroxide and 5 cc. of sodium nitrite were fitted up, one containing air and the other nitrogen. In each case the nitrite was destroyed in 15 minutes, showing that the oxidation depends upon the interaction between peroxide and peroxidase, and not upon the itate which, as was shown above, can only exert its action in the presence of oxygen.

That the oxidation of nitrite cannot be effected by hydrogen peroxide in the absence of peroxidase was shown by rotating a solution of these two substances in water instead of in milk, when no oxidation of nitrite was effected.

Since hydrogen peroxide in an atmosphere of nitrogen, is able to replace acetic aldehyde and oxygen, which by the action of itate produce a peroxide, it may be concluded that the chief function of the itate is to produce the requisite peroxide for interaction with the peroxidase, and that it is not itself concerned directly in the oxidation of the nitrite.

Further evidence for the essential part played by the peroxidase in the oxidation is furnished by the following experiment:

Exp. 5. 850 cc. of whey were divided into two portions, *A* and *B*. Portion *A* was half-saturated with ammonium sulphate and the precipitate was filtered off on a Buchner funnel and redissolved in 100 cc. of water giving solution *A* of fraction 1. Portion *B* was completely saturated with ammonium sulphate and the precipitate, consisting of fractions 1 and 2, was likewise dissolved in 100 cc. of water giving solution *B*. To 75 cc. of each of solutions *A* and *B* were added 1·5 cc. of 10 % acetic aldehyde and 1·5 cc. of sodium nitrite and the two mixtures rotated side by side in the thermostat. After 35 minutes the whole of the nitrite in solution *B* had been destroyed but that in solution *A* had not been destroyed after two hours. Furthermore solution *A*, after 35 minutes, contained no more peroxidase but gave a peroxide reaction, whereas solution *B* still gave a peroxidase reaction but was free from peroxide.

It must be borne in mind that both solutions *A* and *B* contained the same

quantity of itate (supplied by fraction 1) and only differed in their peroxidase content (the major portion of the peroxidase being contained in fraction 2). In view of these facts, the absence of peroxidase from solution *A* at the end of the reaction appears to indicate that the failure to oxidise the whole of the 1·5 cc. of sodium nitrite added was due to an insufficiency of peroxidase; on the other hand the excess of peroxidase found at the end of the reaction in solution *B* indicates that the peroxide formed by the itate was completely used up in oxidising the nitrite with the result that some unchanged peroxidase remained.

The action of peroxides upon itate and peroxidase.

To test the action of hydrogen peroxide on milk peroxidase 75 cc. of milk were rotated with 6 cc. of 0·3 % hydrogen peroxide; at the end of 40 minutes the peroxidase was found to have been entirely destroyed. From the above observation of the destructive effect of hydrogen peroxide upon milk peroxidase, the conclusion appears justified that it is the peroxide generated from acetic aldehyde which is responsible for the inactivation of the peroxidase when once all the nitrite present has been oxidised. It would appear therefore that the peroxide is utilised in the oxidation of the nitrite so long as the latter is in excess, but if insufficient nitrite is present the peroxide destroys the remaining peroxidase. Thus while 75 cc. of milk when rotated for 90 minutes with 1 cc. of 10 % acetic aldehyde and 16 cc. of sodium nitrite still contain unchanged peroxidase but no peroxide, the same quantity of milk rotated with 6 cc. of nitrite for 35 minutes no longer contains any peroxidase, but gives a positive reaction for peroxide.

The fact that the oxidation of nitrite has been shown to be due to the interaction between peroxide and peroxidase explains the failure to oxidise any further nitrite added to milk in which the peroxidase has already been inactivated.

Although the inactivation of peroxidase has been shown to be due to the peroxide produced by the action of itate on aldehyde, there was not sufficient experimental evidence to show whether itate itself is similarly inactivated by peroxide. The addition of more peroxidase to milk in which the peroxidase had been inactivated following on the oxidation of a quantity of nitrite should provide the requisite conditions for the further oxidation of added nitrite by the residual peroxide. If, in these circumstances, itate were still present in an active state, it should be possible to effect the oxidation of further nitrite up to the maximum of 15 cc.

It was shown in an experiment in which 6 cc. of sodium nitrite had been oxidised in 75 cc. of milk containing 1 cc. of aldehyde that, on the addition of fresh peroxidase in the form of fraction 2, it was only possible to oxidise an additional 3 cc. of nitrite. Since the total 9 cc. of nitrite thus oxidised is 6 cc. less than that capable of being oxidised by 75 cc. of milk it must be concluded that the activity of the itate is considerably reduced by the peroxide remaining at the end of the oxidation of the 6 cc. of nitrite.

Conclusions.

Without expressing any opinion on the chemical nature of itate it may be stated that its function in the oxidation of nitrite by milk in presence of acetic aldehyde appears to be an indirect one, inasmuch as it is concerned primarily in the production of the peroxide; that it is not directly responsible for the oxidation of the nitrite is shown by the fact that fraction 2, containing peroxidase but no itate, is able to oxidise nitrite when supplied with a peroxide in the form of hydrogen peroxide.

In effecting the oxidation of the acetic aldehyde to a peroxide the itate is apparently only able to produce a limited amount of peroxide and is itself used up in the process; in the absence of further experimental evidence its mode of action in this respect must remain a matter of speculation. In the meantime it cannot fail to be noted that in its effect it is similar to the hypothetical enzyme "oxygenase" which is assumed by Bach and his collaborators to be an essential constituent of the direct acting oxidase system of plants.

We have made a number of attempts to effect the oxidation of nitrite in the presence of acetic aldehyde and of hydrogen peroxide using the peroxidase of horseradish in place of that of milk but have not so far been able to obtain evidence of any oxidation.

Summary.

1. Itate and peroxidase, while sharing the common properties of being inactivated by boiling and of remaining in the whey on coagulation of milk, are not identical, since it is possible to effect a partial separation of the two substances.

2. The role played by itate in the oxidation of nitrite by milk in the presence of acetic aldehyde and oxygen, is that of producing a peroxide from the aldehyde. In this respect it resembles the hypothetical "oxygenase" of plant oxidases.

3. The oxidation of nitrite by milk results from the interaction of the peroxide so formed with the peroxidase, and does not result from the direct action of the itate, as originally thought. This is shown by the fact that a solution containing a mixture of itate and peroxidase will not oxidise any nitrite in the absence of peroxide, whereas a solution containing peroxidase, but no itate, does oxidise nitrite on the addition of hydrogen peroxide.

4. Attempts to effect a similar oxidation of nitrite by a peroxidase of vegetable origin, in the presence of hydrogen peroxide, have so far been unsuccessful.

During the course of this work we have kept in communication with Mr T. G. Hill who, from unavoidable causes, was prevented from active cooperation.

In conclusion one of us (B.L.) wishes to express her indebtedness to the Department of Scientific and Industrial Research for a grant.

REFERENCE.

Haas and Hill (1923). *Biochem. J.* **17**, 671.