

## EFFECTS OF pH AND HEATING ON REACTION OF NITRITE WITH CYTOCHROME c

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## INTRODUCTION

The reaction of nitrite with the heme protein myoglobin has been studied in regard to the practice of curing (Tarladgis, 1962; Fox, 1966; Bard and Townsend, 1971; Mohler, 1974; Giddings, 1977). Cytochrome c, which is located in the mitochondria, is also a heme protein and it can exist in either the oxidized or reduced form depending upon conditions. The reaction of cytochrome c with nitrite and the stability of the resultant product are aspects which have not been thoroughly investigated especially as pertaining to distribution of nitrite in cured meats and its availability for further reaction.

In the present study, we considered the reaction of nitrite with cytochrome c (oxidized form) under varying conditions of concentration, pH and temperature.

## MATERIALS AND METHODS

Oxidized cytochrome c (purchased from Sigma Chemical Co.) was dissolved in distilled water at pH 3.0 with 0.5 N HCl for 1 hour and the pH was adjusted to 7.0 with 1 N NaOH. All the cytochrome c was present in an oxidized form as confirmed by spectral analysis following the addition of solid potassium ferricyanide. The concentration of the cytochrome c was measured by the magnitude of the  $\alpha$ -peak (550nm) after addition of solid sodium dithionite at pH 6.8 in 0.1 M citrate solution using a millimolar extinction coefficient of 29.5 (Van Gelder and Slater, 1962).

The reaction of nitrite with cytochrome c was carried out in 0.1 M citrate buffer at room temperature. Heat was applied after the initiation of the reaction or after reaction for 24 hours.

## RESULTS

Sodium nitrite reacted with cytochrome c (oxidized form) and the reaction was pH-dependent (Figure 1). The product had peaks at 529 and 562 nm in a Soret region, in agreement with previous work in which gaseous nitric oxide was reacted with cytochrome c (Ehrenberg and Szczepkowski, 1960). The same product is also an intermediate of metabolism of nitrite by muscle mitochondria which are responsible for the reduction of nitrite to nitric oxide (Walters and Taylor, 1964; 1965; Walters, Casselden and Taylor, 1967). This suggests that the reactivity of nitrite is mediated by the effective nitrosating forms of nitrite (NO-ONO or/and NO-OH) which

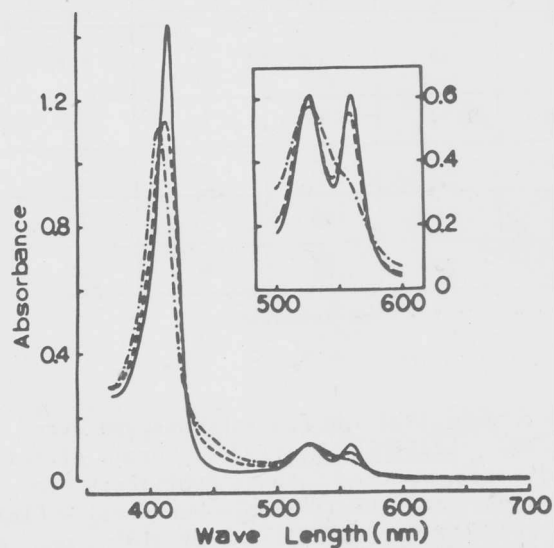


Figure 1 Absorption spectra of the product formed from cytochrome c (oxidized form) and nitrite at various pH values. The reaction medium contained 0.05 mM cytochrome c, 10 mM sodium nitrite and 0.1 M citrate (—, pH 3.0; ----, pH 3.9; - · - · -, pH 5.6). After 24 hours incubation, the scanning was carried out with five times dilution of the reaction mixture or without dilution (inset).

are actively formed at lower pH. The peak at 562 nm was convenient for following the reaction and evaluating the amount of the product formed (Figure 2A). The reaction reached equilibrium within 12 hours over the pH range from 2.8 to 6.0. Even at higher pH (5.6 and 6.0) the reaction occurred although the amount of product formed was small. Likewise, calculation showed that small amounts of nitrous acid would be present at these high pH values (Figure 2B); nitrous acid is directly responsible for the nitrosating forms of nitrite.

When the reaction mixture was heated at 80°C for 1 hour immediately after the reaction was started, the effect produced depended on the pH. At high pH (5.3, 5.6 and 6.0) the heating had no effect. A slight increase in

the amount of product was observed at pH 4.8. At low pH (3.3 and 3.0), however, the heating resulted in decomposition of the product. The reaction mixture became turbid at pH 3.6 to 4.2. This latter effect of heating was quite similar to that observed if the heating was done after 24 hour incubation (Figure 3B). The heat stability of the product at 80°C was examined at pH 3.0 after 24 hour incubation (Figure 3A). The rate of decomposition of the product was approximately 0.235% per minute. The cytochrome c retained its functional properties during this treatment. When a heated reaction mixture (0.0556 mM cytochrome c, 0.111 M citrate, 11.111 mM sodium nitrite) was again incubated with sodium nitrite (0.05 mM cytochrome c, 0.1 M citrate, 20 mM sodium nitrite) for 24 hours at room temperature, some portions of the product which had decomposed then reacted again with nitrite.

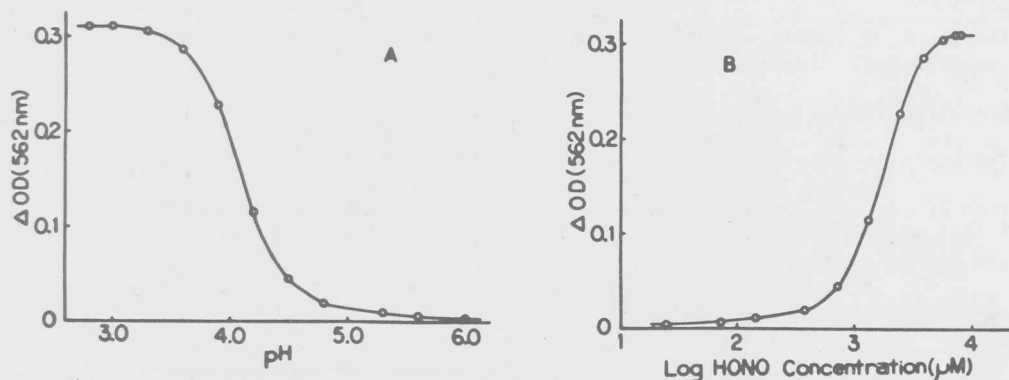


Figure 2 (A) The effect of pH on the formation of ferrocyanochrome c nitrosyl compound. (B) Relationship between ferrocyanochrome c nitrosyl compound and nitrous acid calculated on the basis of pH. Reaction conditions were the same as in Figure 1.

#### DISCUSSION

Cytochrome c in the oxidized form is very stable upon heating but cytochrome c in the reduced form is susceptible to denaturation when heated at lower pH (data not shown). When the product formed from reaction of cytochrome c with nitrite is heated in the presence of nitrite, the following processes may take place.

1. Decomposition of residual nitrite together with an increase in activity of hydrogen ions.
2. Decomposition of the product due to the breakdown of residual nitrite (shift of equilibrium between the product and nitrite).
3. Decomposition of the product by thermal energy.
4. A chance of oxidation of the reduced cytochrome c (which resulted from the decomposition of the product) by residual nitrite.
5. A chance of denaturation of cytochrome c in a reduced form.

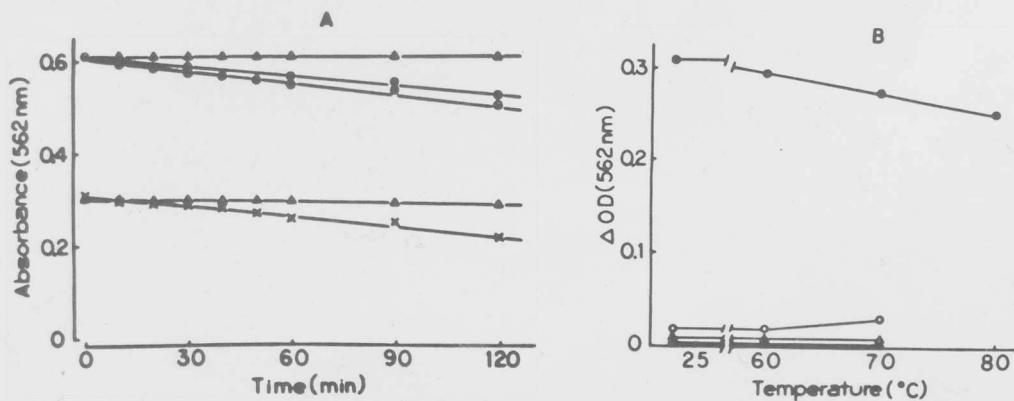


Figure 3 The effect of heating on the formation and decomposition of ferrocyanochrome c nitrosyl compound. Reaction conditions were the same as in Figure 1, and heat was applied after 24 hours of incubation. (A) Effect of heating time at 80°C and pH 3.0 (○ is change in optical density of the reaction mixture; △ is change in optical density of cytochrome c in the oxidized form; x is change in the reaction mixture corrected for change in cytochrome c; ▲ is the reaction of nitrite with cytochrome c in the oxidized form after it was heated; ● is the re-reaction of nitrite with the heated reaction mixture (see text for details). (B) Effect of heating for 1 hour (● is pH 3.0; ○ is pH 4.8; △ is pH 5.3; ▲ is pH 5.6).

At low pH all of the above processes are possible and our results can be so explained. At high pH, however, the system appears to be quite stable.

The slight increase in the product formed at pH 4.8 upon heating suggests it is fairly heat stable. This increase might be due to a higher concentration of nitrous acid as a result of an increase of hydrogen ions during heating. The decomposition of the product at pH 3.0 may be due to the breakdown of nitrous acid resulting in the shift of the equilibrium. However, the difference in stability of the product at different pHs can not be excluded.

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