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HEME-PROTEIN-LIGAND INTERACTIONS

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Recent studies have suggested the use of nitrogenous heterocyclic compounds as color forming reagents in cured meats (Tarladgis, 1967; Howard et al., 1973). During screening in frankfurters of a variety of such compounds, including a series of substituted pyridines, we observed the production of a variety of colors. The principal colors were: purple, produced by strong electron acceptors in the 4 position on the pyridine nucleus; orange, produced by methylpyridine derivatives; and red or pink, produced by all other substituents in the 3 or 4 position. These results led us to a more detailed study of the spectra of these pigments, using bovine myoglobin in pH 5.5 buffer which is approximately the pH of meat.

NATURE OF THE HEMOCHROME

In order to form the hemochrome, it is necessary to denature the protein. The heme of myoglobin is partly buried in the surface of the protein (Figure 1), resulting in steric hindrance of ligand binding to the heme, an effect first observed by St. George and Pauling (1951). The cleft in the protein which contains the heme is too narrow to admit the pyridine nucleus and the protein must be partly denatured. Sodium lauryl sulfate (SLS) not only denatures the protein, producing a soluble hemochrome (Howard et al., 1973) but also solubilizes the heme (Simplicio, 1972). The nature of the SLS-denatured globin hemochrome has been defined only at pH 8.0, by Van den Oord and Wesdorp (1969) who found that the binding of SLS to myoglobin at pH 8.0 required 17 moles of SLS to completely denature a mole of pigment. Our results of the titration of met-myoglobin with SLS at pH 5.5 are shown in Figure 2. The initial

precipitation reaction was linear with SLS concentration and was complete at a ratio of 15 moles of SLS/mole of myoglobin. Upon continued addition of SLS, the pigment was resolubilized. The sigmoid shape of the curve implies an equilibrium process which began at a 28 molar excess and was completed at a 55 molar (0.1% SLS) excess. Katz et al. (1973) recently reported that a point of maximum volume decrease of myoglobin occurs at a molar ratio of 60:1 (SLS:Mb), which is close to the value we have observed for the resolubilization of the pigment. Assuming the protein is still part of the resolubilized hemochrome, the total molecular weight of the micelle is about 32,000 D, composed of 18,000 D of myoglobin and 14,000 D of SLS. We assumed the globin was part of the micelle because: 1. The spectrum of the SLS-denatured globin hemochrome has absorption maxima at 530 and 570 nm at both pH 5.5 and pH 8.0, typical of the diimidazolyl hemochrome (Van den Oord and Wesdorp, 1969). Only the protein can furnish the imidazole groups, probably from the F8 (proximal) and E7 (distal) histidine residues. 2. Only 0.1% SLS was required at pH 5.5 to resolubilize 0.050 mM metmyoglobin (MetMb), whereas Simplicio (1972) found it required 1.0% SLS to solubilize hemin. The difference we attribute to the solubilizing effect of the protein.

FORMATION OF THE HEMOCHROMES

Solutions containing 0.025 mM MetMb and 0.1% SLS in $\Gamma/2 = 50$ mM acetate buffer, pH 5.5, were used to study the kinetics of formation and spectra of the substituted pyridine hemochromes. Ligand concentration varied from 0.1 to 25 mM. The reference was a buffer solution and temperature was controlled to $20.0^\circ \pm 0.1$ in a Cary 14 spectrophotometer. The reaction was started with excess $\text{Na}_2\text{S}_2\text{O}_4$. The spectra of three typical reaction products are shown in Figure 3. The two major bands at ca. 530 and 555-560 nm are due to porphyrin ring π electron excitations and were common to all substituted pyridine hemochromes. Absorption bands at ca. 480 nm were typical of the methyl substituted pyridine hemochromes, whereas bands in the 600-750 nm region were typical of the hemochromes formed from pyridine derivatives with strong electron acceptor groups in the 4 position. These bands are due to charge transfer between the iron and the ligand (Brill and Williams, 1961).

KINETICS

Akoyunoglou et al. (1963) had difficulties obtaining kinetic and equilibrium data for the formation of nitrogenous base-heme complexes, indicating that more than a simple equilibrium between heme and ligand was involved. We had similar difficulties determining the rates of formation of these complexes as well as the

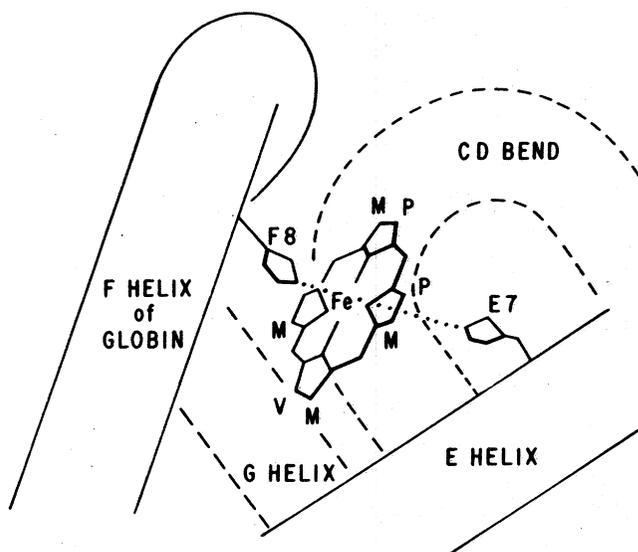


Figure 1. Position of the heme in myoglobin.

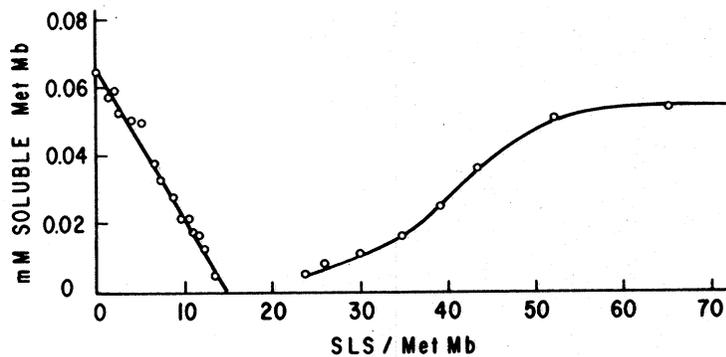


Figure 2. Precipitation and resolubilization of metmyoglobin by sodium lauryl sulfate.

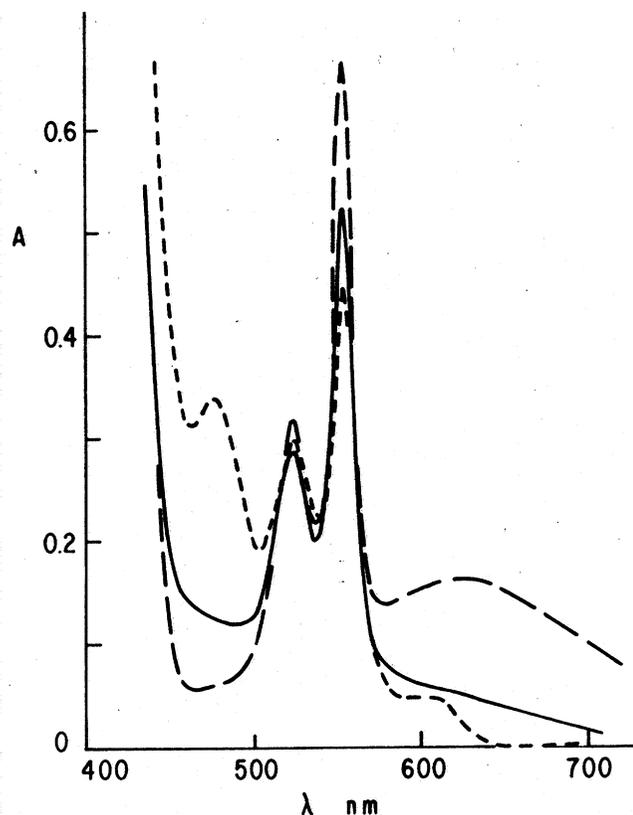


Figure 3. Substituted pyridine SLS-denatured globin hemochromes. —, 3-acetylpyridine (rose), - - -, 4-carboxymethylpyridine (purple), and - · - ·, 3-methylpyridine (orange).

order of the reaction. With large molar excesses of substituted pyridines, the reaction was first order with respect to the heme pigment concentration. These first order rate constants were dependent on the substituted pyridine concentration, but the dependence was greater than third order. If pyridine concentration was less than 1-2 mM, no complex formation was observed, whereas at 25 mM the reaction went too fast to follow. The process of binding 1 or 2 molecules of ligand to the heme is apparently a complex multiple ligand reaction with the SLS-denatured globin heme micelle.

At ligand concentrations of 25 mM and higher, the reaction took place in two stages. The first stage was completed in seconds, and was characterized by the formation of the typical absorption spectra, with the λ_{\max} lying between 554 and 555 nm for all substituted pyridines studied. The second stage took up to an hour to complete. It was characterized by the bathochromic shift of the absorption maxima from the common starting point to final values ranging from 557 to 567 nm, each value characteristic of the particular pyridine derivative.

This reaction sequence suggested three alternative explanations: 1. The initial rapid reaction resulted in an imidazolylpyridyl heme complex, probably through replacement of either the proximal or distal histidine, followed by a slow replacement of the imidazole of a second histidine by another molecule of pyridine derivative; 2. The initial reaction forms a dipyrindine hemochrome, followed by a slow interaction between the ligand and the micelle. Changing the character of the micelle would alter its interaction with the peripheral groups of the heme, resulting in a spectral shift of the absorption bands of the porphyrin as suggested by Caughy et al. (1966); 3. There were multiple forms of the micelle which reacted at different rates with the pyridine. Our evidence indicates that any or all of the explanations may apply, depending on the pyridine derivatives and/or the method of forming the hemochrome.

Alternative 1. Mono to Dipyrindine Hemochrome

All of the hemochromes had essentially the same initial wavelength of maximum absorption. This suggests that the hemochrome π electron structure was basically that of a pyridine complex, modified by an imidazolyl group in the other ligand position. The single pyridine ligand may have affected the fine positioning of the heme peak, but the latter effect was not detectable. With the formation of the dipyrindine hemochrome, the π electron interaction should be much stronger, resulting in a more pronounced shift.

Substituents on the pyridine ring changed the position of the λ_{\max} of the hemochrome, and also affect the positions of the ultraviolet absorption maxima of pyridine, which are the π electron excitation bands of the pyridine ring. To determine if a relationship existed between the λ_{\max} , the hemochrome λ_{\max} were plotted as a function of the substituted pyridine λ_{\max} (Figure 4). The substituents on the pyridine nucleus are differentiated as either electron donors or acceptors. Falk et al. (1966) suggested a positive correlation based on the electron donating character of the substituent, but the intermixing of the two types of substituents in the figure shows that such a correlation is not strictly

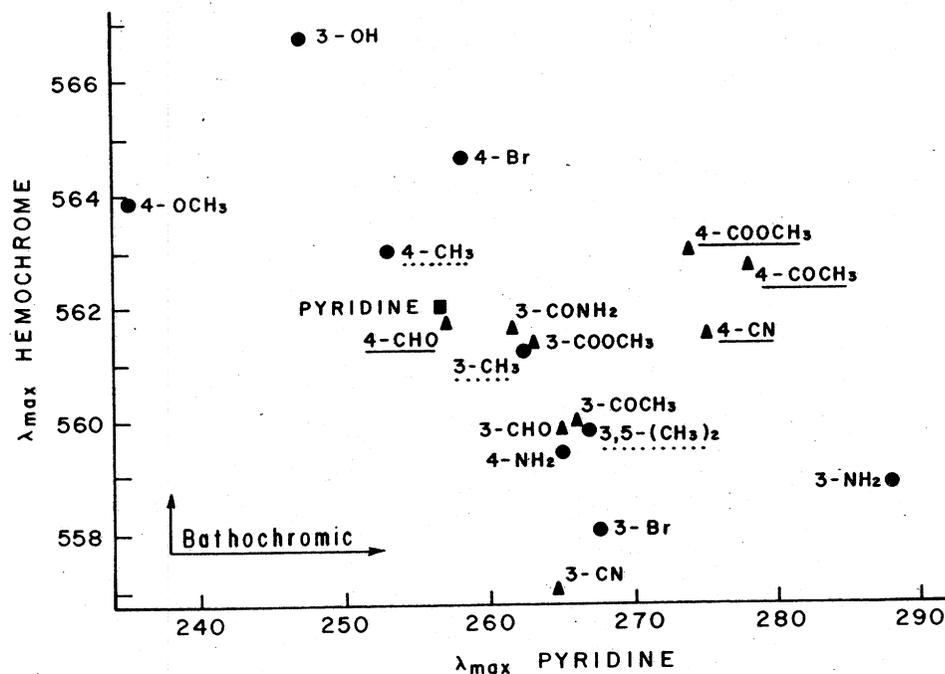


Figure 4. Wavelength of maximum absorption of substituted pyridine hemochromes as a function of the wavelength of maximum absorption of the substituted pyridine. \square , pyridine; \circ , donor and Δ , acceptor substituents. —, purple;, orange. For the data, $r=0.53$, significant at the 1% level.

correct. However, acceptor and donor substituents are also classified as either bathochromic or hypsochromic in their effect on the ultraviolet spectra of the pyridine ring, and it is this characteristic of the substituents that is the basis of the relationship in the plot. The correlation coefficient for the regression is 0.53, which corresponds to a probability of 0.01 (Snedecor, 1946). There was no statistical correlation between λ_{\max} of the hemochrome and either the stability constant of the pyridine hemochrome, as suggested by Falk et al. (1966) or the acid dissociation constant of the pyridine derivative. The spectral correlation may be interpreted in terms of the basic electron structure of the complex. Substituents on the pyridine ring change the energy levels of both the bonding and antibonding orbitals of the π electrons. If the substituent raises the bonding orbital energy level, the effect will

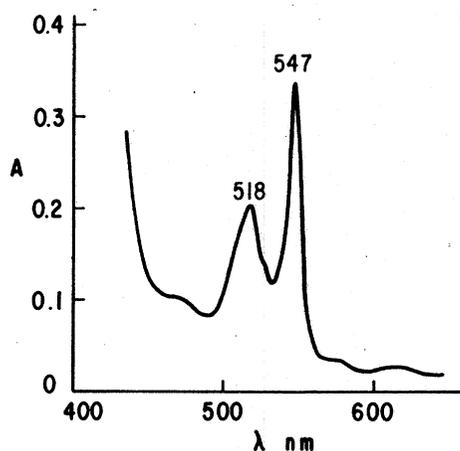


Figure 5. Spectra of the gelatinous liquid from fresh ham cooked either in air or nitrogen at 95°.

be transferred to the $d\pi$ orbitals of the iron with which the pyridine has formed π bonds (Pauling, 1949; Falk et al., 1966). The π electrons of the porphyrin ring also form bonds with the $d\pi$ orbitals of the iron. Energy transfer between the $d\pi$ electrons of the iron would raise the bonding energy levels of the porphyrin ring. Since the antibonding orbitals of the porphyrin are not affected, the energy gap between the bonding and antibonding orbitals would be decreased and the λ_{\max} shifted towards longer wavelengths. Since the correlation is negative, the increase in the bonding orbital energy levels of the pyridine due to substituents must be accompanied by a correspondingly greater increase in the antibonding orbital energy levels.

These energy level shifts are the result of the inductive and conjugative (hyperconjugative) effects of the substituent. If these energy level values were known for the various derivatives,

it seems likely that a better relationship than wavelength* could be established for the data of Figure 4. Chandra and Basu (1959) have calculated the values of the hyperconjugative and inductive effects for 3- and 4-methylpyridine and 3,5-dimethylpyridine. Their results showed that the wavelength shifts were linear with either effect. Since our results are also linear for these three derivatives, no specific correlation to either hyperconjugative or inductive effects can be made.

Alternative 2. Micelle Interaction and Color

Such an interaction was first suggested by Caughey et al. (1966). We have not been able to derive any direct evidence from our studies on this point. However, we have made an observation which bears on the question of detergent-heme interactions. The drip from several ham samples cooked at 95° all had an orange-pink color and the typical spectra of a pyridine hemochrome (Figure 5). Tappel (1957) suggested that the pigment of cooked fresh meats is that of niacinamide hemochrome, partly from the reflectance spectral characteristics and partly because niacinamide is the only nitrogen-containing aromatic ring compound occurring in meat in sufficient quantities to form the complex.

The unusual feature of the spectrum of Figure 5 is that the wavelength maxima occur at 518 and 547 nm, some 10 and 15 nm towards shorter wavelengths than the corresponding maxima of the SLS-denatured globin hemochrome (Figure 4). The heme micelle cannot be the same for the two pigments, and if the ham drip pigment is the niacinamide complex, the difference in spectra must be due to the micelle difference.

Alternative 3. Multiple Reacting Forms

The third alternative was supported by the spectral changes that occurred during the formation of the 3-hydroxypyridine hemochrome (Figure 6). The typical peak at about 555 nm appeared quickly in the first stage, but during the second stage, instead

*The relation between the spectra of the pyridine derivative and the hemochromes formed from them is more accurately expressed as a function of wave number as it is the latter which directly related to the energy of the orbitals involved. During the review of this manuscript, Dr. D. Quimby of E.R.R.C. pointed out that the variability of the data of Figure 4 could be considerably reduced by treating the data of the 3 and 4 position substituted pyridines separately.

of the peak shifting to a longer wavelength, a new peak was formed at 567 nm, both peaks showing simultaneously. Since the 567 nm peak was produced slower, and disappeared faster than the 555 nm peak, it could not have been in equilibrium with the latter and must have been produced de novo. This situation can occur only if there are two different reacting forms of the heme micelle, one of which reacts faster with the pyridine than does the other.

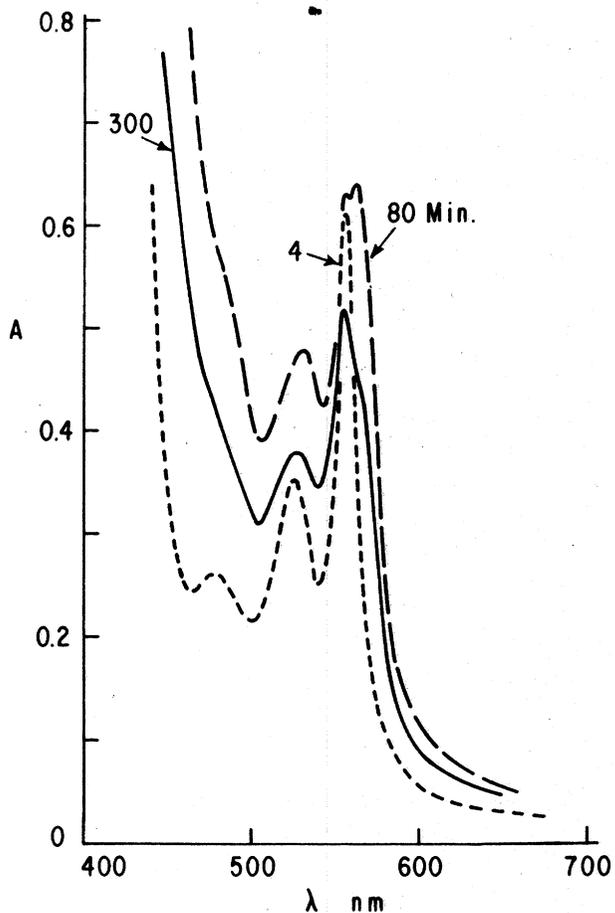


Figure 6. Spectra of the mixture of 3-hydroxypyridine and SLS-denatured globin hemochrome during the slow state of the reaction.

HUE OF THE PYRIDINE HEMOCHROMES

The differences in the λ_{\max} of the various pyridine hemochromes that have just been discussed are not sufficient to account for the observed variations in hue of the hemochromes. Hemochromes in Figure 4 were either orange (dotted underline), purple (solid underline) or various shades of red, inclining to a rose (bluish-red) tint. The orange pigments had strong charge transfer bands in the 480 nm region which, by reducing the amount of blue light transmitted, produces the orange hue. Conversely, the charge transfer bands at 600 to 750, by reducing the amount of red light transmitted, produce a purple hue.

To explain the position of these bands the simple relationship of electron donor or acceptor may be invoked. The electron donating methyl groups raise the energy levels of the antibonding orbitals of the π electrons of the pyridine ring. This increases the energy difference of the transfer of a charge from the $d\pi$ orbitals of the iron to the antibonding π orbitals of the pyridine ring. The absorption bands for this transfer therefore appear at shorter wavelengths. The effect is also observed in the fine positioning of the charge transfer bands of the hemochromes. The data of Table I show that the lower the energy of the π electron excitation of the pyridine ring, the lower the energy of charge transfer. Electron accepting substituents would have the opposite

TABLE I

Absorption Maxima of Various Pyridine
Derivatives and the Corresponding
Hemochromes

Substituent	λ_{\max} , nm	
	Pyridine	Hemochrome
4-methyl	253.0	ca. 472
3-methyl	262.5	477
3,5-dimethyl	267.0	482
4-formyl	257	643
4-carboxymethyl	274	657
4-cyano	275	678
4-acetyl	278	740

effect. Increasing the positive charge on ferric complexes would also have the opposite effect, as suggested by Brill and Williams (1961). In these complexes, the charge transfer is from the ligand to the iron. Raising the energy levels of the pyridine π bonding orbitals decreases the energy gap between them and the antibonding orbitals of the iron, decreasing the energy gap and shifting the absorption bands towards longer wavelengths.

SUMMARY

A negative correlation has been found to exist between the λ_{\max} of various substituted pyridines and the hemochromes formed from them, and we have developed a qualitative theory to explain the phenomenon. Spectral variations in the hemochromes have been observed which are attributed to variations in the heme micelle, either total composition of the micelle, as in the case of the ham exudate, or micro variations in one micelle as in the case of the 3-hydroxypyridine hemochrome.

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