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Structural Effects in Alkyl Nitrite Oxidation of Human Hemoglobin*

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Michael P. Doyle‡, Ruth A. Pickering, and José da Conceição
From the Department of Chemistry, Hope College, Holland, Michigan 49423

Oxidations of hemoglobin in oxygen-saturated and deoxygenated media by 10 structurally variant alkyl nitrites have been examined in kinetic detail. Pronounced structural influences on rate constants, whose values span a range of 80 in oxidations of both oxy- and deoxyhemoglobin, have been observed. *tert*-Butyl nitrite provides the slowest oxidation rate that for deoxyhemoglobin terminates after only half of the available iron(II) heme units have been oxidized. Activation parameters have been determined for oxidations of oxyhemoglobin and deoxyhemoglobin by ethyl, isopropyl, and neopentyl nitrites from kinetic evaluation of these reactions as a function of temperature. The differences in free energies of activation ($\Delta G‡$) between hemoglobin R and T states range from 1.8 to 2.9 kcal/mol for the three alkyl nitrites examined. The composite data portray alkyl nitrite oxidations as inner sphere electron transfer processes whose kinetic characteristics reflect the ligand binding properties of hemoglobin. A sulfhydryl-induced alkyl nitrite oxidation of oxyhemoglobin that is most pronounced in oxygen-saturated media has been observed, and its cause has been traced to nitrosyl exchange of alkyl nitrites with the β-93 cysteine sulfhydryl group of hemoglobin.

The oxidation of hemoglobin to methemoglobin by chemical agents is recognized to be a complex transformation that is dependent on the nature of the chemical oxidant (1–3), on structural features of the protein molecule (1, 4–6), and on the distance over which electron transfer occurs (7–9). Although the literature is replete with examples of oxidants that are capable of electron transfer from hemoglobin, few of these oxidative transformations have been examined in exacting detail, and there is virtually no information on the structural influences of the oxidant in hemoglobin oxidations. The transformation of hemoglobin to methemoglobin can conceptionally occur by either an outer sphere process, in which iron(II) and the chemical oxidant each retains its own full coordination shell, or an inner sphere process, which is characterized by intimate association of the oxidant to the sixth coordination position of hemoglobin. An outer sphere electron transfer process is clearly defined in oxidations that occur with metalloproteins that have an inaccessible metal site (10). However, when the metal site is accessible to ligand association, as is the case for hemoglobin, both inner sphere and outer sphere electron transfer processes are possible.

In reactions with hemoglobin, an inner sphere electron transfer process is suggested if oxidation occurs on both the oxy and deoxy forms with the same kinetic dependence on hemoglobin and on the chemical oxidant and if iron ligands that include dioxygen inhibit oxidation (11–14). We have recently reported results which suggest that the oxidative characteristics of ethyl nitrite fit these criteria and that this system provides an exemplary model for inner sphere metalloprotein oxidations (15, 16). As an inner sphere electron transfer process, structural variation of the oxidatively uninvolved alkyl group of alkyl nitrites is predicted to influence the rate of hemoglobin oxidation as a result of attractive hydrophobic and repulsive steric interactions with the protein. However, until quite recently, a potentially predictive model to which rate constants for inner sphere electron transfer reactions could be compared was unavailable. Reisberg and Olson (17–19) have recently reported their exhaustive examination of the rates and equilibrium constants for isonitrile binding to hemoglobin, and their results offer the first available model with which the structural influences of alkyl nitrites in hemoglobin oxidations can be evaluated.

MATERIALS AND METHODS

Human hemoglobin A (type IV), obtained from Sigma, was reduced with excess sodium dithionite and further purified by passing the resulting aqueous solution through a G-25 Sephadex column using 0.05 M phosphate buffer at pH 7.0. Concentrated solutions of oxyhemoglobin (approximately 1.0 mM heme) were degassed under reduced pressure (less than 0.5 torr), and the resultant deoxyhemoglobin was maintained in a sealed air-tight flask at atmospheric pressure under nitrogen. Reaction of β-93 cysteine residues of the reactant hemoglobin was performed at pH 7.8 with oxyhemoglobin and a 10-fold molar excess of iodoacetamide, relative to heme, according to the procedure of Winterbourn and Carrell (2). The β-93 sulfhydryl blocked hemoglobin was separated from excess iodoacetamide on a G-25 Sephadex column. The absence of accessible sulfhydryl groups was determined with 5,5′-dithiobis(2-nitrobenzoic acid) by the method of Ellman (20). Heme concentrations for deoxyhemoglobin were calculated from the extinction coefficients of Banerjee *et al.* (21). Heme concentrations for oxyhemoglobin samples were calculated from the molar absorptivities of Moore and Gibson (22). Oxygen concentrations in the buffered solutions employed for this study were measured and varied as previously described (16).

Alkyl nitrites were prepared from their respective alcohols by treatment with sodium nitrite in aqueous sulfuric acid (23) and purified by distillation; the physical constants of these synthetically derived reagents were identical to literature values (24), and spectroscopic evaluations confirmed their identity. Hydrolytic rate constants were determined at 25.0 °C in 0.05 M phosphate-buffered solution (pH 7.0) for each of the alkyl nitrites employed in kinetic investigations of hemoglobin oxidation. Half-lives for hydrolysis ranged from 0.8 to 2.4 h for primary alkyl nitrites and from 0.8 to 1.2 h for secondary and tertiary alkyl nitrites. The detailed results of this hydrolytic investigation are reported elsewhere (25). Stock solutions of alkyl nitrites in anhydrous acetonitrile were prepared immediately prior to their use for kinetic measurements. Acetonitrile compositions in the reaction solution never exceeded 1% of the total volume and were not observed to affect reaction rates or product distributions.

Reactions were initiated with the injection, using a gas-tight syringe, of a concentrated alkyl nitrite solution into the hemoglobin

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‡ To whom all reprint requests should be sent.

sample (usually 20–40 μM) contained in 0.05 M phosphate buffer at pH 7.0. Rates for oxyhemoglobin oxidations were determined by monitoring the decrease in absorbance at 576 nm with time using a Pye Unicam SP8-200 spectrophotometer. With deoxyhemoglobin, oxidation rates were determined by monitoring the decrease in absorbance at 552 nm with time. In all kinetic experiments, reactions were carried out under pseudo-first order conditions where alkyl nitrite concentrations were generally in 10-fold molar excess with respect to the total heme groups present. Monophasic time courses were fitted to an integrated single exponential process from which the pseudo-first order rate constants were calculated. Biphasic time courses were fitted to a two-exponential expression from which pseudo-first order rate constants were calculated for both the fast and slow phases. Typically, from 5 to 12 replicate time courses were obtained for each kinetic determination, and the averaged rate constants are reported. The compositions of nitrosylhemoglobin and methemoglobin from alkyl nitrite oxidations of deoxyhemoglobin were calculated from product absorbances observed at 542, 552, and 572 nm in each experiment, and the resulting composition determinations were averaged to obtain the reported values. Deviations in per cent HbNO from calculations at these three wavelengths were less than 1%, and deviations in per cent MetHb[1] between individual reaction determinations amounted to ±2%.

Reaction rates for oxy- and deoxyhemoglobin oxidation by ethyl, isopropyl, and neopentyl nitrites were determined at selected temperatures ±0.1 °C, and their rate constants were obtained from the average of at least four separate measurements. Oxyhemoglobin oxidation rate constants, k_o, were calculated from the previously determined relationship $k_{obs} = 2k_oK^{O_2}/(K^{O_2} + [O_2])$, where K^{O_2} is the equilibrium constant for oxygen dissociation from hemoglobin, using reactions performed at oxygen saturation (16). Activation energies, E_{act}, were determined from the slopes of the lines ($-E_{act}/R$, where R is the gas constant in cal/deg-mol) in plots of ln k_o *versus* 1/T. The estimated error of these determinations for oxidations of oxyhemoglobin were ±1.0 kcal/mol, and for oxidations of deoxyhemoglobin error limits were ±1.5 kcal/mol. Activation enthalpies ($\Delta H‡$), entropies ($\Delta S‡$), and free energies ($\Delta G‡$) were obtained from application of the transition state rate equation evaluated at 25.0 °C. Estimated error limits for oxidations of oxyhemoglobin were ±1.0 kcal/mol ($\Delta H‡$), ±2.0 e.u. ($\Delta S‡$), and ±0.2 kcal/mol ($\Delta G‡$), and those for oxidations of deoxyhemoglobin were ±1.5 kcal/mol ($\Delta H‡$), ±4 e.u. ($\Delta S‡$), and ±0.2 kcal/mol ($\Delta G‡$). Composite errors in $\Delta H‡$ and $\Delta S‡$ cancel in calculations of $\Delta G‡$.

RESULTS

Sulfhydryl-induced Alkyl Nitrite Oxidation—Our prior evaluation of the kinetic characteristics of ethyl nitrite oxidations of oxyhemoglobin identified an inverse first order kinetic dependence on the concentration of molecular oxygen that deviated from linearity when the oxygen concentration approached saturation (16). This same deviation was not observed in similar investigations of oxymyoglobin oxidations by ethyl nitrite, and the existence of an alternate pathway for the oxidation of hemoglobin, but not of myoglobin, was evident. We can now report that this alternate pathway for hemoglobin oxidation is due to the presence of accessible cysteine residues at the 93-position on the β-chains of hemoglobin.

Although alkyl nitrites are hydrolytically stable under reaction conditions employed for hemoglobin oxidation, they are capable of rapid nitrosyl exchange with thiols (25–27). Similar transformations also occur between thiols and nitrous acid or dinitrogen tetroxide (26–29). *S*-Nitrosocysteine (30) is rapidly formed from cysteine upon reaction with ethyl nitrite in aqueous media, although subsequent reactions result in the eventual formation of the disulfide cystine and, presumably, nitric oxide. Since hemoglobin, but not myoglobin, possesses an accessible cysteine residue, conversion of this sulfhydryl group to a derivative sulfide was anticipated to block the alternate pathway for hemoglobin oxidation and to afford linear inverse dependence on the concentration of molecular oxygen in kinetic investigations of oxyhemoglobin oxidations by ethyl nitrite, even at oxygen saturation. Fig. 1 describes the composite results from investigations of the oxygen dependence on oxyhemoglobin oxidations by ethyl nitrite with both unmodified HbO_2 and β-93 sulfhydryl-blocked HbO_2. Clearly, the sulfhydryl group is the active functionality that provides the alternate pathway for hemoglobin oxidation. The composite results fit the two-term rate law

$$\frac{-d[HbO_2]}{dt} = [HbO_2]\,[RONO]\left(\frac{2k_oK^{O_2}}{K^{O_2} + [O_2]} + k_{SH}\right) \quad (1)$$

where k_o for the oxidation of unmodified hemoglobin is 2.53×10^4 $M^{-1}s^{-1}$ at 10.0 °C (16) and k_{SH}, the rate constant for sulfhydryl-induced alkyl nitrite oxidation, is 21 $M^{-1}s^{-1}$. The alternate pathway for oxidation of hemoglobin is not observably dependent on the concentration of molecular oxygen and is specifically facilitated by the cysteine sulfhydryl group at the β-93 position. Free cysteine added to the reaction solution containing β-93 sulfhydryl-blocked HbO_2 did not increase the observed rate constant for hemoglobin oxidation even when a 10-fold molar excess of cysteine was employed.

Alkyl Nitrite Oxidations of Oxyhemoglobin—In order to determine the effect of structure on reactivity in alkyl nitrite oxidations of HbO_2 without competition from sulfhydryl-induced alkyl nitrite oxidation, β-93 sulfhydryl-blocked oxyhemoglobin was employed for kinetic determinations. Since the slope of the line that describes inverse kinetic dependence on oxygen concentration (Fig. 1) for oxidation of β-93 sulfhydryl-blocked HbO_2 is identical to that for oxidation of unmodified hemoglobin, the use of iodoacetamide as the blocking reagent does not influence the kinetic character of oxyhemo-

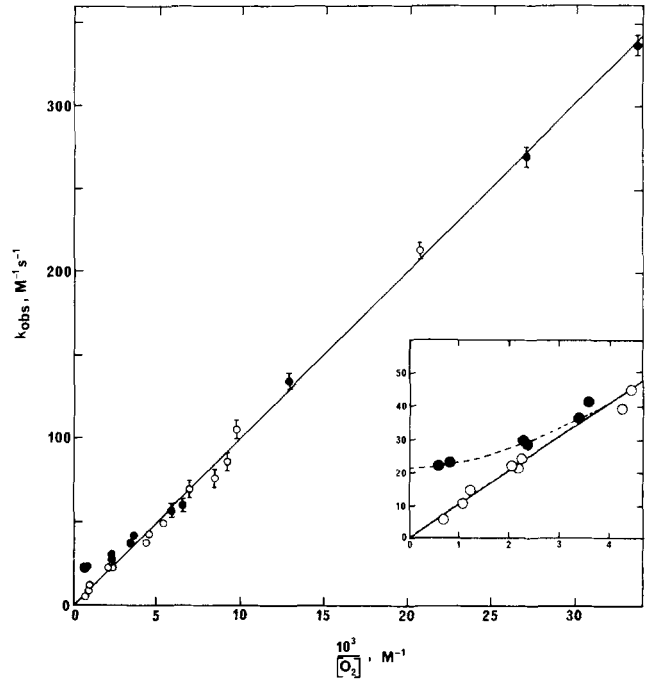


FIG. 1. **Dependence of the rate constant for ethyl nitrite oxidation of unmodified oxyhemoglobin (●) and β-93 sulfhydryl-blocked oxyhemoglobin (○) on the concentration of oxygen.** All reactions were observed at 576 nm in 0.05 M phosphate buffer, pH 7.0, 10.0 °C, and total oxygen concentration is reported. The *inset* is an expansion of this plot for kinetic behavior at oxygen concentrations near solution saturation. Rate constants for the first phase of the reaction are reported.

[1] The abbreviations used are: MetHb, methemoglobin; Hb, hemoglobin; Et, ethyl-; Pr, propyl-; *i*, iso-; Bu, butyl-; *s*, secondary; Pe, pentyl-.

globin oxidation. Oxidations of oxyhemoglobin to methemoglobin were performed at 25.0 °C in oxygen-saturated solutions with ten representative alkyl nitrites. Typical time courses are described in Fig. 2. Like the corresponding oxidations by ethyl nitrite (16), these reactions are biphasic, consisting of a rapid initial phase and a distinctly slower second phase. In the second phase of oxidation, alkyl nitrites generally exhibit reaction rates that are only 0.1–0.3 times as fast as their initial rates. That this behavior does not result from hydrolysis of the alkyl nitrite and subsequent competitive oxidation of HbO_2 by the nitrite ion is known from the hydrolytic rate constants for each of the alkyl nitrites examined (27). The fastest rate for alkyl nitrite hydrolysis projects production of the nitrite ion in concentrations that are equivalent to those of the HbO_2 concentration only after 800 s. As a result, Fig. 2 displays time courses for these reactions only to 800 s.

Oxidation of oxyhemoglobin by *tert*-butyl nitrite provided the only example of hydrolytic influence on the reaction course. In this case, a rapid autocatalytic oxidation was observed subsequent to the initial hemoglobin oxidation. This autocatalytic oxidation is characteristic of the nitrite ion acting upon oxyhemoglobin (31). A similar nitrite ion-induced oxidation was not observed with any other alkyl nitrite.

First order kinetics is observed in each phase of HbO_2 oxidation by these alkyl nitrites with the requisite linear correlation between ln ($c_o^{HbO_2}/c_t^{HbO_2}$) and time extending to the first 50% of the reaction in the first phase and, in a second linear relationship, extending from approximately 60 to ≥85% of the time course of the reaction. Various explanations for this moderate biphasic character have been previously discussed (16), and the possibility that partially oxidized hemoglobin tetramers exhibit differential rates for oxidation (6, 32) should also be considered. The second order rate law for oxyhemoglobin oxidation by these alkyl nitrites, first order in [HbO_2] and first order in [RONO], was confirmed by performing these oxidations with different molar ratios of [RONO]/[HbO_2].

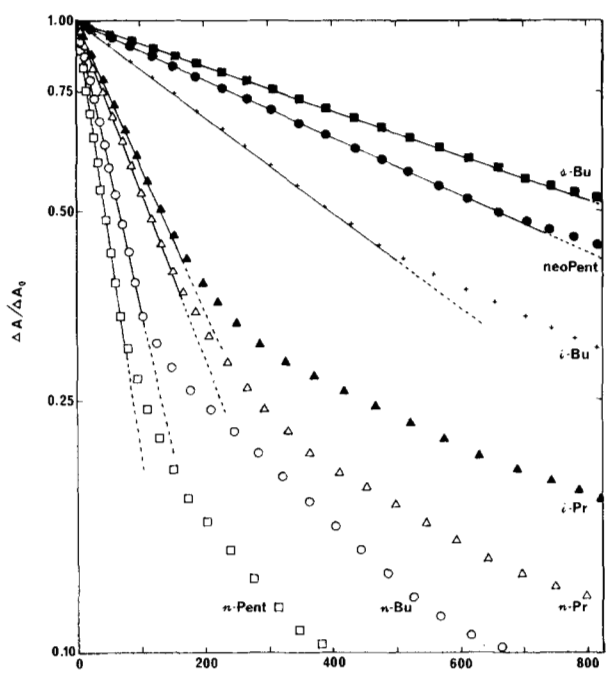


FIG. 2. **Typical time courses for reactions of hemoglobin in oxygen-saturated solutions with representative alkyl nitrites.** Reactions were observed at 576 nm for iodoacetamide-treated oxyhemoglobin in 0.05 M phosphate buffer, pH 7.0, 25.0 °C. *n*-Pentyl nitrite (□), 3.60×10^{-5} M (heme), 3.61×10^{-4} M (*n*-PentONO). *n*-Butyl nitrite (○), 2.43×10^{-5} M (heme), 2.42×10^{-4} M (*n*-BuONO). *n*-Propyl nitrite (△), 3.49×10^{-5} M (heme), 3.49×10^{-4} M (*n*-PrONO). Isopropyl nitrite (▲), 2.75×10^{-5} M (heme), 5.49×10^{-4} M (*i*-PrONO). Isobutyl nitrite (+), 4.01×10^{-5} M (heme), 8.06×10^{-4} M (*i*-BuONO). Neopentyl nitrite (●), 2.54×10^{-5} M (heme), 5.10×10^{-4} M (neoPentONO). *sec*-Butyl nitrite (■), 3.53×10^{-5} M (heme), 7.04×10^{-4} M (*s*-BuONO).

Kinetic results for the oxidation of β-93 sulfhydryl-blocked oxyhemoglobin by a representative series of alkyl nitrites is presented in Table I. Rate constants for the fast initial phase from each of these alkyl nitrites are reported as $k_{obs}^{HbO_2}$. Those for the slow second phase, reported as $k_{obs}^{slow}/k_{obs}^{fast}$, are only provided for primary alkyl nitrites, since complicating secondary reactions due to alkyl nitrite hydrolysis are not competitive when these substrates are employed. In the series of *n*-alkyl nitrites, rate constants for the initial phase of hemoglobin oxidation reach a minimum at *n*-propyl nitrite. Isopropyl and *n*-propyl nitrite oxidize hemoglobin at similar rates, but in the butyl series, the degree of branching produces a dramatic effect on the rate constants for oxidation. A 31-fold change in the rate constants for the initial phase of oxidation is observed with structural variation from *n*-butyl to *tert*-butyl and, overall, an 80-fold rate change is observed from ethyl to *tert*-butyl. The effect of replacing a hydrogen by a methyl group at the position α to the nitrite functionality (ethyl ≫ isopropyl ≫ *tert*-butyl) is greater by a factor of 8 than is the kinetic response to replacement of a hydrogen by a methyl group at the position β to the nitrite functionality (*n*-propyl > isobutyl > neopentyl). Replacement of a hydrogen by a methyl group at the γ-position (*n*-butyl > isopentyl) appears to produce the same kinetic response as does replacement of a hydrogen by a methyl group at the β-position.

Alkyl Nitrite Oxidations of Deoxyhemoglobin—Oxidations of deoxyhemoglobin were performed at 10.0 °C with the identical set of alkyl nitrites as was employed for reactions with HbO_2, and typical time courses are described in Fig. 3. Unlike the corresponding oxidations by ethyl nitrite (15), which exhibit pseudo-first order kinetics through greater than 80% of their time courses, the structurally variant alkyl nitrites normally produce distinctly biphasic kinetic time courses. With the exception of *tert*-butyl nitrite, which causes effective termination of oxidation at approximately 50% oxidation, alkyl nitrites generally exhibit reaction rates in the second phase of oxidation that are only 0.4–0.7 times as fast as their initial rates. Rates for oxidation of β-93 sulfhydryl-blocked hemoglobin were identical with those obtained with unmodi-

TABLE I

Observed and calculated rate constants for oxyhemoglobin oxidation by structually variant alkyl nitrites at pH 7, 25.0 °C

Reaction conditions are given in Fig. 2, k_o was calculated from $k_{obs}^{HbO_2} = 2k_oK^{O_2}/(K^{O_2} + [O_2])$ (16), and $K_{rel}^{HbO_2}$ is the rate for alkyl nitrite oxidation of oxyhemoglobin relative to that for *tert*-butyl nitrite.

Nitrite	$k_{obs}^{HbO_2}$	$10^{-4}\ k_o$	$k_{rel}^{HbO_2}$	$k_{obs}^{slow}/k_{obs}^{fast}$
	$M^{-1}s^{-1}$			
Ethyl	43.0	13.1	80	<1.0
n-Propyl	8.9	2.71	16	0.19
n-Butyl	17.0	5.18	31	0.23
n-Pentyl	22.6	6.89	42	0.17
Isobutyl	2.3	0.702	4.3	0.13
Isopentyl	7.1	2.17	13	0.16
Neopentyl	1.62	0.494	3.0	
Isopropyl	7.2	2.20	13	
sec-Butyl	1.01	0.308	1.9	
tert-Butyl	0.54	0.165	1.0	

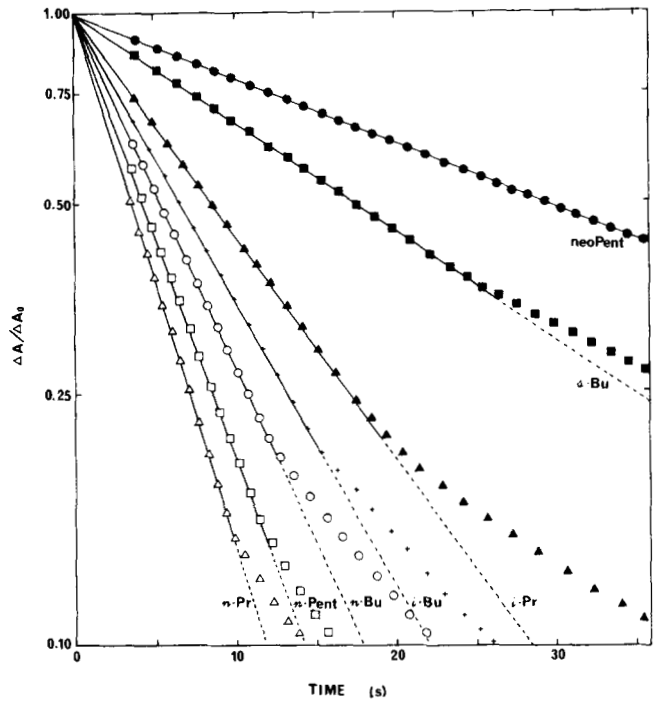


FIG. 3. **Typical time courses for reactions of hemoglobin in deoxygenated solutions with representative alkyl nitrites.** Reactions were observed at 552 nm for iodoacetamide-treated deoxyhemoglobin in 0.05 M phosphate buffer, pH 7.0, 10.0 °C. *n*-Propyl nitrite (△), 4.46×10^{-5} M (heme), 4.44×10^{-4} M (*n*-PrONO). *n*-Pentyl nitrite (□), 3.51×10^{-5} M (heme), 3.50×10^{-4} M (*n*-PentONO). *n*-Butyl nitrite (○), 2.96×10^{-5} M (heme), 2.95×10^{-4} M (*n*-BuONO). Isobutyl nitrite (+), 4.48×10^{-5} M (heme), 4.48×10^{-4} M (*i*-BuONO). Isopropyl nitrite (▲), 3.96×10^{-5} M (heme), 3.95×10^{-4} M (*i*-PrONO). *sec*-Butyl nitrite (■), 4.04×10^{-5} M (heme), 4.04×10^{-4} M (*s*-BuONO). Neopentyl nitrite (●), 2.68×10^{-5} M (heme), 2.68×10^{-4} M (neoPentONO).

TABLE II

Rate constants for deoxyhemoglobin oxidation by structurally variant alkyl nitrites at pH 7, 10.0 °C and the percentage of total hemoglobin converted to nitrosylhemoglobin

Reaction conditions are given in Fig. 3 and k_{rel}^{Hb} is the rate constant for alkyl nitrite oxidation of deoxyhemoglobin relative to that for *tert*-butyl nitrite. Determination of per cent HbNO was made as described under "Materials and Methods."

Nitrite	k_{obs}^{Hb}	k_o	k_{rel}^{Hb}	$k_{obs}^{slow}/k_{obs}^{fast}$	HbNO
	$M^{-1}s^{-1}$				%
Ethyl	1120	560	77	<1.0	28
n-Propyl	812	406	56	0.54	21
n-Butyl	598	299	41	0.64	18
n-Pentyl	755	378	52	0.60	22
Isobutyl	308	154	21	0.58	24
Isopentyl	345	172	24	0.52	32
Neopentyl	45	22	3.1	0.48	23
Isopropyl	174	87	12	0.33	26
sec-Butyl	77	38	5.3	0.38	25
tert-Butyl	14.5	7.2	1.0	<0.05	

fied hemoglobin which suggests the requirement for molecular oxygen in alkyl nitrite oxidations of HbO_2 induced by the sulfhydryl group.

Kinetic results for the oxidation of deoxyhemoglobin by these alkyl nitrites are presented in Table II together with the percentage of total hemoglobin that was converted to nitrosylhemoglobin. Rate constants for the fast initial phase are reported as k_{obs}^{Hb}, and those for the second slower phase are presented as $k_{obs}^{slow}/k_{obs}^{fast}$. In the series of *n*-alkyl nitrites, rate constants for the initial phase of hemoglobin oxidation reach a minimum at *n*-butyl nitrite rather than at *n*-propyl nitrite, as occurs in oxyhemoglobin oxidations (Table I). However, the degree of branching in the butyl series again dramatically affects the rate constants for oxidation, causing a 41-fold change with structural variation from *n*-butyl to *tert*-butyl. The effect of replacing a hydrogen by a methyl group at the position α to the nitrite functionality (ethyl $\gg$ isopropyl $\gg$ *tert*-butyl) is greater by a factor of between 2 and 4 than is the kinetic response to replacement of a hydrogen by a methyl group at the position β to the nitrite functionality (*n*-propyl > isobutyl > neopentyl). Replacement of a hydrogen by a methyl group at the γ-position (*n*-butyl > isopentyl) appears to produce only two-thirds of the kinetic response that is observed with replacement of a hydrogen by a methyl group at the β-position.

Variation of alkyl nitrite structure does not greatly affect the relative yield of nitrosylhemoglobin. However, the overall observed changes in per cent HbNO, from 18 to 32%, are beyond experimental error. That the increased production of methemoglobin is not due to sulfhydryl-induced nitric oxide oxidation (33) was confirmed by separate experiments in which 1:1 molar ratios of nitric oxide, based on NO/heme iron, were employed with iodoacetamide β-93 sulfhydryl-blocked deoxyhemoglobin, identical results to those previously reported for unmodified hemoglobin (15) were obtained.

The reaction of *tert*-butyl nitrite with deoxyhemoglobin is singularly important for its apparent termination after only half of the available iron(II) heme units have been oxidized when this reaction is performed at 10 °C. Oxidation continues after this half-oxidation stage but on a time scale which approaches that of the exceedingly slow nitrite ion oxidation (15) under the same conditions. The time course for this transformation, which is presented in Fig. 4, describes an initially rapid change in absorbance followed by a time period at constant absorbance and, after 500 s, a perceptively slow conversion to the final state. The spectral changes from 650 to 450 nm for the transformation of the initial deoxyhemoglobin to the intermediate and final states (Fig. 5) define the overall uniform conversion of Hb to MetHb and HbNO. Identical results are obtained with β-93 sulfhydryl-blocked hemoglobin. At 25 °C, oxidation of hemoglobin by *tert*-butyl nitrite does not terminate at the half-oxidation stage but continues to completion, although with evident biphasic kinetic character.

Temperature Effects on Alkyl Nitrite Oxidations—Compar-

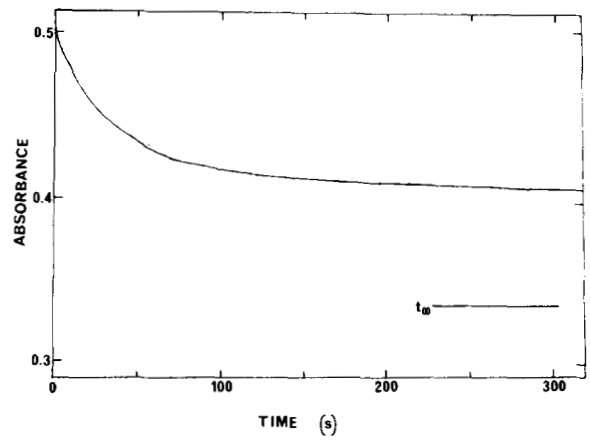


FIG. 4. **Time course for the reaction of deoxyhemoglobin with *tert*-butyl nitrite.** Reaction was performed on the deoxygenated solution in 0.05 M phosphate buffer, pH 7.0, 10.0 °C, and observed at 552 nm, 3.21×10^{-5} M (heme), 6.42×10^{-4} M (*t*-BuONO). The final absorbance reading (t_∞) was obtained after 22 h.

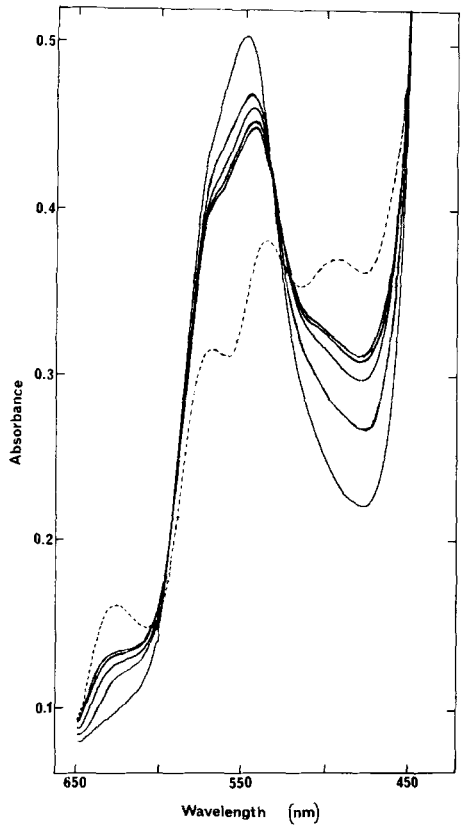


FIG. 5. **Spectral time course for the reaction of deoxyhemoglobin with *tert*-butyl nitrite.** Reaction was performed on the deoxygenated solution in 0.05 M phosphate buffer, pH 7.0, 10.0 °C, 3.21×10^{-5} M (heme), 1.61×10^{-4} M (*t*-BuONO). Spectra were recorded at 80-s intervals. The spectrum at t_∞ (---) was obtained after warming the reaction solution to 25 °C.

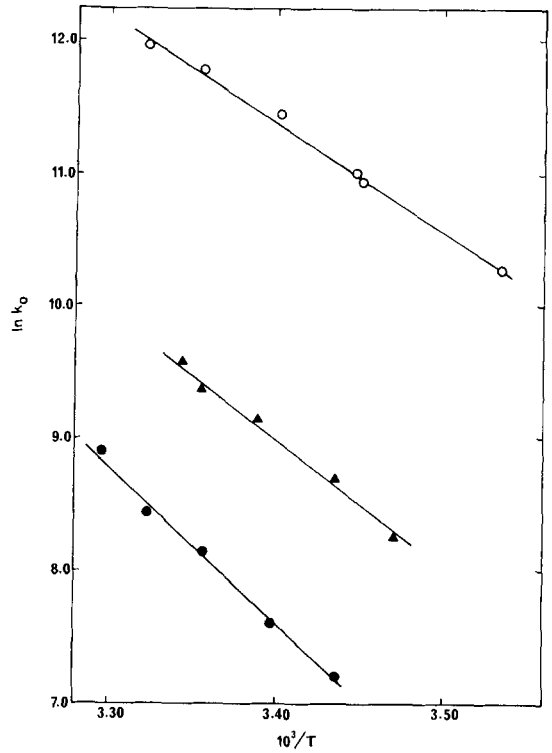


FIG. 6. **Temperature effects on rate constants for oxidations of hemoglobin by alkyl nitrites in oxygen-saturated solutions.** Reactions were observed at 576 nm for iodoacetamide-treated oxyhemoglobin in 0.05 M phosphate buffer, pH 7.0, ○, ethyl nitrite; ▲, isopropyl nitrite; ●, neopentyl nitrite. The averages of at least three kinetic determinations were used to obtain each data point. E_{act} for each alkyl nitrite oxidation was obtained from the slope of line ($-E_{act}/R$).

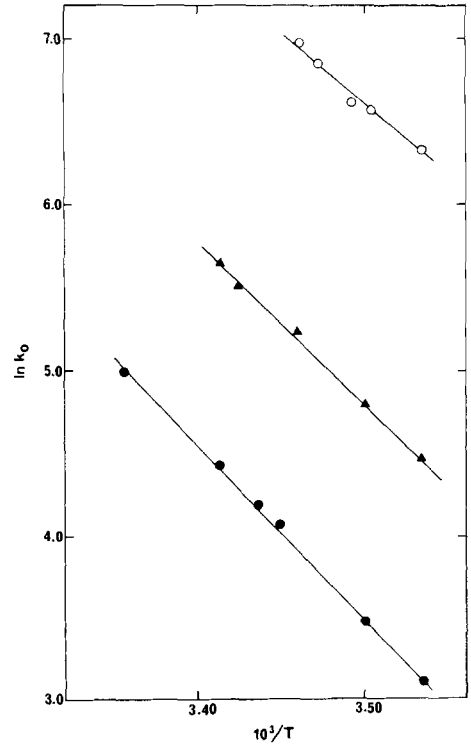


FIG. 7. **Temperature effects on rate constants for oxidations of hemoglobin by alkyl nitrites in deoxygenated solutions.** Reactions were observed at 552 nm for deoxyhemoglobin in 0.05 M phosphate buffer, pH 7.0; ○, ethyl nitrite; ▲, isopropyl nitrite; ●, neopentyl nitrite. The averages of at least five kinetic determination were used to obtain each data point. E_{act} for each alkyl nitrite oxidation was obtained from the slope of the line ($-E_{act}/R$).

ative kinetic results for the ethyl nitrite oxidation of deoxy- and oxyhemoglobin at 10 °C show an oxidative rate enhancement of 45 that has been suggested to result from the T → R change in hemoglobin conformation (16). If hemoglobin oxidation by alkyl nitrites occurs by inner sphere electron transfer, as is now suggested by structural influences on reactivity (Tables I and II), the observed rate enhancement for oxidation of oxyhemoglobin could be a reflection of differential steric barriers in the R and T state conformations (34). Alternatively, this rate enhancement could be caused by the relative electronic accessibility of the heme iron in the R and T states to the alkyl nitrite (35, 36). In order to evaluate the energies associated with alkyl nitrite oxidations of hemoglobin, the temperature effects on rates were determined, and the characteristic energies of alkyl nitrite oxidations were calculated.

Ethyl, isopropyl, and neopentyl nitrites were chosen for this investigation since they span the spectrum of observed relative reactivities and structural variations. *tert*-Butyl nitrite was not included because of its previously discussed special behavior towards deoxyhemoglobin and its hydrolytic instability during reactions with oxyhemoglobin. Rate constants were determined at a minimum of four different temperatures for oxidations of hemoglobin in both oxygen-saturated and deoxygenated media. Plots of ln k_o *versus* $(1/T)$ (Figs. 6 and 7) exhibited strikingly linear correspondance throughout the temperature range, and the values of the corresponding activation energies, E_{act}, were obtained from the Arrhenius equation. The linearity that characterizes the plots in Figs. 6 and 7 suggests that conformational changes in the protein or other aberrant behavior that would have

TABLE III

Activation energies and calculated rate constants for hemoglobin oxidations by alkyl nitrites in oxygenated and deoxygenated solutions

Values of $\Delta H‡$, $\Delta S‡$, and $\Delta G‡$ were calculated at 25.0 °C from values of E_{act} (Figs. 6 and 7).

Nitrite	E_{act}	$10^{-2}\ k_p$	$\Delta H‡$	$\Delta S‡$	$\Delta G‡$
	kcal/mol	$M^{-1}\ s^{-1}$ *(25.0 °C)*	*kcal/mol*	*e.u.*	*kcal/mol*
Oxygenated solutions					
EtONO	16.3	1310	15.7	17.6	10.5
i-PrONO	18.4	122	17.8	19.8	11.9
neoPeONO	23.3	34.2	22.7	33.8	12.6
Deoxygenated solutions					
EtONO	16.8	25.0	16.2	9.5	13.4
i-PrONO	19.4	5.35	18.8	17.0	13.7
neoPeONO	21.2	1.50	20.6	20.4	14.5

altered access of the alkyl nitrite to the heme cavity did not occur in these temperature ranges. The enthalpy ($\Delta H‡$), entropy ($\Delta S‡$), and free energy ($\Delta G‡$) of activation were determined from application of the transition state rate equation evaluated at 25.0 °C. Table III provides the listing of these characteristic energies for alkyl nitrite oxidations of oxy- and deoxyhemoglobin.

The composite data show that in each series of hemoglobin oxidation, activation energies (E_{act}) increase in the order EtONO < *i*-PrONO < neoPeONO, and that the free energy of activation ($\Delta G‡$) determined for each alkyl nitrite is greater for deoxyhemoglobin oxidation than for oxyhemoglobin oxidation. Values of $\Delta S‡$ increase with increasing steric bulk of the alkyl nitrite alkyl group, but they are surprisingly greater for oxyhemoglobin oxidations than for deoxyhemoglobin oxidations. Similarly, values of $\Delta H‡$ also increase in the order EtONO < *i*-PrONO < neoPeONO.

DISCUSSION

Alkyl Nitrite Oxidations of Hemoglobin—Four lines of evidence point to the inner sphere electron transfer mechanism for alkyl nitrite oxidation of hemoglobin: 1) inverse $[O_2]$ dependence on the rate for hemoglobin oxidation (Fig. 1), 2) structural effects of alkyl nitrites on rate constants for hemoglobin oxidation (Tables I and II), 3) the apparent termination of deoxyhemoglobin oxidation in reactions with *tert*-butyl nitrite after only half of the available iron(II) heme units have been oxidized (Figs. 4 and 5), and 4) inhibition of HbNO formation in ethyl nitrite oxidations of deoxyhemoglobin at [EtONO]/[Hb] between 10 and 100 (15). Inverse kinetic oxygen dependence alone does not demonstrate an inner sphere electron transfer process since $Fe(II)O_2$ could be resistant to outer sphere electron transfer, and in hemoglobin oxidations by such agents as ferricyanide (12) or ferricytochrome *c* (6), this latter explanation is most consistent with available data. However, structural effects on hemoglobin oxidation by alkyl nitrites offer a convincing argument for the inner sphere electron transfer process.

Variations of k_o with alkyl nitrite structure (Fig. 8) exhibit trends that are similar, but not parallel, to those observed in kinetic and equilibrium data for isonitrile association with hemoglobin (17–19). Structural effects in isonitrile association with hemoglobin have been interpreted in terms of favorable hydrophobic interactions and unfavorable steric effects in the protein cavity surrounding the ligand at the sixth coordination position of the heme iron, and a similar explanation may be offered for structural effects in alkyl nitrite oxidations. In this latter case, however, association of the

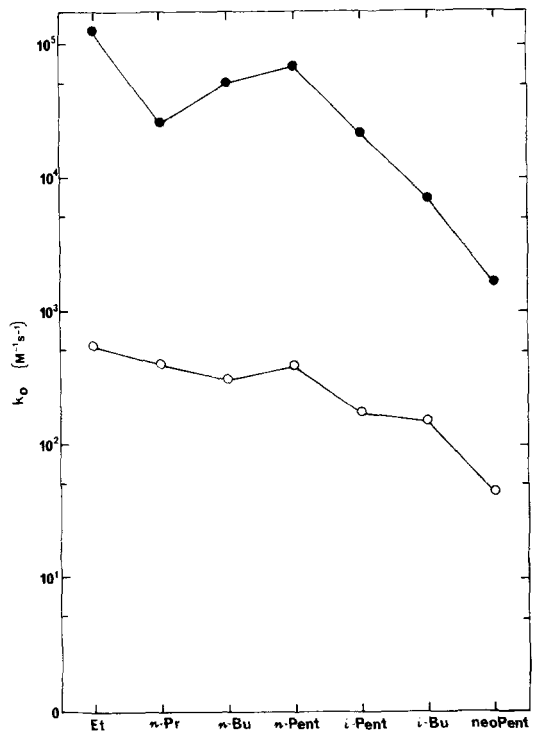
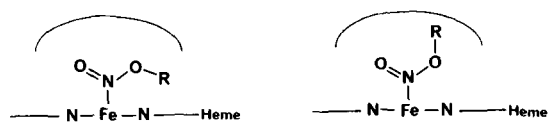


FIG. 8. **Dependence of oxidation rate constants on alkyl nitrite structure.** Data taken from Tables I and II. ●, oxyhemoglobin oxidations; O, deoxyhemoglobin oxidations. Abbreviations for alkyl nitrites are given in Fig. 2.

alkyl nitrite with iron(II) is anticipated to occur at nitrogen (A and B) in accord with the association of hemoglobin with



A B

nitroso compounds (37), and the structural effects that define alkyl nitrite oxidations cannot be expected to parallel those observed for isonitrile association. The alkyl group of nitrite esters is not rigidly affixed to a particular geometry with respect to the nitroso group but is flexible in its orientation within the bonding constraints of the ether linkage. Consequently, conformational orientation A can be expected to contribute to the net structural arrangement of the complex when R is unbranched and be of significantly lesser importance for α-branched alkyl groups. *tert*-Butyl nitrite, which cannot adopt conformation A for steric reasons, is the least reactive of all of the alkyl nitrites examined, and in reactions with deoxyhemoglobin at 10 °C, this nitrite is capable of oxidizing only half of the available heme units, presumably only the β-chains (19) of hemoglobin.

The mechanism of alkyl nitrite oxidations of hemoglobin can be described as involving initial nitrite association with iron(II) followed by electron transfer from iron(II) to the bound alkyl nitrite.

$$\text{Hb} + \text{RONO} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{Hb(RONO)} \qquad (2)$$

$$\text{Hb(RONO)} \xrightarrow{k_3} \text{MetHb} + [\text{RONO}]^- \qquad (3)$$

$$[\text{RONO}]^- + \text{H}^+ \rightarrow \text{ROH} + \text{NO} \qquad (4)$$

Protonation of the resulting alkyl nitrite radical anion results in the formation of alcohol and in the liberation of nitric oxide. That nitric oxide dissociation does not occur in the rate-limiting step is suggested by the absence of a pH dependence on the rates for ethyl nitrite oxidation of either deoxyhemoglobin (15) or oxyhemoglobin (16). However, attempts to electrochemically generate alkyl nitrite radical anions and to establish their reduction potentials by conventional methods (38) were not successful. The fate of nitric oxide in oxygen-saturated and deoxygenated media has previously been discussed (15, 16).

Spectral evidence for a stable complex between alkyl nitrites and hemoglobin has not been obtained. However, an estimate of the equilibrium constant (k_2/k_{-2}) for association-dissociation with ethyl nitrite at 10°C, which was calculated from product data for reactions with deoxyhemoglobin, has been given as 4×10^{-3} M^{-1} (15). Furthermore, the large positive $\Delta S\ddagger$ values determined for alkyl nitrite oxidations of hemoglobin (Table III) point to the formation of a complex between the reactants in both oxygenated and deoxygenated media. Partial oxidation of deoxyhemoglobin by *tert*-butyl nitrite also demands the mechanistic requirement of alkyl nitrite association with iron(II).

Hemoglobin, which under reaction conditions utilized in this study exists principally in its tetrameric form (39, 40), is viewed as undergoing oxidation by alkyl nitrites in a stepwise process. Restrictive oxidation by *tert*-butyl nitrite points to a substantial difference in the accessibility of this alkyl nitrite to the α and β subunits of hemoglobin and suggests, by analogy with conclusions drawn from investigations of isonitrile association with hemoglobin (19), that access by *tert*-butyl nitrite to the α subunits, particularly in the low affinity T state conformation, is blocked. In addition, inhibition of oxidation of α subunits is, as indicated by $k_{obs}^{slow}/k_{obs}^{fast}$ values for oxidation of deoxyhemoglobin by alkyl nitrites (Table II), more pronounced with secondary alkyl nitrites than with primary alkyl nitrites.

Prior results for oxidations of hemoglobin in oxygenated and deoxygenated media have been interpreted in terms of R and T state reactivities (16). The $\Delta G\ddagger$ values obtained for these oxidations provide confirmation of this hypothesis. Oxidations of hemoglobin in oxygenated media are characterized by uniformly lower $\Delta G\ddagger$ values than are oxidations in deoxygenated media (Table III). The energy differences, $\Delta G\ddagger$ (T)-$\Delta G\ddagger$ (R), for alkyl nitrite oxidations are comparable with the 1.5–2.7 kcal/mol for differences in T and R state barrier potentials determined for isonitrile association with hemoglobin (19).

Sulfhydryl-induced Alkyl Nitrite Oxidation of Hemoglobin—The effect of sulfide formation at the β-93 cysteine position of hemoglobin on the rate constant for oxidation of hemoglobin at or near oxygen saturation and the absence of rate enhancement by free cysteine in ethyl nitrite oxidation of HbO_2 demonstrate that the β-93 sulfhydryl residue is intimately involved in the oxidation of iron(II) in the β subunits of hemoglobin. Since nitrosyl exchange with thiols occurs readily (25–27), even in aqueous media, the first step in this oxidation certainly involves thionitrosyl formation at the β-93 position.

$$Hb(\beta\text{-}93\text{-}SH) + RONO \rightarrow Hb(\beta\text{-}93\text{-}SNO) + ROH \quad (5)$$

Although a direct measure of the rate for nitrosyl transfer to the β-93 cysteine residue relative to nitrosyl transfer to free cysteine is not available, comparative influences of the β-93 cysteine residue and free cysteine suggest that nitrosyl transfer with the protein-bound sulfhydryl group occurs at a faster rate. Oxidation of iron(II) by the internal thionitrosyl can be described to occur in several ways but, since neither oxygen inhibition of this electron transfer process nor detectable influences of the β-93 sulfhydryl group in oxidations of deoxyhemoglobin are observed, nitric oxide dissociation from the thionitrosyl followed by oxidation of nitric oxide by the iron(II) bound dioxygen (41, 42) affords a reasonable explanation for the observed oxidative transformation:

$$Hb(O_2)(\beta\text{-}93\text{-}SNO) \rightarrow MetHb(\beta\text{-}93\text{-}S^{\cdot}) + NO_3^- \quad (6)$$

However, since neither the rate for alkyl nitrite oxidation of deoxyhemoglobin nor the percentage of HbNO produced in this transformation are measurably affected by the sulfhydryl group, the direct involvement of iron-bound dioxygen, *eg.*

$$Hb(O_2)(\beta\text{-}93\text{-}SNO) \rightarrow MetHb(\beta\text{-}93\text{-}S^-) + O_2 + NO \quad (7)$$

$$Hb(O_2) + NO \rightarrow MetHb + NO_3^- \quad (8)$$

cannot be excluded from consideration. The absence of any increase in the rate of oxidation of HbO_2 due to the presence of added free cysteine may be due to the occurrence of nitrosative reactions by this thionitrite, transformations that are prohibited for the protein interlocked β-93 cysteine thionitrite.

REFERENCES

1. Kawanishi, S., and Caughey, W. S. (1979) in *Biochemical and Clinical Aspects of Oxygen* (Caughey, W. S., ed) pp. 27–34, Academic Press, New York
2. Winterbourn, C. C., and Carrell, R. W. (1977) *Biochem. J.* **165,** 141–148
3. Tomoda, A., and Yoneyama, Y. (1979) *Experientia* **35,** 15–16
4. Okonjo, K. (1980) *J. Biol. Chem.* **255,** 3274–3277
5. Yamada, T., Marini, C. P., and Cassatt, J. C. (1978) *Biochemistry* **17,** 231–236
6. Tomoda, A., Tsuji, A., and Yoneyama, Y. (1980) *J. Biol. Chem.* **255,** 7978–7983
7. De Vault, D., Parks, J. H., and Chance, B. (1967) *Nature (Lond.)* **215,** 642–644
8. Hopfield, J. J. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 3640–3644
9. Mauk, A. G., Scott, R. A., and Gray, H. B. (1980) *J. Am. Chem. Soc.* **102,** 4360–4363
10. Cummins, D., and Gray, H. B. (1977) *J. Am. Chem. Soc.* **99,** 5158
11. Augustin, M. A., and Yandell, J. K. (1979) *Inorg. Chim. Acta* **37,** 11–18
12. Antonini, E., Brunori, M., and Wyman, J. (1965) *Biochemistry* **4,** 545–551
13. Cassatt, J. C., Marini, C. P., and Bender, J. W. (1975) *Biochemistry* **14,** 5470–5475
14. Rifkind, J. M. (1979) *Biochemistry* **18,** 3860–3865
15. Doyle, M. P., Pickering, R. A., DeWeert, T. M., Hoekstra, J. W., and Pater, D. (1981) *J. Biol. Chem.* **256,** 12393–12398
16. Doyle, M. P., Lepoire, D. M., and Pickering, R. A. (1981) *J. Biol. Chem.* **256,** 12399–12404
17. Reisberg, P. I., and Olson, J. S. (1980) *J. Biol. Chem.* **255,** 4144–4150
18. Reisberg, P. I., and Olson, J. S. (1980) *J. Biol. Chem.* **255,** 4151–4158
19. Reisberg, P. I., and Olson, J. S. (1980) *J. Biol. Chem.* **255,** 4159–4169
20. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82,** 70–77
21. Banerjee, R., Alpert, Y., Leterrier, F., and Williams, R. J. P. (1969) *Biochemistry* **8,** 2862–2867
22. Moore, E. G., and Gibson, Q. H. (1976) *J. Biol. Chem.* **251,** 2788–2794
23. Noyes, W. A. (1943) in *Organic Syntheses,* Collective Vol. II, pp. 108–109, Wiley, New York
24. Berthmann, A., and Ratz, H. (1963) in *Methoden der Organischem Chemie* (Houben-Weyl-Müller) 3rd Ed., Vol. 6/2, pp. 325–362, Georg Thieme Verlag, Stuttgart
25. Doyle, M. P., Terpstra, J. W., Pickering, R. A., and LePoire, D. M. (1983) *J. Org. Chem.* **48,** 3379–3382
26. Lecher, H., and Siefken, W. (1926) *Chem. Ber.* **59B,** 2594–2601
27. Lecher, H., and Siefken, W. (1926) *Chem. Ber.* **59B,** 1314–1321

28. Field, L., Dilts, R. V., Ravichandran, R., Lenhert, P. G., and Carnahan, G. E. (1978) *J. Chem. Soc. Chem. Commun.* 249–250
29. Oae, S., Fukushima, D., and Kim, Y. H. (1977) *J. Chem. Soc. Chem. Commun.* 407–408
30. Schulz, U., and McCalla, D. R. (1969) *Can. J. Chem.* **47,** 2021–2027
31. Doyle, M. P., Pickering, R. A., Dykstra, R. L., Nelson, C. L., and Boyer, R. F. (1982) *Biochem. Biophys. Res. Commun.* **105,** 127–132
32. Tomoda, A., Yoneyama, Y., and Tsuji, A. (1981) *Biochem. J.* **195,** 485–492
33. Pryor, W. A., Church, D. F., Govindan, C. K., and Crank, G. (1982) *J. Org. Chem.* **47,** 156–159
34. Edelstein, S. J., and Gibson, Q. H. (1975) *J. Biol. Chem.* **250,** 961–965
35. Gelin, B. R., and Karplus, M. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 801–805
36. Warshel, A. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 1789–1794
37. Holecek, V., Kopecky, J., and Skramovsky, S. (1979) *Collect. Czech. Chem. Commun.* **44,** 981–985
38. Blankespoor, R. L., Doyle, M. P., Hedstrand, D. M., Tamblyn, W. H., and Van Dyke, D. A. (1981) *J. Am. Chem. Soc.* **103,** 7096–7101
39. Edelstein, S. J., Rehmar, M. J., Olson, J. S., and Gibson, Q. H. (1970) *J. Biol. Chem.* **245,** 4372–4381
40. Barksdale, A. D., and Rosenberg, A. (1978) *J. Biol. Chem.* **253,** 4881–4885
41. Doyle, M. P., and Hoekstra, J. W. (1981) *J. Inorg. Biochem.* **14,** 351–358
42. Doyle, M. P., Pickering, R. A., and Cook, B. R. (1983) *J. Inorg. Biochem.*, in press