simpler spectrum of diallylnitrosamine, it is possible to assign most of the resonances.

The two quartets centered at τ 7.60 and 7.95 correspond to syn and anti forms of the β-methylene group in the butenyl side chain of BPN. Two triplets centered at τ 6.62 and 5.95 are assigned to the syn and anti conformations of the α-methylene group in the butenyl side chain. The latter triplet is overlapping with the resonance of the *syn*-α-methylene of the propylene side chain. The resonance of the corresponding *anti*-α-methylene occurs at lower fields, giving a doublet centering at τ 5.40. The vinylic hydrogens in both groups resonate between τ 3.9 and 5.2.

The yields of BPN from the hydrochlorides of spermidine and spermine were estimated to be 1.7 and 1.4%, respectively, using diallylnitrosamine as an internal standard. The yield of BPN from free spermidine was 2.4%.

In order to substantiate the formation of BPN from spermidine and not from a γ-butenyl-(β-propenyl)amine (BPA) impurity in the spermidine, the following experiment was performed. The purity of free spermidine was examined by gc, with special attention to the possible occurrence of BPA. Since one of the impurities in spermidine cochromatographed with BPA, the effect of the concentration of this impurity on the yield of BPN was determined. Spermidine was purified by vacuum distillation (bp 94° (2 mm)), and the concentration of the impurity was reduced from 370 to 40 ppm. The yield of BPN from the purified spermidine, however, remained the same as before the vacuum distillation, confirming that BPN was a genuine product formed from spermidine.

The mechanism we propose for the formation of BPN from spermidine and nitrite assumes the reaction at the secondary amine function will stop at the nitrosamine stage (Figure 6). Nitrosation of the primary amines produces unstable diazonium ions, which degrade to carbonium ions (Ridd, 1961). Elimination of protons from both carbonium ions produces BPN. The observation that BPN was formed from the reaction between spermine and nitrite was puzzling. As tested by thin-layer chromatography, no spermidine was found in the spermidine·4HCl. Heat stability tests of spermine showed no significant conversion to spermidine under the reaction conditions. Due to lack of information, no mechanism can be suggested for the formation of BPN from spermine at this time.

Besides elimination, nucleophilic additions or rearrangements also may occur at the carbonium ion with the possibility of forming a multiplicity of different nitrosamines upon nitrosation of spermidine and spermine. Experiments are currently underway to determine other nitrosation products of spermidine and spermine.

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Reduced Binding of Nitric Oxide in Irradiated Horse Heart Myoglobin

Pushpa Paul and Umesh S. Kumta*

The ligand binding of nitric oxide with a 0.05 m*M* concentration of horse heart myoglobin has been studied as a function of radiation dose. At 100 krads, the nitrosylmyoglobin (NOMb) peaks at 575 and 545 nm decreased by about 50% as against 20% at 410 nm. Formation of NOMb could not be detected at higher radiation doses. Evidence for conformational changes in the apoglobin moiety caused by irradiation has been presented in terms of increased susceptibility to tryptic hydrolysis and changes in polyacrylamide gel electrophoretic pattern.

The kinetics of ligand binding of nitric oxide with myoglobin has been extensively studied to explain the reaction mechanisms associated with the formation of nitrosylmyoglobin (NOMb), the major pigment of cured meats (Fox, 1966; Fox and Thomson, 1963; Watts and Lehmann, 1952). Some of the processing treatments (Brown and Mebine, 1969; Kraft and Ayres, 1954; Brown and Dolev, 1963) including γ-radiation (Ginger *et al.*, 1955; Tappel, 1958) are known to cause discoloration of meat. Most of the studies on radiation damage to myoglobin relate to the mechanisms of interconversions of met and oxy forms (Tappel, 1950; Satterlee *et al.*, 1971; Madhavan *et al.*, 1973) and loss in spectral characteristics (Ginger *et al.*, 1955; Ginger and Schweigert, 1956; Brown and Akoyunoglou, 1964; Madhavan and Kumta, 1971; Paul *et al.*, 1973). Besides oxidative changes caused by γ-radiation in the protoporphyrin nucleus, in an aqueous system, the apoglobin moiety undergoes initial unfolding followed by aggregation (Brown and Akoyunoglou, 1964; Satterlee *et*

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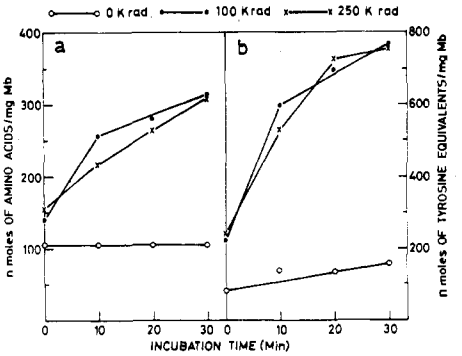

Figure 1. Susceptibility of irradiated myoglobin to trypsin hydrolysis. Tryptic digestion was carried out using 0.05 m*M* aqueous horse heart myoglobin (Nutritional Biochemical Corporation) solution as described in the Experimental Section. Amino acids and peptides released were estimated by the methods of (a) Moore and Stein (1954) and (b) Miller (1959).

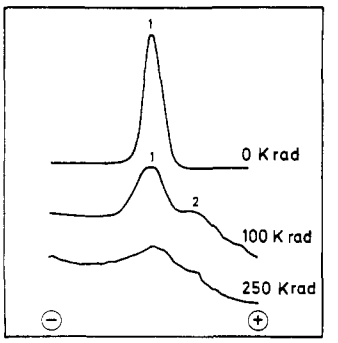

Figure 2. Chromoscanning of polyacrylamide gel electrophoretic bands. Myoglobin solution (horse heart, Nutritional Biochemical Corporation) of 0.05 m*M* concentration was irradiated at 100 and 250 krads doses and 0.02 ml was applied on the gel, unirradiated myoglobin serving as the control.

al., 1971; Satterlee *et al.*, 1972; Paul and Kumta, 1973a). The latter reaction was found to be dose dependent at a 0.5 *M* concentration of myoglobin (Brown and Akoyunoglou, 1964; Paul and Kumta, 1973a). Apparently, two opposite effects could be expected regarding the attachment of the ligands to the protoporphyrin nucleus: the unfolded state may offer steric hindrance or provide increased accessibility to the ligands. The reaction system described by Fox and Thomson (1963) using a 0.05 m*M* concentration of myoglobin is ideal because this concentration does not become rate limiting for nitrosylation reaction and yet it permits evaluation of nitrite binding under conditions where radiation-induced structural alterations of myoglobin become evident without the formation of aggregates.

Lycometros and Brown (1973) have recently reported that irradiated sperm whale myoglobin showed increased accessibility to alkyl isocyanides. The present paper is concerned with the capacity of an irradiated aqueous solution of horse heart myoglobin (0.05 m*M*) to bind nitric oxide.

EXPERIMENTAL SECTION

Materials. Pure horse heart myoglobin was obtained from Nutritional Biochemical Corporation. Ascorbic acid and other reagents were of Analar grade.

Irradiation was carried out under aerobic conditions using a gamma cell-220 (Atomic Energy Canada, Ltd.) at a dose rate of 120 krads/hr.

Preparation of Reactants. *Myoglobin Solution.* A horse heart myoglobin solution of 0.05 m*M* concentration was prepared in distilled water. This concentration was chosen to avoid radiation-induced aggregation occurring at higher concentrations. Myoglobin was in the met form as evidenced from the absorption peaks at 630, 500, and 410 nm.

Nitrite Solution. Sodium nitrite solutions of concentrations varying from 5 to 100 m*M* were prepared in acetate and phosphate buffers to obtain the pH range of 4.5–7.0. These buffers were specifically chosen because they did not show any color reactions with the myoglobin.

Ascorbic Acid. Ascorbic acid solutions of 5–100 m*M* concentrations were prepared fresh, using acetate and phosphate buffers to obtain the pH range of 4.5–7.0.

Preparation of Nitrosylmyoglobin Derivative. Nitrosylmyoglobin was prepared according to the method of Fox and Thomson (1963) and Reith and Szakaly (1967).

The formation of nitrosylmyoglobin was tested with different concentrations of reactants, *viz.* ascorbic acid, 5–100 m*M*, nitrite, 5–100 m*M*, and myoglobin, 0.05 m*M*. The rates of conversion of metmyoglobin to nitrosylmyoglobin were tested over a pH range of 4.5–7.0, using acetate and phosphate buffers. All the experiments were carried out at 20–25°. The stability of the derivative was tested spectrophotometrically under two conditions, viz. (i) ascorbic acid was added to metmyoglobin solution, followed by nitrite, and (ii) nitrite was added to the myoglobin solution prior to ascorbic acid.

Absorption spectra for myoglobin solution and NOMb were determined between 400 and 700 nm using a Beckman DK recording spectrophotometer.

Tryptic Hydrolysis. Pure myoglobin or irradiated myoglobin solutions were used for trypsin digestion by the method described earlier (Paul and Kumta, 1973a). The amino acids and peptides released were estimated by the methods of Miller (1959) and Moore and Stein (1954).

Polyacrylamide Gel Electrophoresis. The method described by Davis (1964) was used for preparing 7.5% polyacrylamide gel in 0.01 *M* Tris–glycine buffer (pH 8.3). A suitable aliquot containing approximately 20 μg of protein, of either the unirradiated myoglobin or the irradiated sample, was applied to the gel. A constant current of 5 mA/tube was maintained throughout the 1-hr run. The gels were stained with 1% Amido Black in glacial acetic acid and the destaining was carried out by using 7% acetic acid solution. A Joyce chromoscan (Joyce-Loebl and Company Ltd., England) equipped with an interference filter with a maximum transmittance at 620 nm was used to make tracings of the polyacrylamide gel pattern.

RESULTS

Alterations in the Apoglobin Moiety. The degree of unfolding in irradiated myoglobin can be indirectly measured by the susceptibility of the protein to tryptic hydrolysis. It can be seen from Figure 1 that the extent of hydrolysis of horse heart myoglobin subjected to 100 krads immediately on addition of trypsin was much more enhanced as compared to that for the native myoglobin and the rate of hydrolysis progressively increased linearly thereafter as a function of time.

Figure 2 shows the tracings of polyacrylamide gels which provide evidence for the formation of an additional band in irradiated myoglobin (100 krads), other than the band corresponding to native myoglobin. At 250 krads, a broad and diffused band was noticed.

Spectral Changes in the Irradiated Myoglobin Solution. Radiation-induced changes in the protoporphyrin nucleus by γ-radiation can be seen from the decrease in the absorbances in the major peaks at 410, 500, and 630 nm. These results are given in Figure 3. The intensity of the Soret peak at 410 nm showed gradual decrease as a function of radiation dose, the loss being 20, 40, and 90–100% at 100, 250, and 500 krads, respectively (Figure 3). The decrease in the intensity of the major peaks at 500 and 630 nm at 100 krads was on the order of 13 and 19%,

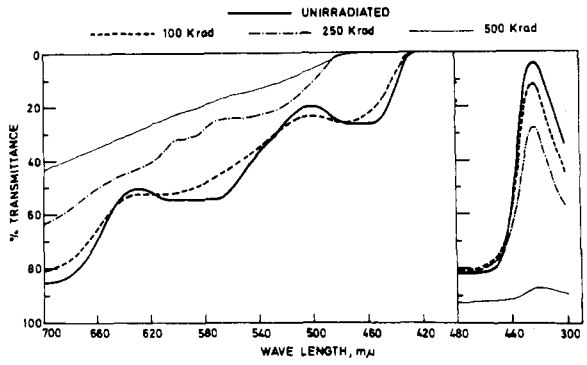

Figure 3. Visible spectra of irradiated horse heart myoglobin solutions.

Table I. Effect of Varying the Concentrations of Reactants[a] on the Formation of Nitrosylmyoglobin (NOMb)

Concn of reactants, m*M*	Color	Time taken for NOMb formation, min	Stability of NOMb, hr	Absorption maxima
5	Faint pink	60 (incomplete)		630, 575, 540, 500 (sh)
15	Pale pink	60	3	630, 575, 545, 500 (sh)
25	Pink	45	6–8	-, 575, 545, 500 (sh)
50	Bright red	10	>24	-, 575, 545, -

[a] Nitrite and ascorbic acid.

respectively, and the total absence of these peaks was noticed at doses of 250 and 500 krads (Figure 3).

Formation of NOMb. It was observed that the formation of NOMb was complete only when the reductant was added after the addition of nitrite. The maximum formation of NOMb was observed when the two reactants (nitrite and ascorbic acid) were used at a concentration of 50 m*M* (Table I) and at pH 5.5 (Figure 4). The NOMb thus formed was stable up to 24 hr as evidenced by the characteristic peaks at 575 and 545 nm. Hence the optimum conditions of 50 m*M* concentration and pH 5.5 of nitrite and ascorbic acid solution were chosen for the preparation of the NOMb derivative in all other experiments.

A decrease in the ability of irradiated myoglobin to form NOMb is shown in Figure 5. A myoglobin solution subjected to 100 krads showed that the peaks corresponding to NOMb (575 and 545 nm) decreased by 50%, while at higher doses (250 krads) formation of NOMb could not be detected. These results, in contrast to those observed with respect to a decrease in the Soret peak (Figure 3) at 100 and 250 krads, thus demonstrate that ligand binding with nitric oxide provides a more sensitive probe for the assessment of damage to the protoporphyrin nucleus.

The yield of NOMb in terms of the absorbances at 575 and 545 nm was examined using varying concentrations of nitrite. These results are incorporated in Figure 6. It can be seen that the absorbancy corresponding to a maximum yield of NOMb at 575 or 545 nm occurs at a 50 m*M* concentration of nitrite, whereas in myoglobin subjected to 100 krads no additional NOMb was formed at concentrations above 30 m*M* nitrite. The yield of NOMb at 30 m*M* nitrite concentration in unirradiated myoglobin was almost twice that in the irradiated myoglobin. If the formation of NOMb with characteristic peaks at 575 and 545 nm is attributed to the presence of native myoglobin molecules surviving after irradiation (Figure 5), the decreased yield of NOMb on irradiation at 100 krads is suggestive of the reduced binding capacity of the molecules damaged by radiation.

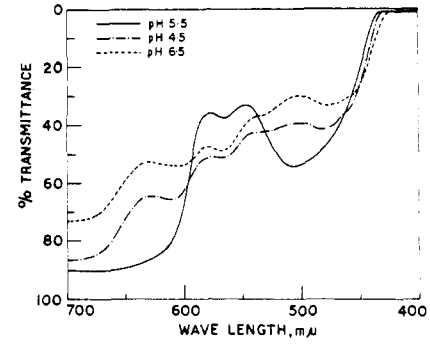

Figure 4. Influence of pH on the formation of NOMb. Kinetics of NOMb was examined at different pH levels (6.5, 5.5, and 4.5) of the reactants. Absorption spectra were determined between 700 and 400 nm, in a Beckman DK recording spectrophotometer.

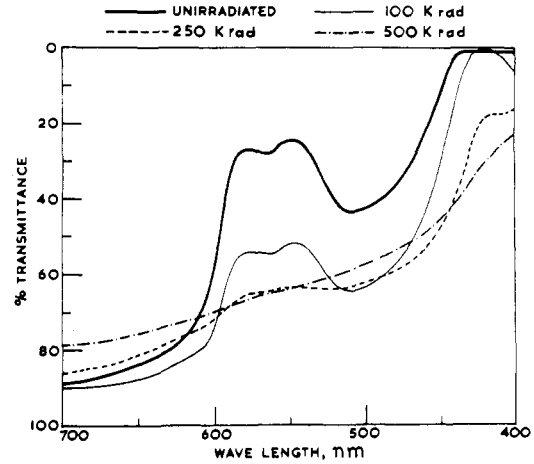

Figure 5. Effect of irradiation on the formation of NOMb. NOMb was prepared from irradiated myoglobin solutions under optimum conditions described in the Experimental Section. Absorption spectra were recorded between 700 and 400 nm in a Beckman DK spectrophotometer.

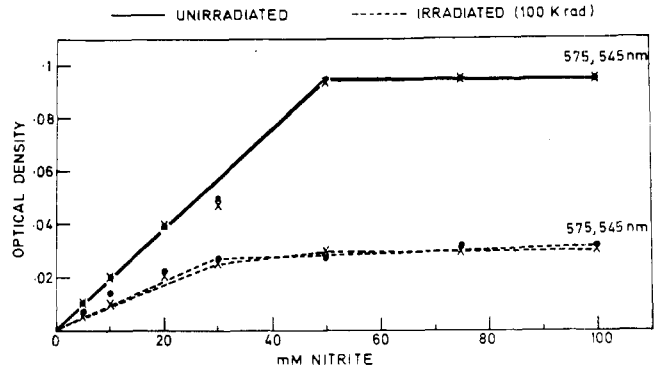

Figure 6. Yield of NOMb with various concentrations of nitrite. The optical density at 575 or 545 nm corresponding to NOMb yield was plotted against varying concentrations of nitrite.

DISCUSSION

It is apparent from the foregoing studies that the apoglobin moiety and the heme prosthetic group form the two major sites susceptible to radiation damage in myoglobin. Irradiation at 100 krads brings about structural changes in protein resulting in the formation of an additional peak as observed on gel electrophoresis. As the radiation dose is increased to 250 krads the two electrophoretic bands prob-

ably merge together to form a diffused band indicative of the polymer formation. Another important structural alteration evident from the susceptibility to tryptic hydrolysis appears to be the degree of unfolding of myoglobin subjected to 100 and 250 krads. However, the extent to which the alterations in the apoglobin moiety may influence the properties of ligand binding of the heme moiety has not been clearly understood. It has been reported (Lein and Pauling, 1956) that in hemoglobin and myoglobin the affinity of the heme prosthetic group for alkyl isocyanides decreased as the bulkiness of the R group increased, suggestive of steric hindrance offered by the protein moiety. Recently, using sperm whale myoglobin, Lycometros and Brown (1973) demonstrated that radiation-induced alterations in the protein moiety, such as "dimer" formation, provide enhanced accessibility of the heme iron to the binding of alkyl isocyanides.

Since ligands such as NO_2, NO, CO, and CN have been shown to protect myoglobin from radiation-induced polymerization (Lycometros and Brown, 1973) it is important to determine whether conformational changes in myoglobin *per se* influence the liganding of nitric oxide to irradiated myoglobin. Our studies on post-irradiation addition of nitrite and ascorbic acid at pH 5.5, which provide optimum conditions for nitric oxide binding, show that irradiation markedly decreases the capacity of horse heart myoglobin to bind nitric oxide (Figure 5). This effect noted consistently with horse heart myoglobin was later ascertained with sperm whale myoglobin (unpublished data). If the damage to the protoporphyrin nucleus, as evidenced from the decrease in the absorbances at 410, 500, and 630 nm at 100 krads analyzes to about 20%, it may be expected that a decrease in NOMb formation should correspondingly decrease commensurate with the damage to the main ligand binding site. The fact that NOMb formation is decreased considerably more is suggestive of other factors which retard the kinetics of nitrosylation reaction. Nitric oxide binding capacity as revealed from the relationship between nitrite concentration and NOMb yield (Figure 6) clearly shows that nitrite concentration was not the limiting factor in the formation of NOMb for irradiated (100 krads) horse heart myoglobin. In fact, maximum NOMb formation occurred with only 30 m*M* nitrite. The nature of the curves for interaction of unirradiated and irradiated myoglobin with nitric oxide is also different as seen from their slopes.

We suggest that in addition to impairment to the protoporphyrin nucleus, conformational changes as revealed from the unfolded state of the myoglobin molecule (Figure 1) and formation of additional species (Figure 2) probably interfere in the nitrosylation reaction. This could be due to the increased steric hindrance caused by unfolding or decreased ability of the radiation-induced microheterogenous species to form NOMb. The occurrence of two major fractions, LMI and LMII, from lamb meat myoglobin differing markedly in their binding to nitric oxide has been reported previously (Paul and Kumta, 1973b). It cannot be generalized, however, that irradiation may reduce binding of other ligands, especially since it has been shown by Lycometros and Brown (1973) that under conditions which form dimer and polymer in sperm whale myoglobin, binding of alkyl isocyanides is indeed increased. These two diverse observations in ligand binding in irradiated myoglobin may probably be related to the nature of the ligands. A knowledge of myoglobin species formed on irradiation and their participation in binding of various ligands may help in elucidating the mechanisms of reactions.

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