The Formation of Green Heme Pigments from Metmyoglobin and Methemoglobin by the Action of Nitrite*

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Received April 6, 1964

A study of the kinetics of formation of the green pigments produced from either metmyoglobin or methemoglobin by the action of nitrite shows the reaction to be bimolecular, involving one molecule of heme pigment and one molecule of undissociated nitrous acid. The reaction is seventeen times as fast with hemoglobin as with myoglobin. A study of the spectrochemical behavior of the green pigments, as well as of the one hemin isolated from the modified heme-globin complexes, indicates that the reaction is a nitrosylation of the heme, probably at the α-methene bridge. An improved technique for the purification of hemins is described. Observations of the state of polymerization of the hemins have been made which indicate that the hemins as isolated are probably monomers, or at most dimers. The implications in the formation of these green heme pigments in meat curing and nitrite toxicology are discussed briefly.

In a previous report from this laboratory (Fox and Thomson, 1963), it was reported that in the presence of high concentrations of nitrite at low *p*H values, metmyoglobin and methemoglobin were converted to green pigments. Similar conditions have been reported by Deibel and Evans (1957) to occur in the condition known as "nitrite burn" in fermented sausages. "Nitrite burn" has always been associated with eventual loss of heme pigment color, and the assumption has been made that the process is oxidative, either initiated or caused by nitrite, with the production of cleaved porphyrin-ring compounds of the choleglobin type (Greenwood *et al.*, 1939). However, the work on "nitrite burn" has been done in whole or comminuted means where identification of specific compounds is impossible.

Green heme pigments as the result of the action of nitrite on hemoglobin have been observed (Brooks, 1938; Havemann, 1941; Bechtold, 1942; Marshall and Marshall, 1945), but of all these only Bechtold described the green pigment as a distinct entity. He did some work on the characteristics of the green heme pigment, but nothing on the mechanism of formation. Because of the possible relation between these green pigments and "nitrite burn," we have extended the studies to include the mechanism of formation, further chemical characterization of the heme-globin products, and the hemin derived therefrom.

Terminology.—There are two methods of naming the nitrite-green heme pigments; the first is a general terminology indicating origin or reactant and the second indicates the structure of the product. The second method of nomenclature is the more desirable, but not in the present case. Although the authors believe the green heme pigments to be nitroso-derivatives, this belief has not yet been substantiated. Second, the prefix "nitroso-" is still widely used for the nitric oxide–ferro(i) heme coordinate covalent complexes, even though the preferred I.U.P.A.C. terminology for these complexes uses the prefix "nitrosyl-." For these reasons, the authors propose an origin terminology, using "nitri-" as the prefix, as is done with the green sulfur heme derivatives, for example, sulfmetmyoglobin, sulfhemoglobin. Thus for the nitrite derivatives, we have nitrimethemoglobin for the green ferric heme–native globin complex of the blood pigment, nitrimyoglobin for the reduced green muscle pigment, and nitrihemin for the heme cleaved from the native globin pigments by acid acetone.

MATERIALS AND METHODS

The preparation of bovine myoglobin and hemoglobin has been described previously (Fox and Thomson, 1963), as well as the spectrophotometric techniques and apparatus used to follow the reaction.

Kinetics.—The course of the reaction between nitrite and the heme pigments was followed by the change in optical absorption at 615 mμ and 611 mμ when using myoglobin and hemoglobin, respectively, as substrates. The change was followed by means of time scan at constant wavelength in a Beckman DK recording spectrophotometer.

Preparation of Protohemin.—Several methods of removing the heme from the globin were used. A perusal of the literature has disclosed over a dozen different published methods of preparing and purifying hemes from heme-globin complexes. All the methods can be categorized according to the method of initial cleavage: (1) the hot glacial acetic acid method first reported by Nencki and Zaleski (1900); (2) the hydrochloric acid–organic solvent method (Hill and Holden, 1926; Morrison and Stotz, 1955; Lewis, 1954); and (3) the organic base–chloroform method (Caughey and York, 1962; Banerjee, 1962). In our study, the hemes were cleaved from the globin by acid-acetone as described by Lewis (1954). A typical preparative procedure for protohemin was as follows: Myoglobin or hemoglobin was precipitated by addition of acetone at *p*H values between 4.5 and 7.0, that is, high enough to avoid cleavage (cf. Lewis, 1954), decanting and discarding the supernatant. The precipitate was washed twice with acetone. After the last washing, the precipitate was obtained in a small volume by careful decantation. The precipitate could not be dried, for neither the myoglobin nor the hemoglobin dry powder would cleave in subsequent steps. Two volumes of acetone were added to one volume of the precipitate

* This work was supported in part by a grant (A 3700) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health. Part of this paper was presented at the Research Reports Meeting of the American Meat Institute Foundation, March, 1963, and has been assigned Journal Paper number 272 of the American Meat Institute Foundation.

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TABLE I

ABSORPTION MAXIMA AND COEFFICIENTS FOR THE SEVERAL NITRITE-GREEN HEME PIGMENTS[a]

Compound	Soret				Visible					
	λ	ε	λ	ε	λ	ε	λ	ε	λ	ε
Metmyoglobin	407	73.5			505	9.7			635	3.6
Metmyoglobin nitrite										
100:1									*615*[b]	*3.0*
300:1									*615*[b]	*3.7*
*p*H 4.5										
Nitrimetmyoglobin	385	66.0					568	11.0	615	10.9
Nitrimethemoglobin[c]	380	64.9					515–565	10.9	610	10.8
Nitrimyoglobin							557	11.6	619	15.2
Nitrihemoglobin							551	11.4	615	14.9
*p*H 4.0										
Nitrimetmyoglobin	359	21.6			*500*	*10.5*			*600*	*6.5*
Protohemin IX	388	96.9			512	9.5	543	9.4	642	4.5
Nitrihemin	368	58.0	435	50.5			562	7.9	660 (s)[d]	4.6

[a] λ_{max} in mμ and ϵ_{max} in mM^{-1}/cm. [b] *615* = no inflection; given for reference only. [c] For purposes of comparison, the absorption coefficients in this table are per mole of heme. The correct coefficient per mole of hemoglobin will be four times as great. [d] (s) = shoulder.

and the hemes were cleaved by addition of 1 ml concentrated hydrochloric acid per 25 ml of suspension. The cleaved protein was allowed to settle and the supernatant was decanted. The last amounts of supernatant were recovered by centrifugation. To 50 ml of supernatant were added 15 ml of chloroform and 100 ml of 0.5 N HCl in 1:1 MeOH–H_2O. The hemin concentrated in the chloroform layer. The chloroform solution of hemin was washed with the acid-methanol-water solution until the absorption spectrum met the criteria to be described later.

To precipitate the hemin from the chloroform-methanol-HCl phase, petroleum ether was added until the solution became turbid. Protohemin was precipitated by centrifugation. The precipitate was dried in air or *in vacuo*, but was not heated, as the hemins tended to polymerize above room temperature.

On occasion during this study, protohemin was prepared from ox blood and it was found that upon final precipitation of the hemin an oily residue was obtained, rather than a dry hemin powder. It was suspected that some lipid material had survived the acetone washing of the precipitated protein, or was cleaved from some complex during the acid cleavage. To rid the heme solution of the suspected lipid material, an equal volume of methanol and about 0.25 volume of water were added to the acid-chloroform solution of the heme, and excess petroleum ether was added until two phases were formed. The lower methanol-water phase containing the heme was washed at least twice with petroleum ether. The hemin was then transferred back into chloroform-methanol-HCl by first adding an equal volume of chloroform to the methanol-water hemin solution and then adding excess acid methanol-water until two phases were formed.

Isolation of Nitrihemin.—The procedure for the isolation of nitrihemin was essentially the same as for protohemin, except that it was necessary to add 0.1 volume of methanol to the acetone and acid-acetone used for precipitating and cleaving the heme-globin complexes. If this was not done, the nitrihemin isolated had an absorption spectrum which indicated that the hemin had polymerized. Apparently this problem was restricted to nitrihemin, for whether or not methanol was added in the preliminary steps of protohemin isolation made no difference in the absorption spectrum of purified protohemin.

Preparation of Porphyrins.—The iron content was removed from the hemins by the dry HCl–methanol–oxalic acid–ferrous sulfate method of Grinstein (1947). It was found satisfactory in our study to mix the last three reagents together, dissolve the hemes in the mixture, and add dry HCl. The mixture has kept indefinitely in the laboratory.

Instrumentation.—Infrared spectra of the hemins were taken in a Perkin-Elmer 421 spectrometer and the fluorescent spectra of the porphyrins were read in an Aminco-Bowman recording spectrophotofluorometer.

RESULTS

Kinetics.—In order to determine the mechanics of a reaction, it is best to have the reactants in approximately equal molar ratios. In the present study, usable data could be obtained only with 100-fold-plus excesses of nitrite over heme. Under these conditions the reaction was found to be first order, that is, monomolecular, with respect to the heme pigment. The first-order rate constant calculated from the absorption change at 615 or 611 mμ was a linear function of the nitrite concentration, indicating that the reaction was also monomolecular with respect to nitrite, and that the complete reaction was bimolecular. The equations that describe the dependence of the first-order (pigment) rate constants on nitrite concentration at *p*H 4.5 are:

$$\text{myoglobin: } k_{1st} = (2.00 \pm 0.24)10^{-1} M^{-1}\,\text{min}^{-1} \times [NO_2^-]$$

$$\text{hemoglobin: } k_{1st} = (3.36 \pm 0.11) M^{-1}\,\text{min}^{-1} \times [NO_2^-] - 0.0055\,\text{min}^{-1}$$

where the concentration of nitrite is in terms of moles per liter. It will be observed that hemoglobin reacts about 17 times as fast as myoglobin.

Finally, the reaction involves only undissociated nitrous acid for, as seen in Figure 1, the reaction increases linearly with increasing hydrogen-ion concentration, or what is equivalent in this *p*H range, the undissociated nitrous acid concentration.

Spectral Characteristics.—The spectra of nitrimetmyoglobin and nitrimethemoglobin as formed at *p*H 4.5 and higher are shown in Figures 2 and 3. The absorption maxima and coefficients are given in Table I. The usual red shift in the absorption maxima of myoglobin as compared to those of hemoglobin is observed. Nitrimethemoglobin has a plateau from 520 to 550 mμ, where nitrimetmyoglobin shows a minimum at 545 mμ; otherwise the spectra are alike. If the normal pigments were reacted with nitrite at *p*H 4.0, or if the green pigments, formed at *p*H 4.5, were allowed to stand for long periods with nitrite, the featureless curve shown in

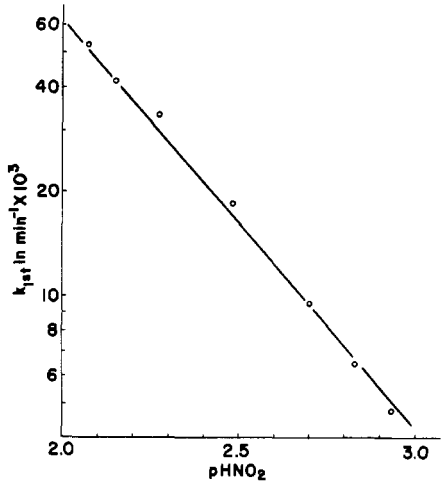

FIG. 1.—The first-order rate constants for the formation of nitrimetmyoglobin plotted against the negative log of the undissociated nitrous acid concentration. Both ordinate and abscissa are logarithmic.

Figure 2 was obtained. These latter compounds are called the "*p*H 4.0 nitrimethemoglobin" and "*p*H 4.0 nitrimetmyoglobin" pigments, for, as will be discussed later, they appear to have essentially the same modified porphyrin-ring structures as the *p*H 4.5 compounds, even though they do not have the characteristic absorption bands.

The kinetics of the formation of the *p*H 4.0 compounds could not be defined either at *p*H 4.0 or by prolonged exposure to nitrite at higher *p*H values. Their formation at low *p*H values probably proceeds through the formation of the *p*H 4.5 nitriheme pigments. This was indicated from the appearance of a low peak at 615 mμ during the reaction.

Spectrochemical Behavior.—Both *p*H 4.5 nitrimetmyoglobin and *p*H 4.5 nitrimethemoglobin were reduced by dithionite to form spectrally distinct compounds (Fig. 3, Table I). Since the spectrum of nitrihemoglobin was the same as that of nitrimyoglobin except for the usual blue shift, it is not shown. Neither cysteine nor ascorbate would effect the reduction indicated by the spectral changes in the *p*H 4.5 compounds, nor would any of these three reducing agents cause any change in the spectra of the *p*H 4.0 pigments. These observed spectral changes indicate that the heme had reacted. Since the solutions still had an excess of nitrite, which is very swiftly reduced by dithionite to nitric oxide, the absorption maxima of the nitrosyl complexes would have been observed if the heme had not been modified.

It was observed that not only was absorption in the red increased, but the wavelength of maximum absorption was shifted toward the red. Since these changes bear a resemblance to those observed upon reduction of sulfmetmyoglobin and sulfmethemoglobin, it is of interest to compare the pigments. The sulfur-containing ferric-heme pigments have low absorption maxima in the red, which are greatly increased by reduction to the ferrous state. When the reduced pigments are reacted with carbon monoxide the maxima shift 3–4 mμ toward the blue and may rise slightly. This shift in wavelength characterizes the formation carbon monoxide complex of the sulfheme compounds. Since the nitrimet-heme pigment solutions contained excess nitrite, reduced to nitric oxide upon the addition of dithionite, the reduced green pigments might have been nitrosylnitrimyoglobin and nitrosylnitrihemoglobin. To test this possibility, solutions of nitrimetmyoglobin and nitrimethemoglobin were dialyzed

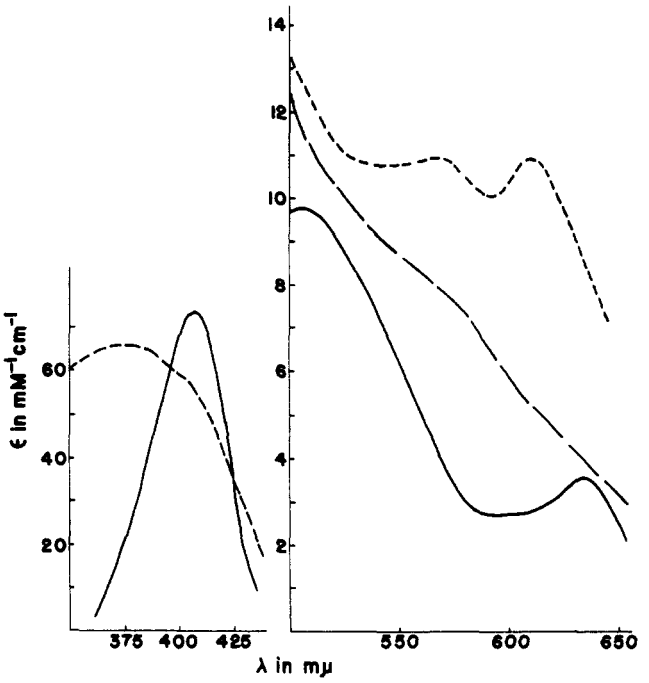

FIG. 2.—The spectra of *p*H 4.5 nitrimethemoglobin (- - - - -), methemoglobin (———), and *p*H 4.0 nitrimethemoglobin (— —).

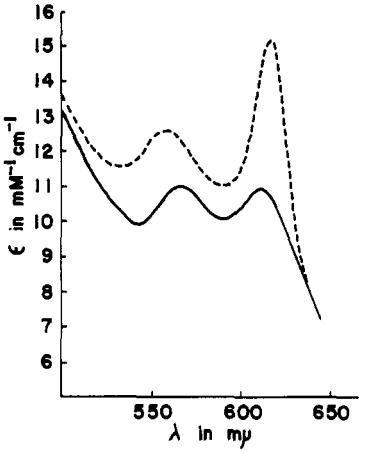

FIG. 3.—The spectra of nitrimetmyoglobin (———) and nitrimyoglobin (- - - - -). The latter was produced from the former by reduction with dithionite.

until the Griess test for nitrite (Official Methods of Analysis of the A.O.A.C., 1960) indicated that the concentration of residual nitrite was less than 0.1 the molar concentration of pigment. Reducing the dialyzed pigments with dithionite resulted in the typical reduced nitrimyoglobin and nitrihemoglobin spectra. Since the spectra both with and without nitric oxide were the same, it is tentatively assumed that the chemical modification involved both porphyrin ring and iron, thus interfering with the formation of other iron-ligand complexes.

Cleavage of the Hemins.—The hemes cleaved from both nitrimyoglobin and nitrihemoglobin were identical spectrally, confirming the view that the observed spectral difference between nitrimyoglobin and nitrihemoglobin was a function of the binding of the heme to the globin and did not reflect a difference in the structure of the nitrite-heme compound. The nitrihemin thus obtained was spectrally distinct from the protohemin IX obtained from metmyoglobin or methe-

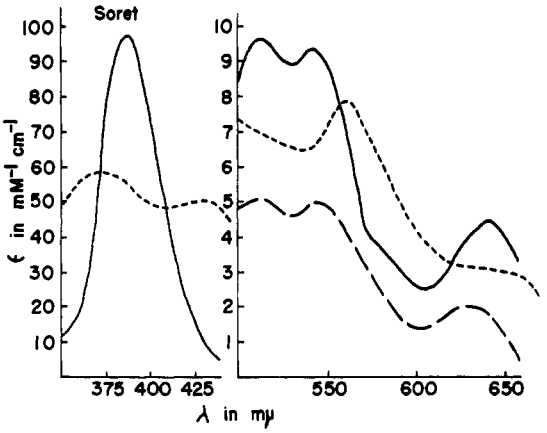

FIG. 4.—The spectra of protohemin IX (———), nitrihemin (- - - - -), and polymerized protohemin IX (— —). The hemins were in solution in chloroform, originally in equilibrium with 0.5 N HCl in 1:1 methanol-water.

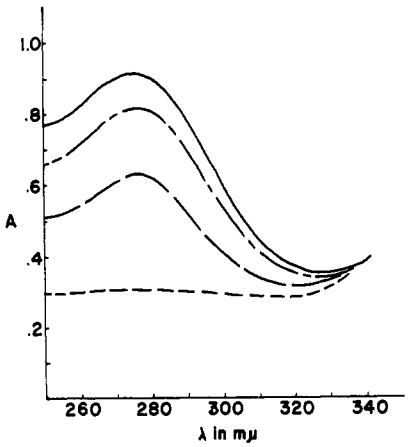

FIG. 5.—The effect of exhaustive washing with 0.5 N HCl in 1:1 methanol-water on the ultraviolet-absorption spectra of protohemin IX in chloroform. Freshly cleaved (———); 2 washings (— — —); 4 washings (— —); 8 washings (- - - - -).

moglobin, Figure 4, Table I. Although the *p*H 4.0 nitriheme-globin pigments were spectrally distinct from the *p*H 4.5 compounds, the nitrihemin obtained by cleavage was the same from both.

During the course of this study of nitrihemin and protohemin, some observations were made on the physical state of the heme that are pertinent to the identity of these pigments. These are discussed in the following paragraphs.

Purity.—The use of alcohol in the preparation of hemins has been the subject of observations made by many workers. Dhéré and Vegezzi (1916) first noted the solubilizing effect of ethyl alcohol on hemins and, subsequently, Hill and Holden (1926) made the claim that alcohol was a necessary adjunct in preparing hemins. Recently Connelley *et al.* (1958) noted that alcohol released a white pasty material from their hemin preparations. It seemed likely that the material was a protein or peptide residue. Upon spectral examination of crude preparations, it was found that hemin prepared either from blood or muscle extracts showed low absorption maxima at 276 mμ (Fig. 5). Maehly and Åkeson (1958) showed a protohemin spectrum which had a low peak between 270–280 mμ, which may be presumed to be a small peptide. They titrated this protohemin with alcohol and found an uptake of 2 moles of ethanol per mole of hemin. The conclusion from these findings then is that in purification procedures alcohol is replacing these residual ligands. Continuing these observations in this study, the solubility of the methanol complex was tested in various solvents, and it was found that the complex was soluble in chloroform. After testing several different solvent systems, it was found that washing the chloroform solution of heme with 0.5 N HCl in 1:1 water-methanol gave the most satisfactory results. The exact proportions were not critical, but lowering either the normality of the hydrochloric acid or the proportion of methanol caused the hemin to precipitate from the chloroform layer. Repeated washings of the heme with this system did result in the eventual removal of the 276-mμ absorption band (Fig. 5). The washings tested ninhydrin positive, indicating removal of protein or peptide material.

Examination of protohemins prepared by the hot glacial acetic acid and pyridine-chloroform methods showed some 276-mμ-absorbing and ninhydrin-positive material in the hemins prepared by the first method, but none in the hemins prepared by the second method.

Polymerization.—The question of polymerization also arises when considering the properties of hemins. The extent of aggregation of the hemin preparations of this study was investigated, using a valve-type synthetic-boundary cell in a Spinco Model E ultracentrifuge. Protohemin, freshly cleaved into acid-acetone, was found to aggregate to varying extents and over varying time periods. Since hemin absorbs in the visible range of wavelengths, it was possible to observe very slow polymerizations in the ultracentrifuge by following the decrease in optical absorption, even where no schlieren peaks appeared. The state of aggregation was highly polydisperse and some of the hemin in almost all samples studied failed to polymerize over centrifugation periods of up to 48 hours. On the other hand, methanol-chloroform preparations, washed free of 276-mμ absorbing or ninhydrin-positive material, showed no movement of the boundary for periods of up to 44 hours of centrifugation. There was no detectable aggregation of any of the hemin present, as densitometer tracings of the photographic plates showed no significant diminution of the optical absorption of the hemin solution below the boundary. Similarly, the protohemin prepared by pyridine-chloroform cleavage of metmyoglobin had no apparent impurities absorbing in the ultraviolet, and when centrifuged in the synthetic-boundary cell did not sediment, i.e., did not aggregate. It is apparent that the impurities present can have an effect on the physical state of the hemin, perhaps by providing a "bridge" between successive hemins.

Further evidence of polymerization of hemins was obtained by the study of commercial preparations and the insoluble residues from the ultracentrifugal studies. None of these hemin samples was soluble in acidic-methanolic-chloroform. If the suspensions in chloroform were made alkaline with ammonium hydroxide, a few crystals of sodium cyanide added, and the mixture allowed to stand for one-half hour or more, the hemin would go into chloroform solution upon reacidification. Since cyanide has been shown to depolymerize hematin (Hogness *et al.*, 1937; Shack and Clark, 1947), it was assumed that cyanide was depolymerizing a chloroform-insoluble hemin polymer.

Polymerization of Nitrihemin.—It was mentioned previously that the polymerization of acid protohemin was time dependent. This polymerization could be followed spectrophotometrically not only by the decrease in the Soret band absorption, but also by a general slurring of the distinctive hemin spectrum in the visible range (Fig. 4). The same spectrum was obtained with

nitrihemin prepared without methanol or ethanol and with nitrihemin preparations aged several days. From solubility and sedimentation characteristics, the nitrihemin in these preparations was found to be highly polymerized. It is concluded that the slurred spectrum is typical of polymerized hemins. The apparent ease with which nitrihemin polymerizes is strong presumptive evidence for a nitrosylation reaction between heme-globin complexes and nitrite, for King and Bisnette (1963) have shown that nitroso groups readily form such bridges in metallo-organic compounds. The function of methanol during the preparation of nitrihemin therefore would be the prevention of formation of such bridges.

In summary, from the ultracentrifugal experiments and the cyanide depolymerization experiments it is clear that the acid hemin soluble in methanolic chloroform was not a highly polymerized material. Some question remains as to whether the chloroform-soluble hemin is a monomer or dimer. It has been noted that the Soret peak is sensitive to the state of polymerization (Maehly and Åkeson, 1958; Blauer and Rottenberg, 1963). The Soret peak of the chloroform-soluble hemin has an absorption coefficient, $\epsilon_{388\ m\mu} = 96.9$, intermediate between that of the dialcoholic complex $\epsilon_{398\ m\mu} = 131$ and that of acid protohemin, *ca.* 80 (Maehly and Åkeson, 1958). Boeri *et al.* (1953) have suggested that chloride ions will form bridges between hemins. Since the chloroform-soluble hemin is likely an alcohol-chloride complex, it may exist in solution as a dimer, the monomers linked by chloride. Because of the solubility differences between the high and low polymers of the hemins, the chromatographic results to be presented are somewhat uncertain, although it is believed both hemins were in the dimeric form when put on the columns. It is the opinion of the authors that further assessment of the effect of polymerization is necessary before definitive conclusions may be drawn from hemin chromatographic studies.

Hemin Chromatography.—Protohemin IX and nitrihemin could be separated on silicic acid using the system described by Morrison and Stotz (1955). It was found necessary to add methanol (4 drops/25 ml of chloroform) to move the bands down the column. In this system, protohemin moved first with nitrihemin following. However, the nitrihemin band after elution did not have the specific absorption spectrum associated with the nitrihemin monomer, but had the diffuse protoheminlike spectrum associated with polymerized or decomposed nitrihemin. Further chromatographic studies on paper using the systems described by Connelly *et al.* (1958) were carried out, but the two hemins moved with essentially the same R_F values. Considering the instability of the nitrihemin and/or the likelihood that the nitrite modifies an integral part of the resonant system of the heme, thus masking any specific reactivity the modification might have, this lack of resolution is not surprising. Several modifications of the solvent systems were tried, as well as the use of modified cellulose papers, but no significant separations could be achieved.

The Porphyrins.—Removal of the iron from nitrihemin yielded protoporphyrin IX as determined by absorption and fluorescent spectroscopy. The method used to remove the iron evidently removed the nitrite modification, but it is evident that the porphyrin ring had not been cleaved by the reaction with nitrite.

Infrared Spectra.—No significant differences were noted in the infrared spectra of the two hemins. The conclusion is that the modification in nitrihemin is fully incorporated into the resonant structure of the hemin. This fits well with the conclusion that the nitrogenous modification is complexed to the iron in some way and the observation that nitrihemin chromatographs the same as protohemin.

TABLE II

THEORETICAL AND ANALYZED PERCENTAGES FOR IRON AND NITROGEN IN PROTOHEMIN AND NITRIHEMIN[a]

	Protohemin		Nitrihemin	
	Theoretical	Analyzed	Theoretical	Analyzed
Iron	8.17	8.41	7.84	7.45
		8.72		7.75
Nitrogen	8.20	7.51	9.84	9.47
		7.70		9.67

[a] Protohemin-Cl-methanol ($C_{36}H_{36}O_5N_4FeCl$), mw 683.97; nitrihemin-Cl-methanol ($C_{35}H_{35}O_6H_5FeCl$), mw 712.97.

Chemical Analysis of Protohemin and Nitrihemin.—Samples purified by the acid-methanol-water washing procedure were analyzed for nitrogen and iron. The molecular weight of protohemin with 1 mole each of chloride and methanol would be 683.97. If nitrihemin were a nitroso derivative, with chloride and methanol complexed to the iron, the molecular weight would be 712.97. The theoretical and analyzed values are shown in Table II.

The analyzed values are in good agreement with the theoretical values, especially in view of the uncertainties as to the exact form of the complex and in view of the resistance of the hemins to total degradation. From the tabulated values, nitrihemin does contain more nitrogen, showing that nitrihemin was protohemin modified by the addition of a nitrogenous compound. Oxygen analyses could also have been useful, but such analyses could not be obtained in the presence of iron.

DISCUSSION

The evidence accumulated on the characteristics of the nitriheme compounds indicates a modification of the chromophoric group by the action of nitrous acid on the porphyrin ring. Although the modification does not directly involve the protein, the latter apparently does play an indirect role, for as yet we have been unable to produce a modified hemin by the action of nitrite on globin-free hemin. The nearest analogy to the nitrite reaction under study is the reaction between nitrous acid and aromatic nuclei, where the acid attack is electrophilic. According to Pullman and Perault (1959), a high electron density occurs at the α-methene bridge of hemins. The tentative structure suggested for the nitriheme pigments, then, is one in which the porphyrin ring has been nitrosylated at the α-methene bridge. The nitroso group itself may be complexed to the iron to form a ring structure involving a coordinate-covalent bond. It is the interruption of the normal resonance state of the heme molecule which causes the absorption-band shift and makes these pigments green. The authors have mentioned the analogy of these pigments to the green sulfheme pigments, and it is with respect to the structure of this class of pigments (unoxidized-ring green hemes) that the nitriheme pigments are of greatest interest. The size and definitive character of nitrihemin, as compared to the heme-globin complexes, render it much more amenable to study. Defining the structure of nitrihemin may well help in defining the structure of the sulfheme compounds, concerning which there is still some discussion (Lemberg and Legge, 1949).

With regard to the questions of "nitrite burn" or "greening" in cured meats, it is clear that there is a green pigment produced by the action of nitrite on the

heme of native heme pigments. It has been suggested (Chaumet, 1955) that "nitrite burn" is the result of "xanthoprotein"-type reactions, but the contribution of these reactions to discoloration would appear to be somewhat doubtful from the results presented here. It was observed that the denatured proteins produced in the acidification of the acetone suspensions of nitrimetmyoglobin or nitrimethemoglobin were quite pale or colorless, especially with respect to the extracted hemin, and therefore could contribute little to total color. It is evident that controlled studies in cured meat products are indicated for there is no clear correlation between the chemistry of formation of nitriheme pigments and the discolorations observed in practice.

A further consequence of this study relates to the relatively rare incidence of nitrite poisoning. Because of studies which show that hemoglobin is oxidized by nitrite to methemoglobin, cases of nitrite poisoning are usually treated as methemoglobinemia. However, in view of the possibility of the formation of a modified heme-protein complex, no longer capable of complexing oxygen or reconversion to hemoglobin, it may prove that a new approach to the treatment of such cases is necessary. Here, investigations are indicated on the effect on the blood of large doses of nitrite.

Acknowledgments

The authors wish to express their gratitude to the following people: Miss Evelyn M. Sabino and Miss Victoria Bisson for their assistance; Drs. L. M. Kozloff and A. Heller for taking fluorescent spectra on their spectrophotofluorometers. We also acknowledge the work of the Galbraith Laboratories of Knoxville, Tenn., in obtaining the elemental analyses of the hemin samples.

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