REACTIONS OF NITRITE IN MEAT

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When nitrite is added to meat, it may react with many of the constituents, and the environment of the meat including such factors as concentration of reactants, pH and temperature influences the reactions. Obvious interest has centered on the reaction of nitrite with the proteins of meat not only because numerous potential reaction sites are present but also because experimental studies with ^{15}N labelled nitrite have shown that a large proportion /20-50%/ of the label added is recoverable from the meat proteins. We investigated the reaction of nitrite with tryptophyl residues of protein because it is well known that tryptophan is easily nitrosated given appropriate conditions of pH and concentration. On the basis of spectrophotometric evidence, we found it difficult to nitrosate tryptophyl residues of myosin even when the myosin was denatured and the reaction was conducted at low pH /3.0/. Lysozyme, which is soluble at low pH and high in tryptophan, nitrosated rather easily. Additional work with model systems showed that nitrosated protein could transnitrosate a receptor molecule such as myoglobin.

Reaktionen von Nitrit im Fleisch

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Nitrit kann im Fleisch mit vielen Einzelkomponenten reagieren, wobei bestimmte Umweltbedingungen wie Substratkonzentration, pH-Wert und Temperatur diese Reaktionen beeinflussen. Das besondere Interesse konzentriert sich nun auf die Reaktion von Nitrit mit den Proteinen im Fleisch, nicht nur weil es hier zahlreiche potentielle Bindungsstellen gibt sondern weil auch Untersuchungen mit ^{15}N markiertem Nitrit gezeigt haben, dass sich ein beträchtlicher Anteil der zugesetzten Menge, ca. 20-50%, in den Fleischproteinen wiederfindet. Wir untersuchten die Reaktion von Nitrit mit tryptophanhaltigen Proteinresten, da bekannt ist, dass Tryptophan bei entsprechenden pH-und Konzentrationsbedingungen sehr leicht Nitrit bindet. Bei unseren spektrophotometrischen Untersuchungen fanden wir kaum eine Bindung von Nitrit an tryptophanhaltige Myosinreste, auch dann nicht, wenn das Myosin denaturiert und die Reaktion bei sehr niedrigem pH-Wert /3.0/ durchgeführt wurde. Das bei niedrigen pH-Werten lösliche und sehr tryptophanreiche Lysozym verbindet sich dagegen ziemlich leicht mit Nitrit. Weitere Arbeiten an Modellsystemen zeigen, dass an Protein gebundenes Nitrit auf ein Rezeptormolekul wie Myoglobin transferiert werden kann.

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RÉACTIONS DU NITRITE SUR LA VIANDE

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Le nitrite ajouté à la viande peut réagir avec beaucoup de ses composants, et l'environnement de la viande comprenant des facteurs comme la concentration des réactifs, le PH et la température, contrôle les réactions. La réaction du nitrite avec les protéines de la viande a soulevé un intérêt indéniable à cause des nombreux sites potentiels de réaction mais aussi parce que des expériences avec le ^{15}N-nitrite ont montré qu'une forte proportion /20 a 50%/ de la radioactivité ajoutée est récupérable des protéines de la viande. Nous avons examiné la réaction du nitrite avec les résidus tryptophyles des protéines car il est bien connu que le tryptophane est facilement nitrosé dans des conditions appropriées de pH et de concentration. Base sur l'expérience spectrophotométrique, il était difficile de nitroser les résidus tryptophyles de myosine même quand la myosine était dénaturalisée et quand la réaction était produite à un bas PH/3./. La lysosyme, qui est en solution à bas PH et forte en tryptophane, a été nitrosé assez facilement. Des études supplémentaires avec des systèmes modèles ont montré que des protéines nitrosées peuvent transnitroser une molécule réceptrice telle que la myoglobine.

Реакции нитрита в мясе

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Когда нитрит прибавляется к мясу, он может реагировать со многими составными частями и окружающая обстановка, включая такие факторы как концентрацию реагентов, pH и температуру, оказывают влияние на реакции. Очевидный интерес сосредоточен на реакцию нитрита с белками мяса не только потому, что являются многочиленные местоположения для реакции, а и потому, что опытные исследования меченным ^{15}N нитритом показали, что высокая пропорция (20-50%) меченного возместимая с белком мяса. Мы исследовали реакцию нитрита с триптофильными осадками белка так как известно, что триптофан легко нитризирован, данные подходящие условия pH и концентрации. На сонование спектрофотометрических данных, нам трудно было нитризировать триптофильные осадки миозина даже когда миозин был денатурирован и реакция была проведена на низком pH (/,0). Лицозим, который растворим при низком pH и высокий триптофаном, был довольно легко нитризирован. Дополнительная работа модельными системами показала, что нитризированный белок мог перенитризировать приемную молекулу как миоглобин.

Reactions of nitrite in meat

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Introduction

It is well known that some of the nitrite added to meat for the purpose of curing becomes unavailable when analyzed for by current analytical methods. The amount detected is called residual nitrite. The amount of residual nitrite in a given cured meat depends on the composition and type of product as well as on the procedure used for making it. In general, more than 50% of the added nitrite is lost from detection, and in many cases, residual nitrite is very low, being in the range of 5-15 ppm at the time the product is offered for sale.

Concern about nitrite as a meat curing agent has arisen because of the possibility for formation of nitrosamines in cured meat. Also, the residual nitrite in cured meat when consumed by humans may react in the gastric environment. Most research effort in the area has been directed at analytical detection of nitrosamines and study of their toxicology especially at low dose levels. Assessment has also been made of the contribution of cured meat to total intake of nitrite and found to be low (<20%). Tannenbaum et al. (1978) have suggested recently that endogenous nitrification occurs in humans.

Even though nitrite is known to be an extremely reactive chemical, only few attempts have been made to study its fate in a meat system (Sebranek et al., 1973; Olsman, 1974; Emi-Miwa et al., 1976 ; Frouin, 1977). It seems especially important, in view of the conclusion of Cassens et al. (1977) that 25-40% of the nitrite added to meat in the usual curing procedure becomes in some way associated with the non-heme proteins, to elucidate and quantitate the reaction products formed. Not only the complexity of the meat system but also the changing reaction environment during processing, storage and preparation present genuine roadblocks to production of realistic and reliable information. A review of the chemistry of nitrite, with emphasis on a discussion of reaction mechanisms in a meat environment has been written recently (Cassens et al., 1979).

If attention is limited to reactions of nitrite with protein, then the literature reveals several possibilities; however, quantitation of reaction products in cured meats is lacking. Nitrite may react with the α-amino group (Van Slyke reaction) to liberate N_2 and also with the amide linkage, although sluggishly, to form a nitrosamide (Bonnett and Holleyhead, 1974). Nitrosothiols are formed from the reaction of nitrite with sulfhydryl groups, and this has been documented in cured meat (Mirna and Hofmann, 1969; Kubberød et al., 1974). It has also been established (Kurosky and Hofmann, 1972) that nitrite can react with the ε-amino group of lysine, the ring nitrogen of tryptophan and tyrosine (C-nitrosation).

The formation of N-nitrosotryptophan from tryptophan and nitrite reacted under appropriate conditions is well documented (Kurosky and Hofmann, 1972; Bonnett and Holleyhead, 1974; Brown and Stevens, 1975), and a characteristic absorption peak at 330 nm is formed thereby providing a possibility for quantitation. Work by Nakai et al. (1978) confirmed the above reports, and the authors suggested from the model systems used that the conditions for the reaction to occur in cured meat were unfavorable.

The objective of the present work was to investigate the nitrosation of tryptophyl residues in a meat protein. A secondary objective was to obtain information about transnitrosation. A detailed account of some aspects of the work has been published (Ito et al., 1979).

Materials and Methods

The proteins used were myosin, lysozyme and myoglobin. Myosin was prepared from skeletal muscle of rabbit (Nauss et al., 1969) and lysozyme and myoglobin were purchased from Sigma Chemical Company.

In general, experiments were conducted by mixing buffered protein and buffered nitrite solutions (see Ito et al., 1979 for details). Protein was prepared for reaction by dialyzing it overnight against 100 volumes of buffer to equilibrate it to a given pH and concentration of buffer. Measurements were made with a Cary 118 spectrophotometer, and a double cell system was employed in some cases. Some reactions were carried out with denatured protein; denaturation was accomplished with sodium dodecylsulfate, urea or guanidine - HCl by dissolving the protein in the denaturing solution and then dialyzing it against the same solution.

For transnitrosation experiments, nitrosated lysozyme was prepared by incubating it with sodium nitrite and then separating the nitrosated protein from the reaction mixture by chromatography on Sephadex G-15. The nitrosated protein was incubated with myoglobin. The reaction mixture was then heated at 75°C for 30 minutes, cooled in ice, extracted with acetone and absorbance was measured at 540 nm (essentially the procedure of Hornsey, 1956).

Results and Discussions

Numerous attempts were made to nitrosate myosin and measure the production of nitrosated tryptophyl residues by increased absorbance at 330 nm. Unequivocal evidence was not obtained with either native or denatured myosin (1.3 to 2.0 mg/ml) at pH 6.0 and a nitrite concentration of 10 mM (see Figure 1). At pH 3.0 increased absorption at 330 nm was obtained but it was not clearly evident as a peak. When nitrite concentration was

increased to 0.1 M, then obvious changes in the protein occurred (Figure 2). A new peak at 353.3 - 355 nm appeared and it was probably due to nitrosation of tyrosyl residues (Kurosky and Hofmann, 1972).

Lysozyme was studied because, in contrast to myosin, it is soluble at low pH and has a much higher content of tryptophan. Clear evidence for nitrosation of tryptophyl residues in lysozyme was obtained as detected by appearance of a characteristic peak at 330 nm (Figure 3). The reaction was pH dependent, increasing as the pH decreased in the range 6 to 3, and as shown in Figure 3, heating also promoted the reaction.

It was found that nitrosated lysozyme, separated from the reaction mixture, decomposed spontaneously as measured by decreasing absorption at 330 nm over a 24 hour period. The decomposition was pH dependent with a loss of about 60% of the original 330 nm absorption occurring in 24 hours at pH 2.8. The loss was less at higher pH. We determined that the protein fraction, following gel filtration, contained bound nitrite which could be transferred to myoglobin.

Our work was undertaken because the nitrite reacted with or bound to non-heme proteins in cured meats represents unknown and non-quantitated compounds, and also may represent a pool of nitrosating ability. Both of these factors have implications in regard to cured meat and therefore information must be obtained on them before the question regarding continued use of nitrite is resolved.

Proteins have numerous sites which may react with nitrite provided conditions are appropriate. We studied nitrosation of the tryptophyl residue (reaction at the indolic nitrogen); with myosin, we did not observe nitrosation to take place unless extreme conditions were employed but with lysozyme clear evidence for nitrosation was obtained, at least with the spectrophotometric methods used. The chemistry of transnitrosation is known (Challis and Osborne, 1973; Buglass et al., 1975), and our preliminary results in a simple system of nitrosated lysozyme and myoglobin confirmed it can occur. The results must be extended to meat systems before the significance can be assessed.

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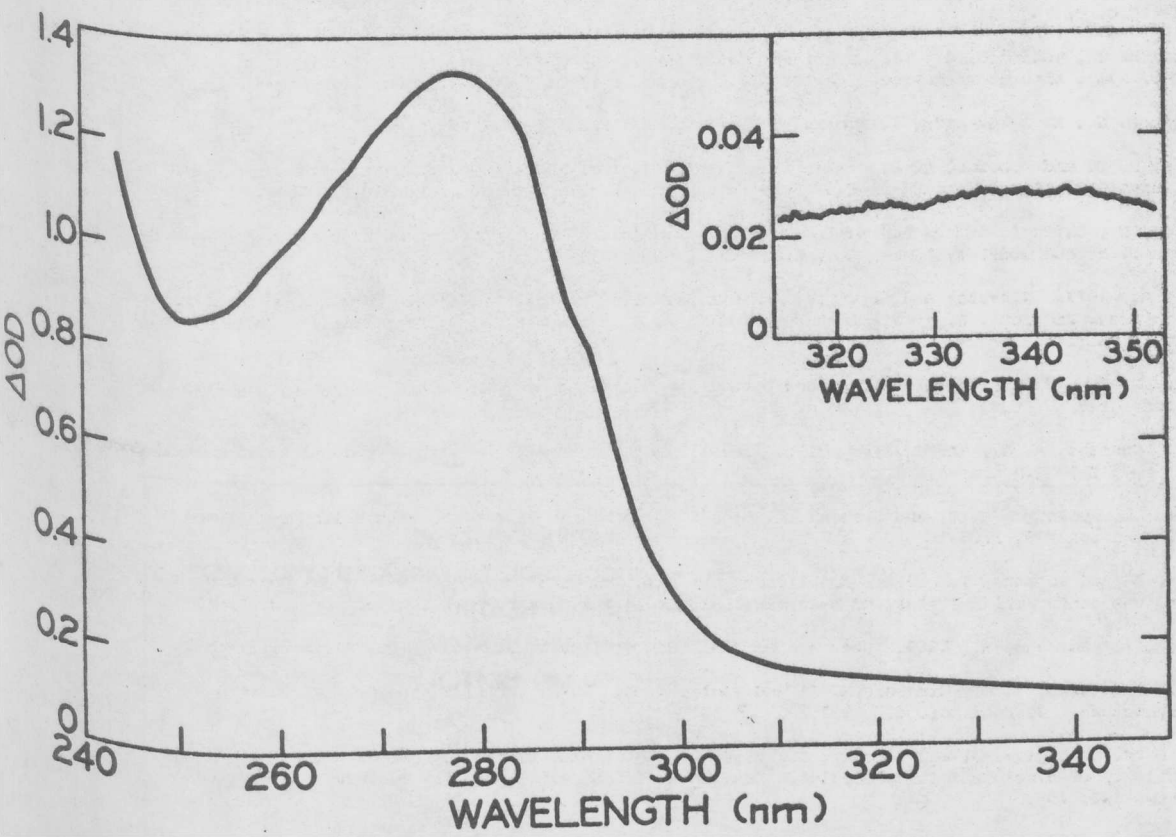
List of Figures

Figure 1. Difference spectrum of reaction mixture containing myosin and sodium nitrite versus dialyzing solution. The reaction of myosin (2mg/ml) with sodium nitrite (10mM) was made in 0.5M NaCl, 10mM phosphate (pH 6.0) by dialyzing the reaction mixture against 100 vol of 0.5M sodium chloride, 10mM $NaNO_2$ and 10mM phosphate (pH 6.0) at 23 ± 2°C for 24 hr. The inset shows a difference spectrum of the reaction mixture between myosin and sodium nitrite by using a double cell system.

Figure 2. Difference spectrum of reaction mixture containing myosin and high concentration of sodium nitrite versus dialyzing solution in the presence of SDS. The reaction of myosin (1.5mg/ml) with sodium nitrite (0.1M) was made in 1% SDS and 20mM phosphate - 10mM citric acid (pH 6.0) at 23 ± 2°C for 24 hr. by dialyzing the reaction mixture against 100 vol. of 0.1M sodium nitrite, 1% SDS and 20mM phosphate -- 10mM citrate (pH 6.0).

Figure 3. Time-dependent effect of heating on reaction of lysozyme with sodium nitrite at pH 3. The reaction of lysozyme (0.37mg/ml) with sodium nitrite (10mM) was made in 0.2M phosphate-0.1M citric acid (pH 3) at 70°C by incubating the reaction mixture in a water-bath for 5, 30 or 60 minutes. After heating, the mixtures were cooled in ice and then let stand at 23 ± 2°C until measurement. Difference spectra of the mixtures were obtained 3 hr after the mixing of lysozyme and sodium nitrite.



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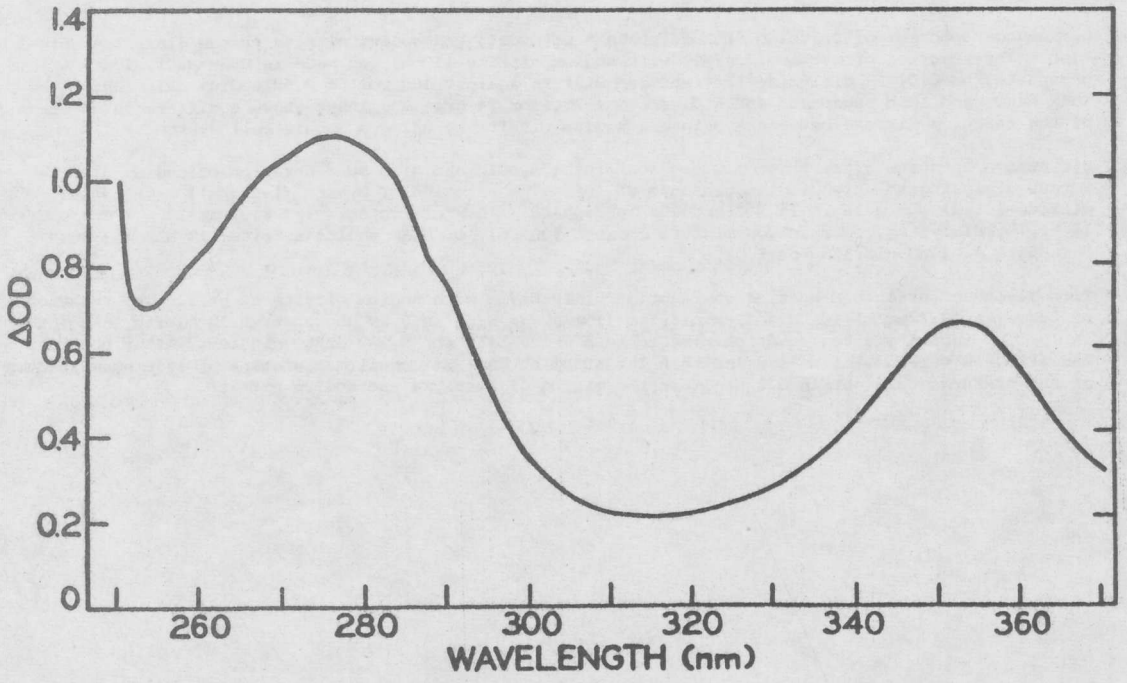
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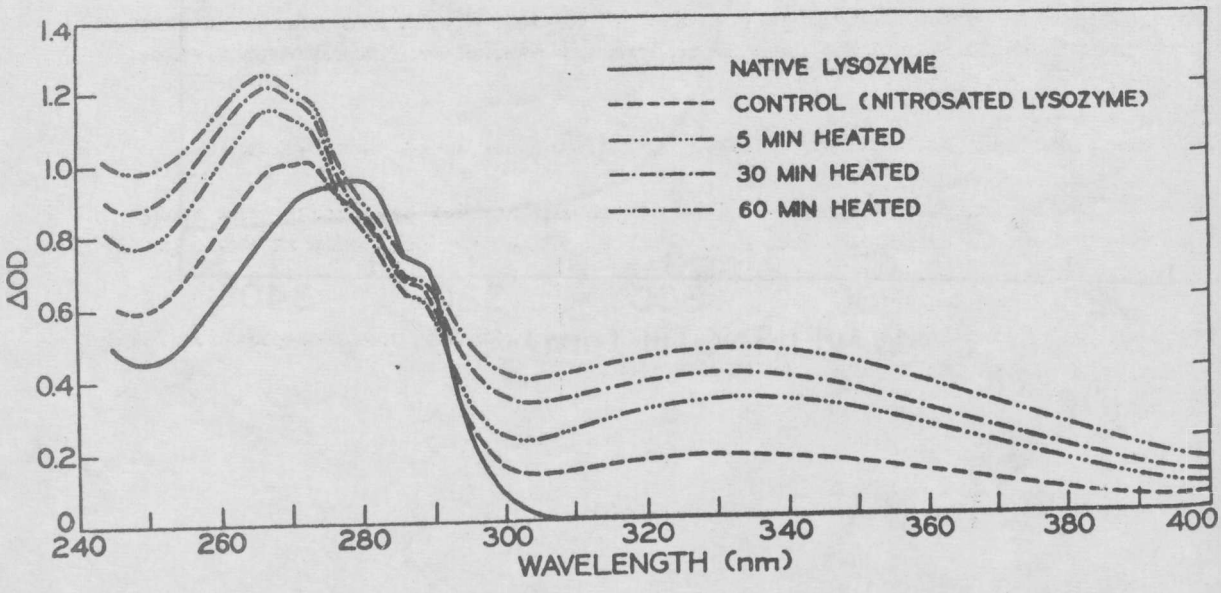
NATIVE LYSOZYME
CONTROL (NITROSATED LYSOZYME)
5 MIN HEATED
30 MIN HEATED
60 MIN HEATED
1.4
1.2
1.0
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0.6
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0.2
0
ΔOD
240
260
280
300
320
340
360
380
400
WAVELENGTH (nm)