data, we hope that our current studies of the temperature dependence of the unfolding will shed some light on this question.

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Contribution of Aromatic Residue Interactions to the Stability of Myoglobin. IV. Delineation of Binding Forces between Aromatic Compounds and Myoglobin*

John R. Cann

ABSTRACT: Aromatic compounds exert their enhancing effect on the rate of urea denaturation of myoglobin by complexing generally with two sites in the hemoprotein. The complexes are fundamentally of the electron donor–acceptor type with the aromatic compound acting as the donor, but hydrophobic interactions make a substantial contribution to their stability. Steric configuration of the aromatic compound and coulombic repulsion of donors with cationic side chains are also of importance. In fact, the relative rate enhancement of certain compounds is determined by a delicate balance of these forces. Whereas the urea denaturation of apomyoglobin is unaffected by a representative aromatic compound, reconstituted myoglobin shows the same sensitivity as myoglobin. Reversible complex formation has been demonstrated between hemin and β-naphthoate or 3-indolebutyrate and between hematoporphyrin and β-naphthoate. The above results are synthesized within the context of the three-dimensional structure of myoglobin with special reference to the heme moiety and its environs.

Finally, some interpretative comments are made on the band structure of spectra of model charge-transfer complexes.

As reported previously (Cann, 1965, 1967) aromatic compounds as diverse as benzene and chlorpromazine have an enormous and specific enhancing effect upon the rate of reaction of Zn^{2+} with myoglobin and the rate of urea denaturation of the protein. Kinetic and spectroscopic evidence has been advanced in support of our interpretation that these compounds exert their effect by forming electron donor–acceptor (charge-transfer) complexes with the heme moiety of myoglobin, the aromatic compounds serving as donors. Charge transfer is not the only force involved, however. Thus, there is an important hydrophobic contribution to the stability of the complex. Indeed, subsequent experiments suggested that in certain instances the balance of forces is such that hydrophobic interactions play a decisive role in determining the relative strength of complexing with the heme of two compounds having different electron-donating tendencies. A case in point is illustrated in Figure 1, which compares the effectiveness of benzene, naphthalene, and indole in enhancing the rate of urea denaturation of myoglobin. If charge transfer were the only force that need be considered, one would expect the effectiveness of these compounds to increase in the aforementioned order which is their order of increasing electron-donating tendency.[1] Ac-

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[1] The theoretical ordering of these compounds as electron donors was verified experimentally by comparing the wavelengths of maximum absorption of their charge-transfer complexes with the acceptor molecule, chloranil, in carbon tetrachloride. The maximum wavelength of the charge-transfer band generally increases with decreasing molecular ionization potential of the donor, *i.e.*, with increasing tendency to donate electrons. The maximum wavelength and color of the complexes are: benzene, 347 mμ, yellow; naphthalene, bimodal with maxima at 384 and 478 mμ, orange; indole, 505 mμ, magenta. The benzene and naphthalene data are given in Table 7, p 30 of Briegleb (1961), the latter being confirmed in this investigation; indole, this investigation.

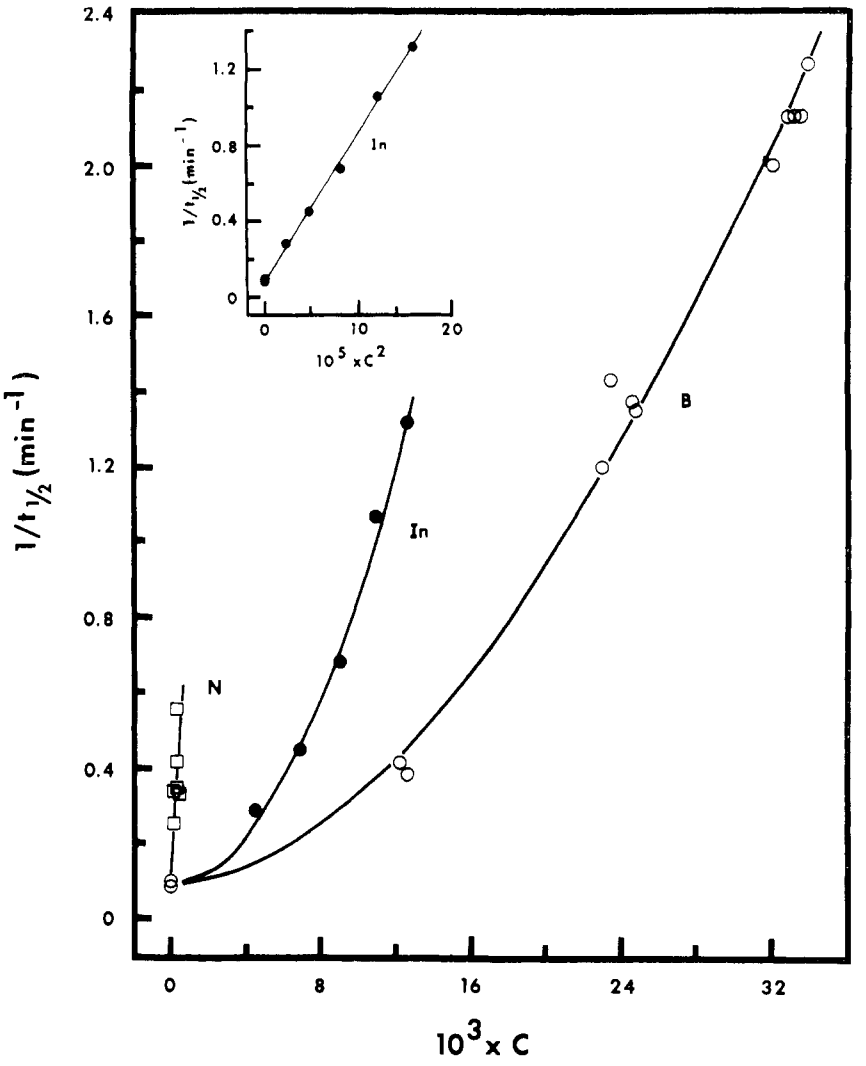

FIGURE 1: Comparison of the enhancement of the rate of urea denaturation of ferrimyoglobin by benzene (B), naphthalene (N), and indole (In). Plot of the reciprocal of the half-time of denaturation, $1/t_{1/2}$, *vs.* the molar concentration, C, of added aromatic compound. Order of reaction with respect to added aromatic compound: B, 1.8; N, uncertain because of limited concentration range; In, 2 (see insert), B and N data from Cann (1967).

tually, the ordering with respect to effectiveness is benzene < indole < naphthalene. This apparent discrepancy is seemingly resolved by noting that naphthalene, which is a more hydrophobic compound than indole, would consequently have a greater preference for the nonpolar environment of the heme moiety (Kendrew, 1962). Evidently, the difference in hydrophobicity more than balances the difference in electron-donating ability as a result of which naphthalene is more effective than indole.

The above considerations point up the need for a more precise delineation of the several forces involved in the complexing of aromatic compounds with myoglobin. Toward this end we have examined selected series of homologous compounds of approximately the same electron-donating tendencies but progressively increasing hydrophobicity, and *vice versa*, for their ability to enhance the rate of urea denaturation of the protein. Steric and electrostatic factors have also been explored. Lastly, experiments on apomyoglobin, reconstituted myoglobin, and the molecular complexes of hemin and hematoporphyrin with two representative aromatic compounds have been made in order to define as clearly as possible the binding sites in the hemoprotein.

Methods

The sperm whale ferrimyoglobin and hematoporphyrin (free base) were obtained from Mann Research Analytical Laboratories. The hemin was Eastman's recrystallized protohemin chloride. Apomyoglobin prepared by the method of Breslow (1964) was stored at 0° and used within a week. Its combining capacity for hemin (1.02 moles of hemin/mole of globin) was determined as described by Breslow and coworkers (1965) except that absorbances were measured at 408 mμ. Judging from the absorbance at the equivalence point, the globin preparation was about 99% native protein. Reconstituted ferrimyoglobin for urea denaturation studies was prepared by the 1:1 addition of hemin (in 0.16 ionic strength sodium borate buffer, pH 9.2) to a 0.1% solution

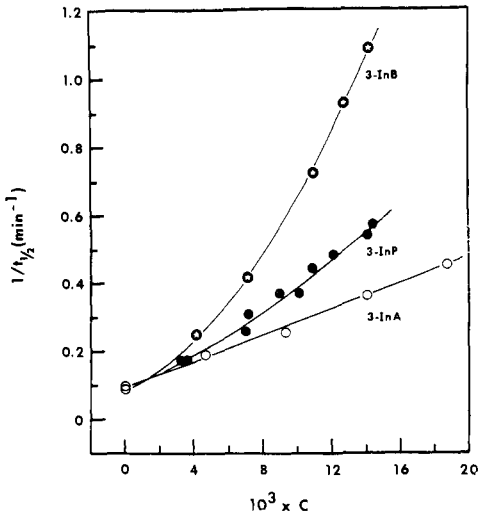

FIGURE 2: Comparison of the effectiveness of 3-indoleacetate (3-InA), 3-indolepropionate (3-InP), and 3-indolebutyrate (3-InB) in enhancing the rate of urea denaturation of myoglobin. Order of the reaction with respect to aromatic compound: 3-InA; 1; 3-InP, 1.3; 3-InB, 1.6. The 3-InP data are better fitted to a 1.3-order relationship than a least-squares straight line, the standard error of estimate being about 35% less for the former. In the case of 3-InB, the standard error of estimate is a factor of about 3 smaller for 1.6 order than 2 order.

of globin in the same buffer followed by dialysis in the cold against three changes of 0.05 M sodium acetate buffer–10^{-4} M EDTA (pH 5.82).

Analytical reagent grade urea was recrystallized from 70% ethanol. The highest quality, commercially available aromatic compounds were purified further by standard procedures and their melting points were checked against literature values. Amines such as phenethylamine were converted into their hydrochlorides and recrystallized; serotonin hydrogen oxalate was converted into the hydrochloride on an anion-exchange resin. Racemic hydratropic acid (2-phenylpropionic acid) was resolved into its optical antipodes by the method of Pettersson (1956–1957) and their melting points and optical rotations were checked against literature values.

The time course of urea denaturation was followed by measuring the decrease in Soret absorbance in a thermostated Beckman DK-2 spectrophotometer as described previously (Cann, 1967). Rate measurements were made at 28 ± 0.01° on solutions containing 0.01% myoglobin, 6.4 M urea, and 0.05 M sodium acetate buffer; pH of reaction mixture, 5.86 ± 0.02 except for the naphthalene derivatives which were examined at pH 5.98. The ionic strength of reaction mixtures containing aromatic salts was maintained at 0.05 M by downward adjustment of the acetate buffer. Results are displayed as plots of the reciprocal of the half-time of denaturation, $1/t_{1/2}$, *vs.* the molar concentration, C, of added aromatic compound. The order of the reaction with respect to the added aromatic compound was determined by statistical analysis and is given in the figure legend. Unless otherwise indicated, the curve through the data points is the least-squares curve for the given order. In the case of compounds showing simple second-order kinetics, the corresponding plots are sometimes included in the figures as inserts.

Optical rotatory dispersion measurements on the urea denaturation of apomyoglobin were made using the Cary Model 60 instrument at 27° with a path length of 1 cm on samples containing about 2 mg of protein/ml.

Spectroscopic measurements on the colored molecular complexes formed between donors such as 3-indolebutyrate and the acceptor, methyl viologen, were made in the thermostated DK-2 spectrophotometer using either tandem cells with 0.44-cm light path/compartment or, when the temperature was to be varied, standard cells of 1-cm light path. In the former case, the front compartment of the sample cell contained the reaction mixture and the rear compartment, solvent. The front compartment of the reference cell contained methyl viologen and the rear compartment, the donor. When standard cells were used, the reference contained methyl viologen. Actually, both donor and acceptor were transparent in the region of the charge-transfer band. Experiments were made by varying the methyl viologen concentration at constant donor concentration and *vice versa*, with the methyl viologen always in large excess. Reversibility was tested by dilution. A reversible 1:1 complex was indicated in each case, and the data were analyzed in accordance with the corresponding mass action relationship

$$\frac{(\text{donor})l}{A} = \frac{1}{K\epsilon(\text{methyl viologen})} + \frac{1}{\epsilon}$$

where the parentheses denote molar concentration; K, association constant; ϵ, molar extinction coefficient of the complex; and A, absorbance of the complex observed with light path, l. Values of K and ϵ were determined by the method of least squares.

Molecular complexes of hemin and hematoporphyrin were investigated using a technique devised previously (Cann, 1967) to avoid complications arising from aggregation of these materials in free solution. This is achieved by allowing complex formation to occur between aromatic compounds and hemin or hematoporphyrin bound to serum albumin. Hemin or hematoporphyrin dissolved in 0.1 M NaOH was diluted into a buffered solution of Armour's crystallized bovine plasma albumin (Lot No. C70710). These were diluted in turn with a buffered solution of the aromatic compound to give a final reaction mixture containing 0.05 M acetate buffer (pH 6.0). Its spectrum was measured with either the DK-2 instrument or a Cary Model 14 spectrophotometer.

Finally, a few words are in order concerning the methods used to test reversibility of complex formation. In the case of the several donors with methyl viologen, the spectra of 25-fold dilutions of the most concentrated reaction mixtures (buffer concentration and pH held constant, 10-cm light path) were compared with the original spectra (0.44-cm light path), and where the requisite information had been determined, the absorbance of the diluted mixture was compared with that predicted by the mass action relationship. In the case of aromatic compounds with hemin and hematoporphyrin, the Soret band of a 25-fold dilution of the reaction mixture (buffer concentration, pH, and albumin concentration held constant, 10-cm light path) was compared with control spectra of the same low concentration of hemin or hematoporphyrin and admixtures with varying concentration of aromatic compound. With hemin, the original reaction mixtures contained less aromatic compound than used in the

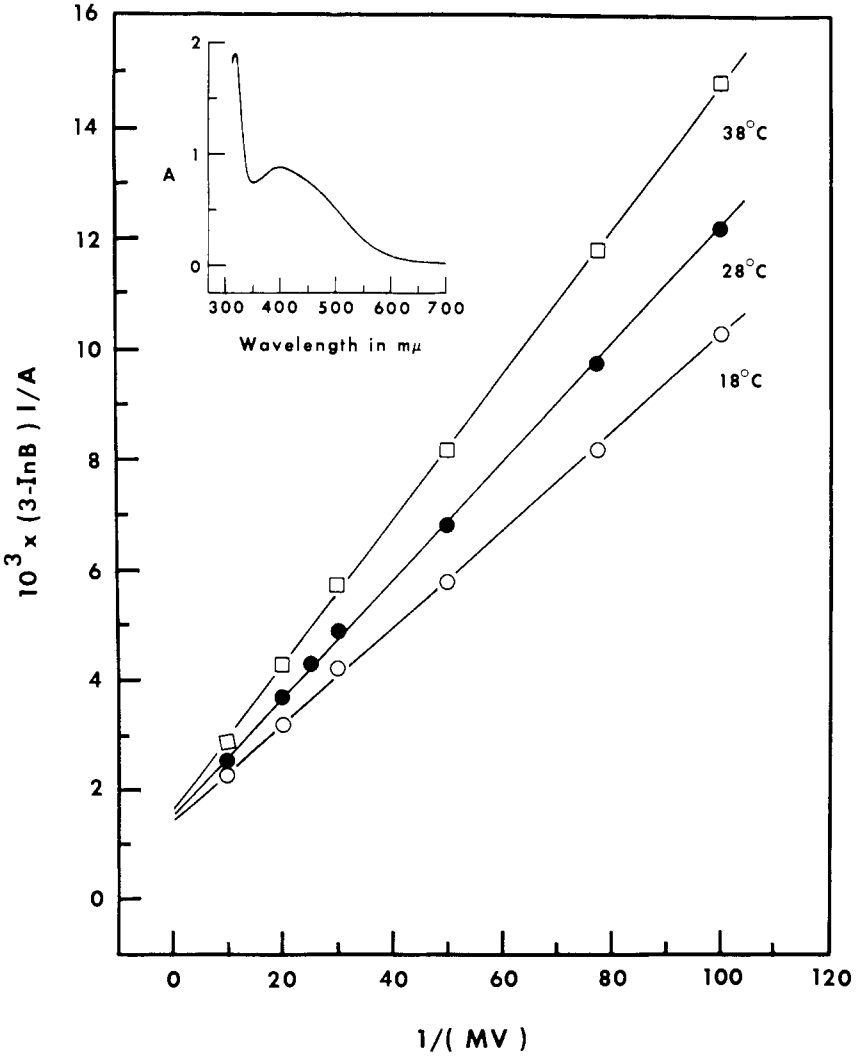

FIGURE 3: Molecular complexing between 3-indolebutyrate (3-InB) and methyl viologen (MV) in 0.5 M acetate buffer (pH 6.00 ± 0.05) at indicated temperatures. Absorbance was measured at 400 mμ, 1-cm light path. Values of K, ϵ, and ΔH° given in Table I. Insert is spectrum of complex formed at 28° in solution containing 5×10^{-3} M 3-indolebutyrate and 0.1 M methyl viologen; 0.44-cm light path. About 90% dissociation of the complex occurred upon 25-fold dilution; the effect of temperature was also reversible.

illustrative examples quoted in the text but sufficient to have a very marked effect on the Soret band.

Results

Hydrophobic Interactions. The first series of compounds examined were homologs of 3-indolecarboxylate in which the carbon straight chain separating the indole moiety from the carboxylate group becomes progressively longer on going from 3-indoleacetate to 3-indolebutyrate. As shown in Figure 2 these compounds have a rather strong enhancing effect upon the rate of urea denaturation of myoglobin. Moreover, they exert their action on a limited portion of the protein molecule. Thus, the reaction is first order with respect to 3-indoleacetate and intermediate between first and second order for the other two compounds. The latter indicates that these compounds bind at two sites in the hemoprotein with different association constants such that the reaction mixture contains uncomplexed protein and its complexes with one or two aromatic molecules, each species denaturating at a different rate. Of greater importance for this investigation is the ordering of these compounds according to their effectiveness in enhancing the rate of denaturation, 3-indoleacetate < 3-indolepropionate < 3-indolebutyrate.

On chemical grounds it does not seem likely that the ordering simply reflects increasing electron-donating tendency, although this may be a contributing factor. To assess this factor more fully, comparative measurements were made on the colored molecular complexes of 3-indoleacetate and 3-indolebutyrate with the acceptor molecule, methyl viologen. As illustrated by the measurements presented in Figure 3 and the spectral and thermodynamic parameters summarized in Table I, these complexes have properties characteristic of electron donor–acceptor complexes, *i.e.*, a broad structureless absorption band of low molar extinction coefficient and a negative standard enthalpy of formation. (It is of some interest that in addition to the usual charge-transfer band, the spectra also show a sharp, considerably more intense band at shorter wavelength.) The results for 3-indoleacetate and 3-indolebutyrate are compared in Table I. Although the wavelength

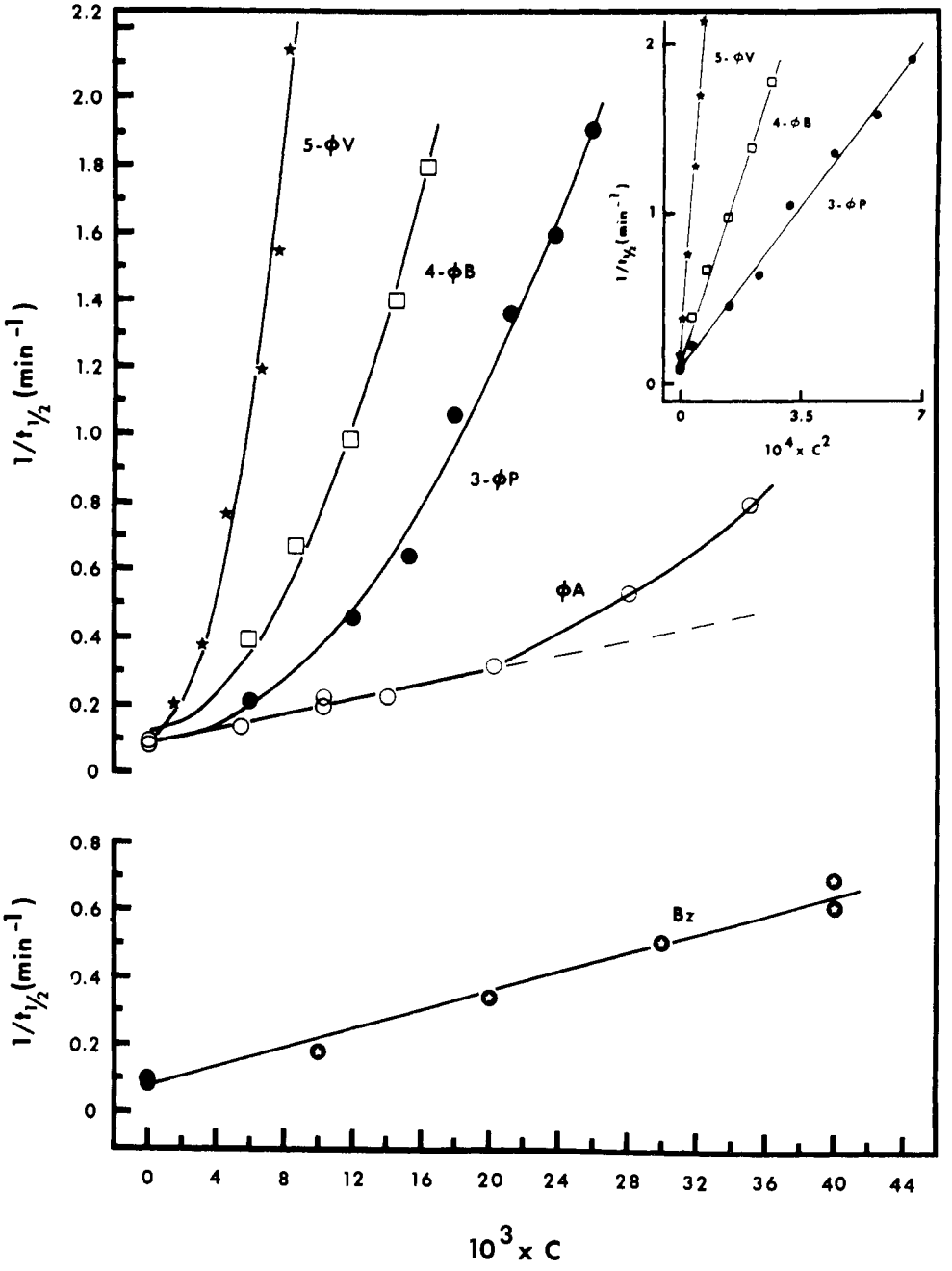

FIGURE 4: Comparison of the effectiveness of benzoate and its homologs in enhancing the rate of urea denaturation: Bz, benzoate; φA, phenylacetate; 3-φP, 3-phenylpropionate; 4-φB, 4-phenylbutyrate; 5-φV, 5-phenylvalerate. Order of reaction with respect to aromatic compounds: Bz, first order; 3-φP, 4-φB and 5-φV, second order (see insert). In the case of φA the rate is evidently first order for concentrations up to 0.02 M, departing markedly therefrom at higher concentrations; and the curve through the points is drawn to stress this feature. However, the data does give quite a good least-squares fit to a second-order relationship over the entire concentration range, although the standard error of estimate is almost twice as large as for the straight line through the lower concentration points. Bz data from Cann (1967).

of maximum absorbance of the charge-transfer band differs somewhat for the two donors, the values of K are the same within experimental error. This is so in urea solution as well as in acetate buffer. The conclusion seems justified that differences in the electron-donating tendency of the homologs cannot account for the huge differences in their rate enhancement. Accordingly, two alternative explanations must be entertained; namely, increasing hydrophobicity due to increasing length of the carbon chain separating the indole ring from the carboxylate group or a purely geometrical effect on protein binding due to increasing separation of the indole and carboxylate moieties *per se*. In order to decide between these two possibilities appeal was made to another series of compounds which lend themselves to a more penetrating analysis.

The compounds in question are homologs of benzoate in which a carbon straight chain separates the phenyl ring from the carboxylate group. Their effect on the rate of urea dena-

TABLE I: Some Spectral and Thermodynamic Parameters of Molecular Complexes of Several Electron Donors with Methyl Viologen.

Donor	λ_{max}[a] (mμ)	ϵ[b] (l. mole^{-1} cm^{-1})	K[b,c] (l. mole^{-1})	$\Delta H°$[d] (kcal mole^{-1})
Solvent: 0.5 M sodium acetate buffer, pH 6.00 ± 0.05				
3-Indoleacetate	390	660 ± 36	13.5 ± 0.78	−2.5 ± 0.4
3-Indolebutyrate	400	640 ± 26	14.7 ± 0.61	−2.5 ± 0.6
α-Naphthoate	375	420 ± 24	9.1 ± 0.54	
α-Naphthol	433	490 ± 14	13.2 ± 0.38	
Solvent: 6.4 M urea–0.05 M sodium acetate buffer, pH 5.90 ± 0.05				
3-Indoleacetate	395	370 ± 71	14 ± 3.0	
3-Indolebutyrate	405	390 ± 49	12 ± 1.6	

[a] Maximum wavelength of the charge-transfer band except for α-naphthoate whose charge-transfer band does not show a maximum due to overlap with the more intense band at shorter wavelength. [b] Value plus and minus standard deviation. [c] Value at 28 ± 0.01°. [d] Value plus and minus mean deviation.

turation is shown in Figure 4. Three conclusions can be drawn: (a) Whereas the rate of denaturation is first order with respect to benzoate, it is second order with respect to its homologs (see legend for analysis of the phenylacetate data). (b) The ordering of these compounds with respect to effectiveness in enhancing the rate is benzoate < phenylacetate < 3-phenylpropionate < 4-phenylbutyrate < 5-phenylvalerate. (c) The effectiveness of the homologs approximately doubles upon each addition of a methylene group to the carbon chain. The latter is the key to our problem, for we recall that the standard free energy of transfer of the lower, straight-chain aliphatic hydrocarbons from aqueous solution to the liquid phase (or nonpolar solution) increases linearly with increasing chain length. Since the homologs of benzoate evidently exert their action by binding at two sites, comparison should be made between the second-order dose rate defined as $k_2 = d(1/t_{1/2})/dC^2$ and the quantity, exp $(-2\Delta F°/RT)$, where $\Delta F°$ is the standard free energy of transfer per mole of hydrocarbon. The comparison is made in Figure 5, which presents semilogarithmic plots of each quantity against the number of carbon atoms in the straight chain. It is immediately apparent that there is an intimate relationship between the second-order dose rate for a given homolog and the hydrophobicity of its aliphatic carbon chain. The difference in the slope of the two lines in the figure is interpreted to mean that the free energy of transfer of the hydrocarbon moiety from urea solution to protein is less than from aqueous solution to liquid hydrocarbon. These results constitute strong support for increasing hydrophobicity as the major force responsible for the ordering not only of the benzoate but also, by induction, the 3-indolecarboxylate homologs with respect to their rate enhancement. This conclusion is in accord with previous observations (Cann, 1967) on alkylbenzene homologs of approximately the same molecular ionization potential but different hydrophobicity. Their ordering with respect to rate enhancement, toluene < ethylbenzene < *n*-propylbenzene, is the same as for increasing hydrophobicity.

Charge-Transfer Forces. The foregoing demonstration of the importance of hydrophobic forces for the interaction of aromatic compounds with myoglobin prompted further inquiry into the contribution of charge-transfer forces. Thus, measurements were made on a series of naphthalene derivatives having about the same hydrophobicity but quite different electron-donating tendencies. The results displayed in Figure 6 show that these molecules also act on a very limited

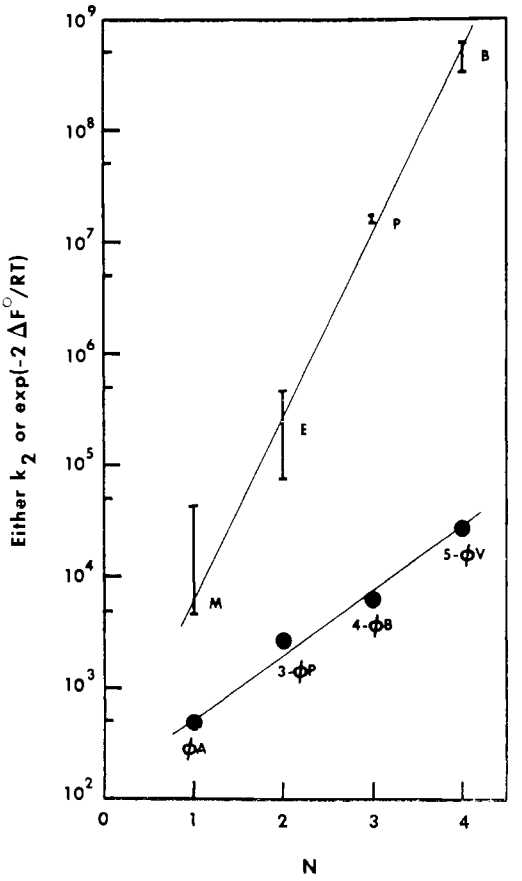

FIGURE 5: Semilogarithmic plots of the second-order dose rate, k_2 (l.^{2} mole^{-2} min^{-1}), for benzoate homologs (lower line) and the quantity, exp $(-2\Delta F°/RT)$, for aliphatic hydrocarbons against the number of carbon atoms, N, in the straight chain: M, methane; E, ethane; P, propane; B, butane; other symbols as in Figure 4. Values of $\Delta F°$ from Nemethy and Scheraga (1962).

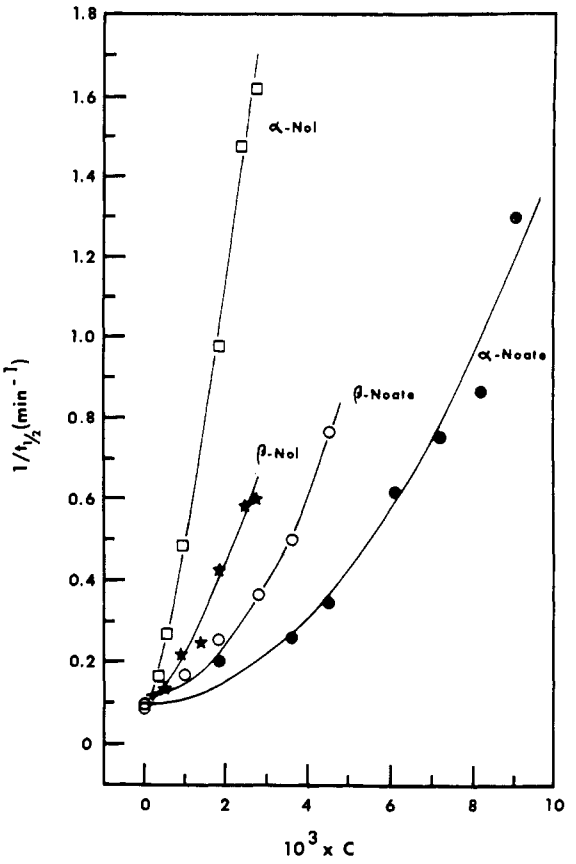

FIGURE 6: Comparison of the effectiveness of naphthalene derivative in enhancing the rate of urea denaturation: α-Noate, α-naphthoate; β-Noate, β-naphthoate; β-Nol, β-naphthol; α-Nol, α-naphthol. Order of reaction with respect to added aromatic compound: α-Noate and β-Noate, 2; β-Nol, 1.4; α-Nol, 1.3. Standard error of estimate for β-Nol is about 40% lower for 1.4-order than first or second order; α-Nol, a factor of 2 lower for 1.3-order than first or second order.

portion of the myoglobin molecule and that their ordering with respect to increasing rate enhancement is α-naphthoate < β-naphthoate < β-naphthol < α-naphthol. This is also their ordering with respect to increasing electron-donating tendency as judged both on chemical grounds and from the progressive red shift of the charge-transfer band of their molecular complexes with methyl viologen on going from α-naphthoate to α-naphthol (Figure 7). Moreover, the association constant for complex formation is greater in the case of α-naphthol than α-naphthoate (Table I). These observations are in complete accord with our previous conclusion (Cann, 1967) that the complexes of aromatic compounds with myoglobin are fundamentally of the electron donor–acceptor type.

Concerning the spectra of the molecular complexes shown in Figure 7, we note that in the case of α-naphthol there are two well-resolved bands: the broad, structureless charge-transfer band at 433 mμ and a sharp, much more intense band at 327 mμ. The values of the association constant determined from measurements on each band are the same within experimental error. The spectra of the complexes of the other three donors also show two such bands, although they are not as well resolved. The characteristic parameters of the

TABLE II: Characteristic Parameters of the Short-Wavelength Absorption Band of Molecular Complexes and the First Discernable Band of Uncomplexed Donor.

Donor	λ_{max} (mμ)		$\Delta h\nu^b$ (kcal mole^{-1})	$10^{-3} \times \epsilon$ (l. mole^{-1} cm^{-1})	
	Mol Complex[a]	Uncomplexed Donor		Mol Complex[a]	Uncomplexed Donor
α-Naphthoate	320	313	−1.9	0.96	0.76
α-Naphthol	327	322	−1.5	1.89	2.34
β-Naphthol	337	328	−2.3		1.78
β-Naphthoate	338	329	−2.3		0.89

[a] With the acceptor, methyl viologen. [b] Difference in energy between the band of the complex and the uncomplexed donor.

short-wavelength band and those of the first discernable absorption band of uncomplexed donor in the same solvent are presented in Table II.

Steric Factors. While experiments on the enhancement of the rate of denaturation by the optical antipodes of 2-phenylpropionate showed no stereospecificity of interaction, they did reveal a pronounced influence of steric configuration. Thus, as can be seen from Figure 8, the branched-chain molecule, 2-phenylpropionate, is two- to threefold less effective than its straight-chain isomer, 3-phenylpropionate. Likewise, 3-phenylbutyrate is about twofold less effective than 4-phenylbutyrate. It is interesting that 2-phenylbutyrate, although considerably less effective than the straight-chain isomer, is significantly more effective than 3-phenylbutyrate.

Electrostatic Interactions. All 34 nonionic or anionic aromatic compounds examined to date cause a marked (albeit graded) enhancement of the rate of urea denaturation of myoglobin. In contrast, all but one of the seven tested donors with cationic side chains had little, if any, effect on the rate. These include phenethylamine-HCl, γ-phenylpropylamine-HCl, *N*-methylphenethylamine-HCl, and serotonin-HCl. The sole exception is the exceedingly strong electron-donor chlorpromazine-HCl, which is highly rate enhancing (Cann, 1967). The zwitterions L- and D-phenylalanine and L-tryptophan are also without effect. Nor do the ineffective cations inhibit denaturation or interfere with the action of rate-enhancing compounds. Thus, the rate of denaturation in a solution containing 8.55×10^{-3} M 4-phenylbutyrate and 3.15×10^{-2} M phenethylamine-HCl was the same (about sevenfold enhancement) as with 8.55×10^{-3} M 4-phenylbutyrate alone. Clearly, coulombic repulsion between aromatic cations and the protein binding sites or their environs is a force of major consequence.

Apoprotein and Reconstituted Myoglobin. Urea denaturation experiments have also been made on apomyoglobin and reconstituted myoglobin. Although the reversible denaturation of apomyoglobin proceeds too rapidly for conventional-type kinetic measurements, the final conformation of the molecule can be characterized by optical rotatory dispersion (Harrison and Blout, 1965). The experiments presented in

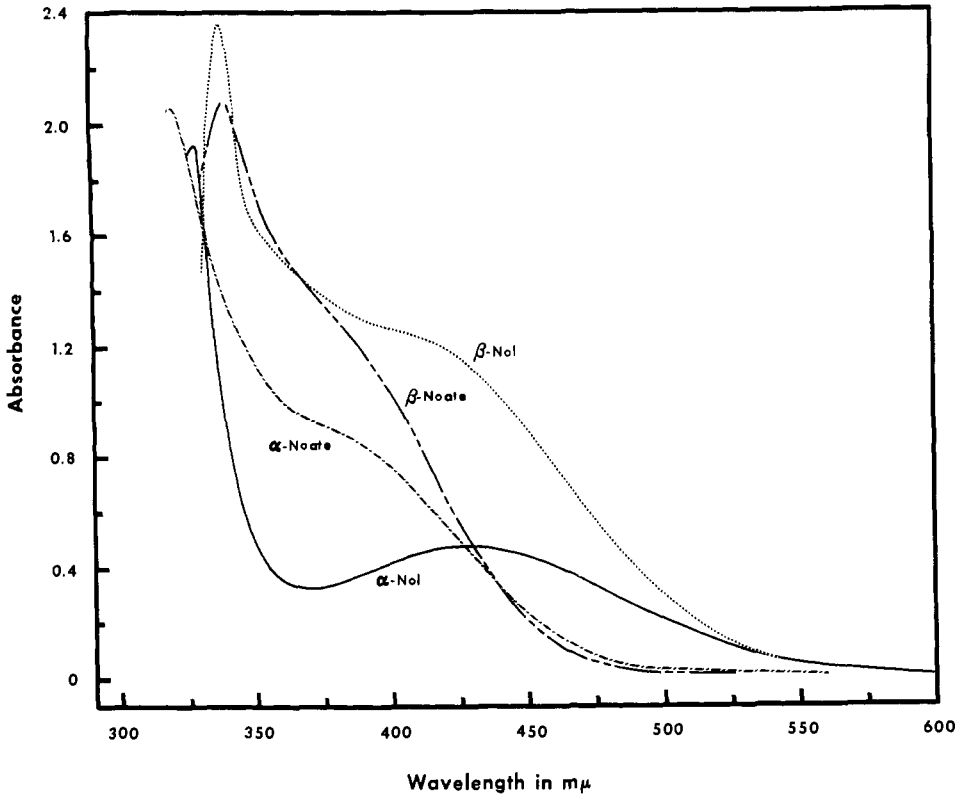

FIGURE 7: Absorption spectra of molecular complexes of naphthalene derivatives with methyl viologen. Symbols as in Figure 6. Absorbance scale shown for α-Nol; divide by 2 for α- and β-Noate and by 4 for β-Nol. Reaction mixtures contained 0.1 M methyl viologen and 4.93×10^{-3} M α-Noate, 4.66×10^{-3} M β-Noate, 2.08×10^{-3} M β-Nol, or 3.88×10^{-3} M α-Nol; 0.5 M sodium acetate buffer (pH 6.06), 28°, 0.44-cm light path. About 90% dissociation of each complex occurred upon 25-fold dilution.

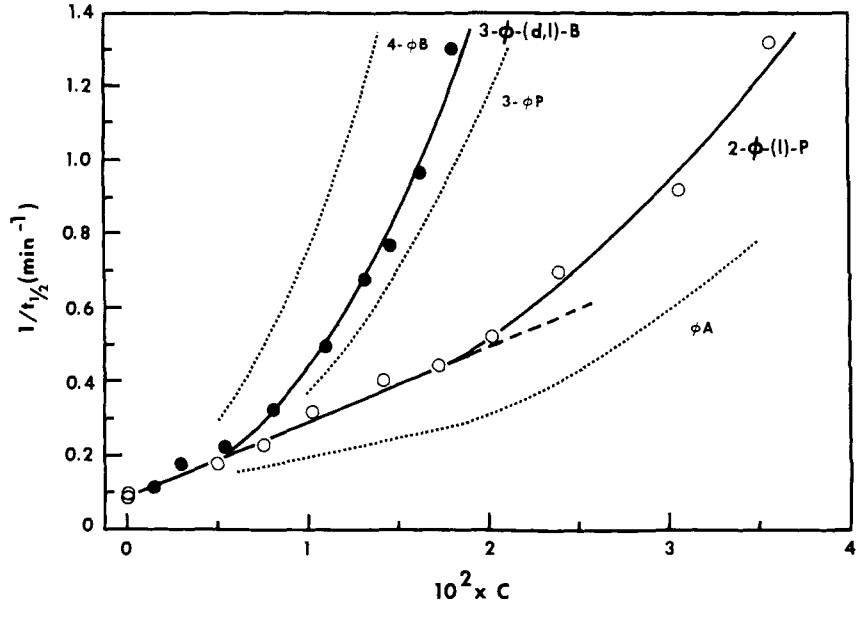

FIGURE 8: Comparison of steric isomers of benzoate homologs with respect to their effectiveness in enhancing the rate of urea denaturation: 2-φ-(*l*)-P, 2-phenyl-*l*-propionate; 3-φ-*dl*-B, 3-phenyl-*dl*-butyrate; other symbols as in Figure 4. In the interest of clarity, data for 2-phenyl-*d*-propionate (only slightly more effective than its antipode) and for 2-phenyl-*dl*-butyrate (considerably less effective than 4-φB but significantly more so than 3-φ-*dl*-B) are not shown. Although the 2-φ-*l*-P data give a good least-squares fit to a second-order relationship, the curve shown is meant to stress the first-order nature of the reaction over the lower half of the concentration range. The standard error of estimate for a least-square straight line through all but the three higher concentrations points was one-fourth (one-seventh in the case of the antipode) of that for a second-order curve through all the points.

Figure 9 show that 3-phenylpropionate at a concentration which causes about a tenfold enhancement of the rate of denaturation of myoglobin, has no effect on the conformational state of partially unfolded apomyoglobin in 4 M urea. When myoglobin is reconstituted by addition of hemin to globin, its denaturation once again proceeds at a measurable rate. Moreover, the rate is enhanced by 3-phenylpropionate (Figure 10) and to the same extent as observed with native myoglobin never subjected to the rigors of globin preparation. These results eliminate any possibility of an artifact such as dissociation by aromatic compounds of a protein-bound, low molecular weight stabilizer of native conformation and

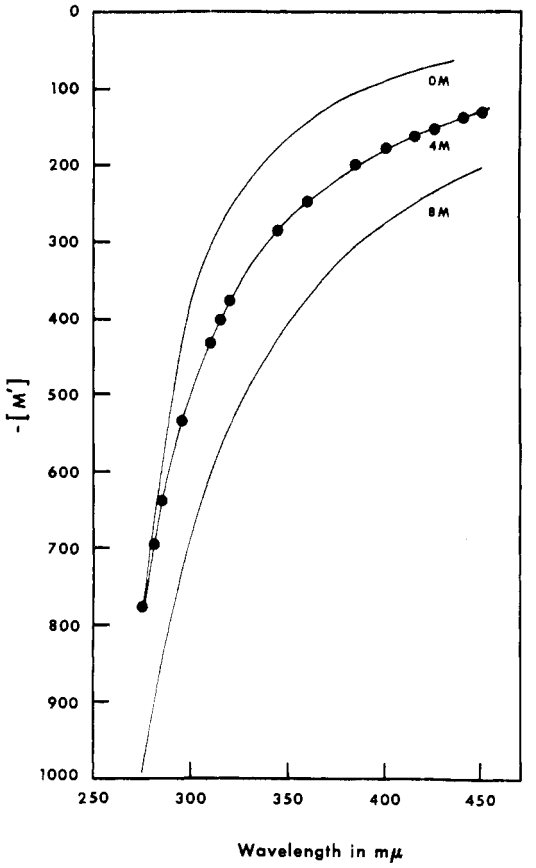

FIGURE 9: Demonstration that the urea denaturation of apomyoglobin is unaffected by 3-phenylpropionate. Plot of mean residue rotation of the protein, [M'], against wavelength: the three dispersion curves are for apomyoglobin in 0, 4, and 8 M urea; (●) 4 M urea solution containing 0.02 M 3-phenylpropionate. Values of the Moffitt parameters describing the three curves are in good agreement with literature values (Harrison and Blout, 1965; Urnes and Doty, 1961). Those for 4 M urea + 0.02 M 3-phenylpropionate are the same, within experimental error, as for 4 M urea alone.

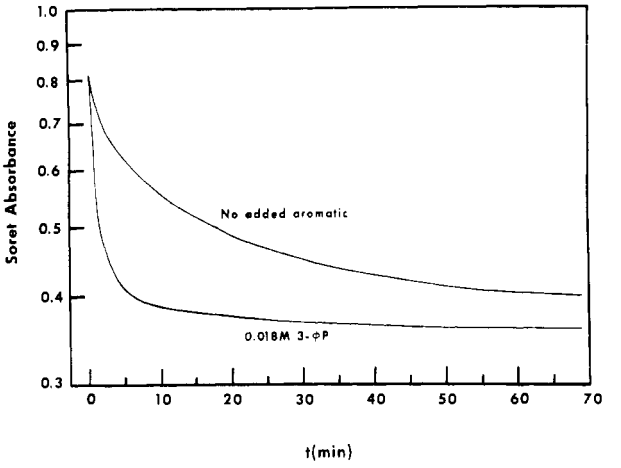

FIGURE 10: Enhancement of the rate of urea denaturation of reconstituted myoglobin by 3-phenylpropionate (3-ϕP). Conditions same as for myoglobin. The half-times in the absence and presence of the indicated concentration of 3-ϕP, 8 ± 0.6 and 0.9 ± 0.03 min, respectively, are to be compared with the corresponding values for myoglobin, 11 ± 0.7 and 1.1 min.

provide further support for the view that they act by complexing with the heme moiety.

Molecular Complexes of Hemin and Hematoporphyrin. The various kinetic measurements described above were complemented with some observations on the molecular complexes of hemin with β-naphthoate and 3-indolebutyrate. These spectral observations were made on hemin bound to serum albumin in order to avoid complications arising from aggregation of hemin in free solution, but control experiments on hemin in free solution are still required in order to minimize ambiguity. Thus, for example, it is conceivable that the spectral effects produced by an aromatic compound on hemin bound to albumin might really be exerted by the binding of the aromatic directly to the protein in such a way that it alters secondarily the interaction of the protein with hemin. This possibility was eliminated previously (Cann, 1967) in the case of chlorpromazine which was demonstrated to form molecular complexes with hemin (or hematoporphyrin) in free solution accompanied by changes in the Soret band virtually the same as produced by chlorpromazine on hemin (or hematoporphyrin) bound to albumin. Supporting evidence for formation of the complexes in free solution is their eventual precipitation. The corresponding control experiment has now been done with β-naphthoate. In contrast to chlorpromazine, β-naphthoate does not cause precipitation of hemin from free solution, but it does produce spectral changes. Thus, upon addition of 1.1×10^{-2} M β-naphthoate to 1.3×10^{-5} M hemin in pH 6 acetate buffer, ionic strength maintained at 0.06 M, the Soret absorbance increased about 7%; the absorbance in the spectral region (480–566 mμ) increased about 8%; the absorbance in the region (566–618 mμ) decreased about 4%; and the 634-mμ charge-transfer band was delineated and intensified by about 8%. While less striking than with chlorpromazine, this result does indicate complex formation and provides support for a similar interpretation of experiments with β-naphthoate and 3-indolebutyrate using hemin bound to albumin.

As illustrated in Figure 11 these compounds cause pronounced and reversible changes in the spectrum of albumin-bound hemin.[2] Consider first the Soret band, whose bimodality is apparently due to different vibrational states (0–0 and 0–1) of the same electronic state[3] (Gouterman, 1959). The band is also mixed in the presence of the aromatic compounds; but the relative contribution of each state is considerably altered over that in uncomplexed hemin, with conservation of the integrated intensity of the band as indicated by approximate constancy of its dipole strength (Gouterman, 1959, 1961). With β-naphthoate the relative contribution of the 0–0 transition is considerably increased, shifted red, and sharpened. In contrast, with 3-indolebutyrate it is the 0–1

[2] Color change produced by β-naphthoate, greenish yellow to amber; by 3-indolebutyrate, greenish yellow to yellowish brown.

[3] The degeneracy of the pair of $\pi-\pi$ transitions giving rise to the Soret band of symmetrical porphyrins and metal porphyrins is generally unbroken (Gouterman, 1959, 1961; Mason, 1963). But it is conceivable that with hemin and hematoporphyrin the bimodality of the Soret band could be due to a split in electronic state rather than to different vibrational states. This would not change the gist of our argument, however, since in either case a change in character of the band can be ascribed to differences in the geometry of the complexes.

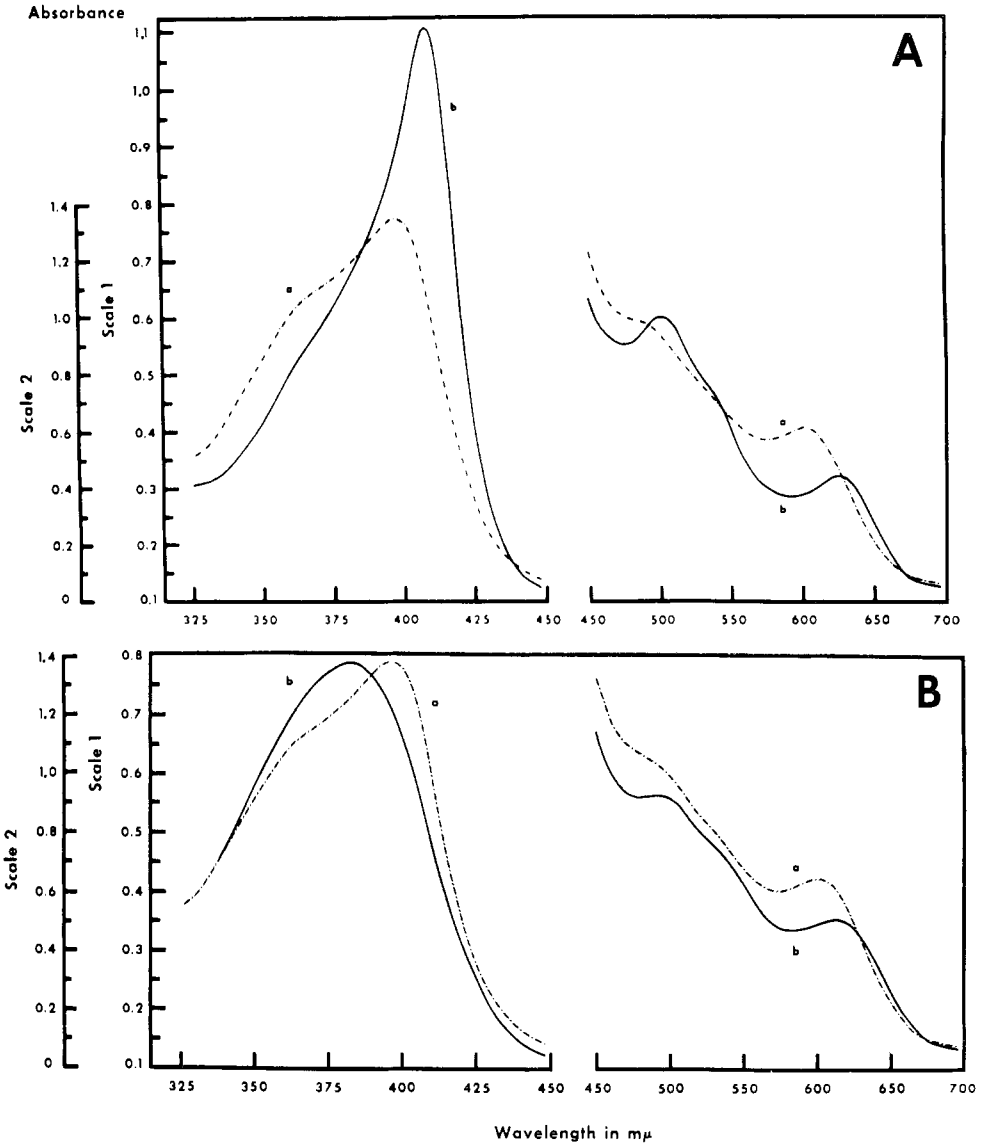

FIGURE 11: Molecular complexes of hemin with (A) β-naphthoate and (B) 3-indolebutyrate. Effect of these compounds on the Soret band and visible spectrum of 1.3×10^{-5} M hemin bound to 2.9×10^{-5} M bovine plasma albumin: (a) In the absence of aromatic compounds; (b) 5.10×10^{-3} M β-naphthoate to 1.50×10^{-2} M 3-indolebutyrate. Scale 1, Soret band, 1-cm light path; scale 2, visible spectrum, 10-cm light path. Dipole strength of Soret band in the absence of aromatic compounds, 5.4; hemin–naphthoate complex, 4.8; hemin–indolebutyrate complex, 5.3. Reversibility established by dilution.

transition whose contribution is increased thereby giving the superficial impression of a blue shift of the Soret band. In this instance the spectral change was followed by varying the concentration of 3-indolebutyrate over the range 1.2×10^{-5}–6.0×10^{-5} M at the same time holding the concentration of hemin constant at 5.2×10^{-7} M. As the concentration of the aromatic molecule was increased, the contribution of the 0–1 transition increased progressively with concomitant decrease in the 0–0 transition. The difference in response of the Soret band to the addition of β-naphthoate or 3-indolebutyrate is indicative of a difference in the geometry of the complexes which these compounds form with hemin.

Provocative changes also occur in the visible spectrum of hemin upon complex formation, more so with β-naphthoate than 3-indolebutyrate. Addition of either aromatic compound causes delineation of the 500-mμ band and a strong red shift of the 603-mμ charge-transfer band. The latter is of considerable interest since the extent of the band shift correlates with the effectiveness of the corresponding compound in enhancing the rate of urea denaturation of myoglobin. Thus, β-naphthoate, which is the more effective rate enhancer, causes a shift of 23 mμ as compared with 12 mμ for 3-indolebutyrate.

One is struck by the similarity between the spectrum of the hemin–naphthoate complex and that of myoglobin. The wavelength of the several bands are the same, or nearly so, and the molar extinction coefficients of the 500- and 630-mμ bands are about the same for the complex as for myoglobin. The chief difference is that the Soret band of the complex is only about 60% as intense as for myoglobin. The question naturally arises as to whether β-naphthoate simply serves as a ligand for the iron. This possibility seems to be eliminated by the fact that β-naphthoate also forms a molecular complex

with hematoporphyrin (color change, peach to beige). Thus, addition of 3.89×10^{-3} M β-naphthoate to 5×10^{-5} M hematoporphyrin bound to 2.8×10^{-5} M albumin caused a marked change in the Soret band curiously similar to that produced by complexing of 3-indolebutyrate with hemin, *i.e.*, the contribution of the 0–1 transition was increased at the expense of the 0–0 transition. Changes also occurred in the visible spectrum as revealed by experiments in which 3.48×10^{-3} M β-naphthoate was added to 1.85×10^{-4} M hematoporphyrin bound to 2.7×10^{-4} M albumin. The maximum absorbances of bands IV and III (502 and 535 mμ, respectively) were reduced by about 14% without a change in band position; the maximum absorbances of bands II and I (565 and 618 mμ) were reduced by about 6% with a small (3 and 2 mμ) but significant red shift. All of these spectral changes were reversed, or nearly so, by a 25-fold dilution of the reaction mixtures.

Discussion

The results described above and those reported previously (Cann, 1965, 1967) can be summarized as follows. (a) Enhancement by aromatic compounds of the rate of reaction of Zn^{2+} with myoglobin and the rate of urea denaturation of the protein is reversible and involves complex formation between two aromatic molecules and two sites in the hemoprotein as judged from the order of reaction with respect to added compound. There are, however, some restrictions exhibited by at least one of the sites. Thus, compounds like benzene, indole, 3-phenylpropionate, and many others bind at both sites. In contrast, isopropylbenzene, 3-indoleacetate, and benzoate bind at only one. Still others, *e.g.*, 3-indolebutyrate and chlorpromazine, bind at both sites but with distinguishable affinities. In fact, the data suggest that in some instances binding at one site either increases the affinity for the second one or causes it to become accessible for complex formation. (Note in Figure 4, for example, the anomalous transition from first- to second-order kinetics with respect to added aromatic on progressing through the series of compounds: benzoate, phenylacetate, and 3-phenylpropionate.) (b) The complex is fundamentally of the electron donor–acceptor type with the aromatic compound serving as donor, but hydrophobic interactions make an important contribution to its stability. Steric configuration of the aromatic compound and coulombic repulsion of donors with cationic side chains are also forces of consequence. In fact, the relative effectiveness of certain compounds in enhancing the rate of denaturation is determined by a delicate balance of these forces. (c) Phenomenologically, enhancement of the rate of reaction with Zn^{2+} reflects a decrease in activation energy of about 7 kcal mole^{-1} which is of the order of the standard enthalpy of the exoergic formation of two moles of a typical π complex (Briegleb, 1961). (d) Whereas the urea denaturation of apomyoglobin is unaffected by a representative aromatic compound, reconstituted myoglobin shows the same exquisite sensitivity as myoglobin. (e) Hemin forms molecular complexes with chlorpromazine, β-naphthoate, and 3-indolebutyrate. Nor do these compounds merely serve as ligands for the iron. Thus, hematoporphyrin also complexes with chlorpromazine and β-naphthoate as well as with iodobenzene and nitrobenzene, indicating that in the case of hemin complex formation is with the protoporphyrin ring system and/or its side-chain vinyl groups. Moreover, chlorpromazine complexes with the heme moiety of myoglobin. Hematoporphyrin has been shown to be a good electron acceptor, and the new visible absorption bands exhibited by the complexes of chlorpromazine with hemin and with myoglobin may be charge-transfer bands. Finally, there is a nice correspondence between the relative strength of complexing of aromatic compounds with hemin or hematoporphyrin and their degree of synergism with urea.

These results are readily synthesized within the context of the three-dimensional X-ray crystallographic structure of myoglobin with particular reference to the heme moiety and its immediate environment (Kendrew, 1962). The iron of the heme is bonded to the imidazole group of the F8 histidyl residue. Except for its propionic acid groups which are at the surface of the macromolecule and probably involved in salt linkages, the heme is tucked away in a pocket where it is surrounded almost entirely by nonpolar residues. There are some 90 Van der Waals contacts between the heme and neighboring atoms, and two aromatic rings (phenylalanine residues, CD1 and 15H) are arranged parallel, or nearly so, to its pyrrole rings or vinyl groups. Kendrew points out that π-bonding interactions must be significant here. He also notes that while rather large ligands (*e.g.*, *p*-phenylisocyanide) can combine with the iron without a change in the conformation of the molecule as a whole, a substantial rearrangement of side chains in the vicinity of the heme must accompany attachment of at least the larger ligands. (This latter observation is quoted to illustrate the confines of the heme and in no way implies that the aromatic compounds used in our investigations simply serve as ligands for the iron.)

We visualize the aromatic compounds as forming charge-transfer complexes directly with the heme moiety of myoglobin, thereby relieving its π-bonding interactions with the two aromatic rings of phenylalanine residues, CD1 and 15H. This, in turn, labilizes the macromolecular conformation to the disrupting action of agents like Zn^{2+} and urea. Now, from the geometrical structural details concerning the disposition of the heme in the protein, it is understandable that hydrophobic interactions make such a large contribution to the stability of the charge-transfer complex. Consider, for example, the homologs of benzoate. As the length of the straight chain separating the phenyl ring from the carboxylate group is increased the amount of highly structured water surrounding the hydrophobic portion of the compound in aqueous solution also increases. Upon removal of the compound from solution this highly structured water assumes the less ordered structure of bulk water with a comcomitant increase in entropy. Accordingly, the larger increase in entropy accompanying the transfer of 5-phenylvalerate from aqueous solution to the nonpolar environment of the heme provides greater stabilization for its complex with heme than in the case of the lower homologs. We believe that the heme provides both of the binding sites of myoglobin,[4] but that

[4] While comparative spectroscopic measurements on the chlorpromazine–hemin and chlorpromazine–myoglobin complexes (Cann, 1967) have established that the heme moiety provides at least one of the binding sites, there are two other possible sites in the protein molecule (Cann, 1967). However, the simplest interpretation of our data makes the assumption that the heme provides both sites. This view finds some support in the experiments on apomyoglobin and reconstituted myoglobin.

they differ in physical accessibility (and perhaps nonpolar character). Thus, for example, it appears that binding of phenylacetate or 2-phenylpropionate (Figures 4 and 8) at one of the sites may cause sufficient rearrangement of protein side chains in the vicinity of the heme to make the second site accessible to the aromatic molecule. Indeed, it is conceivable that this is so for the great majority of compounds examined and may account for the effect of steric configuration of aromatic acid anions upon their rate enhancement. These considerations are in accord with the snug fit of the heme in the protein pocket. As for the ineffectual donors with cationic side chains, *e.g.*, γ-phenylpropylamine-HCl, coulombic repulsion by the large positive charge on the heme iron may prevent entry into the pocket. In the exceptional case of chlorpromazine-HCl the unusually strong electron-donating tendency of the phenothiazine ring system evidently more than compensates for coulombic repulsion between the positively charged tertiary amino group and the heme iron. This balance of forces is underscored by the observation that neither tryptamine-HCl nor serotonin-HCl are rate enhancers, indole being a weaker electron donor than phenothiazine.[5]

The preceding discussion illustrates one way in which the geometrical details of the three-dimensional crystallographic structure of a protein can be translated into physicochemical terms for the purpose of delineating conformation-determining forces. Thus, it is evident that aromatic residue interactions play a significant role in maintaining the structural integrity of myoglobin. Our experiments also bring into sharper focus the cooperative action of the heme moiety and protein part of myoglobin in determining macromolecular structure.

Finally, a few words are in order concerning the spectra (Figures 3 and 7) of the electron donor–acceptor complexes formed between the several indole or naphthalene derivatives and the acceptor molecule, methyl viologen. In each instance there is in addition to the charge-transfer band a sharper, much more intense band at shorter wavelength. Measurements on the α-naphthol system indicate that both bands belong to the complex; the comparison made in Table II between the short-wavelength band and the first discernable absorption band of uncomplexed donor leaves little doubt that in the case of the four naphthalene derivatives the former is the first absorption band of the donor in the complex itself (Briegleb, 1961). In the case of 3-indoleacetate and 3-indolebutyrate, on the other hand, the short-wavelength band (317 mμ) of the complex is most certainly not the analog of the first *discernable* band (288 mμ) of the uncomplexed donor. The large value, -9 kcal mole^{-1}, of $\Delta h\nu$ is not commensurate with the value, -2.5 kcal mole^{-1}, of the standard enthalpy of complex formation, and there is a large disparity in molar extinction (about 1.4×10^3 l. mole^{-1} cm^{-1} for the complex as against 4.7×10^3 for the uncomplexed donor). The spectra of the uncomplexed indole derivatives are rather broad, however, and extend as far into the red as 320 mμ (for 3-indolebutyrate, ϵ 6×10^2 l. mole^{-1} cm^{-1} at 305 mμ and 2×10^2 at 310 mμ). Possibly, a broad band hidden in the near edge of the spectrum is shifted red and sharpened upon complex formation.

[5] This is so both theoretically and experimentally. Thus, the wavelength of maximum absorbance of the charge-transfer complex of indole, 3-indoleacetate or 3-indolebutyrate with methyl viologen is about 400 mμ (Figure 3 and Table 1) as compared with 500 mμ for the chlorpromazine–methyl viologen complex (Figure 7 of Cann, 1967).

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