Parker, C. W. (1967), *in* The Handbook of Experimental Immunology, Oxford, Blackwells Scientific Publications (in press).
Parker, C. W., Godt, S. M., and Johnson, M. C. (1966), *Biochemistry 5*, 2314.
Parker, C. W., Yoo, T. J., Johnson, M. C., and Godt, S. M. (1967), *Biochemistry 6*, 3408 (this issue; preceding paper).
Paul, W. E., Siskind, G. W., and Benacerraf, B. (1966), *J. Exptl. Med. 123*, 689.
Robbins, J. B., Harmovich, J., and Sela, M. (1967), *Immunochemistry 4*, 11.
Scheidigger, J. J. (1955), *Intern. Arch. Allergy Appl. Immunol. 7*, 103.
Sela, M., and Mozes, E. (1966), *Proc. Natl. Acad. Sci. U. S. 52*, 285.
Singer, S. J., and Doolittle, R. F. (1966), *Science 153*, 13.
Velick, S. F., Parker, C. W., and Eisen, H. N. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1470.
Weber, G. (1952), *Biochemistry J. 51*, 155.
Weber, G. (1965), *in* Molecular Biophysics, New York, N. Y., Academic, p 369.

Contribution of Aromatic Residue Interactions to the Stability of Myoglobin. II. Enhancement by Aromatic Compounds of the Rate of Urea Denaturation*

John R. Cann

ABSTRACT: As in the case of the zinc–myoglobin reaction, benzene and other aromatics have a strong and specific enhancing effect upon the rate of urea denaturation of this protein.

Aromatic compounds exert their action on a very small portion of the myoglobin molecule, and one of the two sites with which they complex exhibits certain restraints. Both charge-transfer and hydrophobic forces are implicated in complex formation, which is believed to disrupt π-bonding interactions between the two aromatic rings of the phenylalanine residues, CD1 and 15H, and the heme.

Reaction of zinc ions with sperm whale myoglobin causes major changes in the physical and chemical properties of the protein, the most characteristic spectral change being a marked reduction in Soret-band intensity. Studies made in this laboratory (Cann, 1963, 1964a,b) have led to the conclusion that the rate-controlling step in suppression of the Soret band involves macromolecular conformational changes concomitant with rupture of the otherwise inaccessible Fe^{3+}–F8 imidazole linkage and occupancy of the imidazole group by zinc. Thus, attention is focused upon the structural complex involving the heme and adjacent portions of the protein moiety as the critical site of attack by zinc. Kendrews' 2-A model of myoglobin (Kendrew *et al.*, 1961; Kendrew, 1961, 1962; Stryer *et al.*, 1964) reveals the heme resting snugly in a hydrophobic pocket in the protein with two aromatic rings (phenylalanine residues, CD1 and 15H)[1] arranged parallel to its pyrrole rings or vinyl groups. Kendrew points out that π-bonding interactions must be significant here. That being the case, reaction of myoglobin with zinc would almost certainly necessitate rupture of these π bonds. In that event, the addition of aromatic hydrocarbons such as benzene or naphthalene to the reaction mixture should increase the rate of reaction. Such compounds might be expected to relieve the intramolecular π-bonding interactions, thereby decreasing the activation energy. Previously reported experiments designed to test these ideas (Cann, 1965) demonstrated that low concentrations of benzene and other aromatic compounds have an enormous and specific enhancing effect upon the rate of reaction. Enhancement of the rate is reversible; phenomenologically it reflects a decrease in activation energy, and mechanistically, evidently involves formation of electron donor–

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[1] In accordance with the slightly altered nomenclature to be introduced shortly by Kendrew and his co-workers (private communication from Dr. John C. Kendrew), 15H is the new designation for the phenylalanine residue formerly called H14.

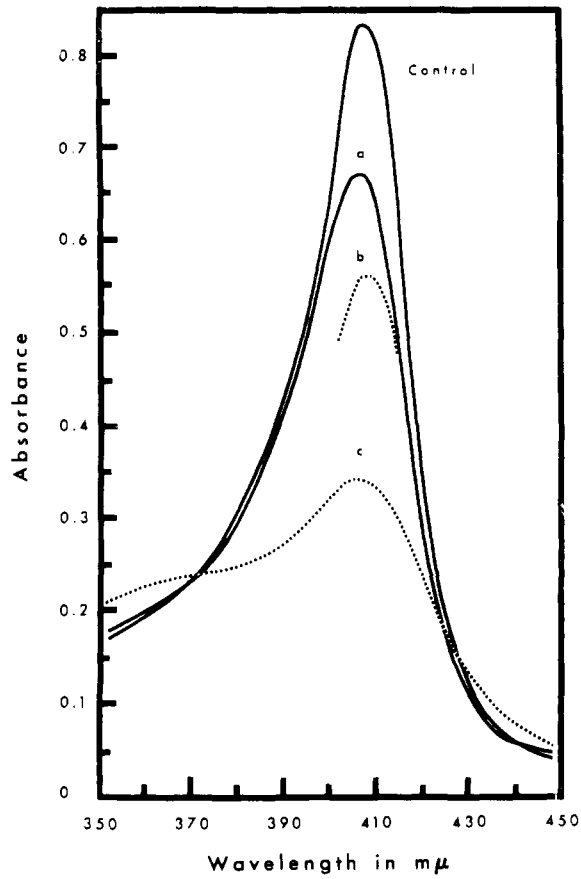

FIGURE 1: Enhancement of the rate of urea-induced suppression of the Soret band of ferrimyoglobin by addition of benzene to the reaction mixture. (a) No benzene, 5.5 min after preparation of reaction mixture. (b and c) 0.035 M benzene, 0.3 and 4.9 min after preparation of reaction mixture, respectively.

acceptor complexes between two aromatic molecules and two sites in the heme protein.

These results indicate that aromatic side-chain interactions play a significant role in maintaining the structural integrity of myoglobin. They also bring into sharper focus the cooperative action of the heme moiety and protein part of myoglobin in determining macromolecular structure. The next logical step in this investigation is to inquire whether or not rupture of π-bonding interactions labilizes the protein to the denaturing action of agents such as urea. Indeed, preliminary experiments showed that, as in the case of the zinc–myoglobin reaction, near-saturation of the reaction mixture with benzene increases, by more than an order of magnitude, the rate of urea-induced suppression of Soret intensity. It has been found that a large number of other aromatic compounds are also effective. The detailed experiments reported below indicate that they act by forming molecular complexes generally with two sites in the macromolecule. Both charge-transfer and hydrophobic forces are implicated in complex formation. Pertinent to these considerations is the spectroscopic demonstration reported in the companion paper (Cann, 1967) of molecular complexing between several of the aromatic compounds and hemin, hematoporphyrin, or myoglobin.

Methods and Background Information

The sperm whale ferrimyoglobin was obtained from Mann Research Analytical Laboratories. Analytical reagent grade urea was recrystallized from 70% ethanol. Most of the aromatic compounds were Spectrograde, analytical reagent, gas chromatographically pure, zone refined, Fisher's Certified, or other high quality materials. When indicated, they were further purified by passage through a column of activated aluminum oxide (Woelm basic activity grade 1), or, as in the case of Mann's methyl viologen, by recrystallization from ethanol. Passage of even the highest grade toluene, *m*-xylene, and ethylbenzene through aluminum oxide or scrubbing with ferrous sulfate solution was found essential for removal of hydroperoxides which are catalytically converted to their corresponding aldehydes by myoglobin. The crystalline chlorpromazine hydrochloride was kindly supplied by Smith Kline and French Laboratories.

Preliminary experiments showed that 6.4 M urea at about pH 5.8 denatures myoglobin as judged qualitatively by the classical criterion of insolubility in 1.6 M NaCl and quantitatively by changes in sedimentation behavior and spectral properties, notably drastic suppression of the Soret band (Figure 1). The time course of denaturation was followed by measuring the decrease in Soret absorbance in a thermostated Beckman DK-2 spectrophotometer using appropriate blanks. Repetitive scanning of the Soret band was chosen in preference to direct recording of absorbance at a given wavelength *vs.* time because as denaturation proceeds the Soret band shifts progressively into the blue from a maximum at 408 mμ to one at 399 mμ. This behavior may be related to growth of the weak band at 360–370 mμ (Figure 1). During the very last stages of reaction, the whole spectrum slowly shifts back toward the red by a few millimicrons. These spectral shifts also occur in the presence of added aromatic compounds but are much less pronounced with benzene and toluene.

Rate measurements were made at 28 ± 0.01° on solutions containing about 0.01% myoglobin, 6.4 M urea, and 0.05 M sodium acetate buffer; pH of reaction mixture, 5.84 ± 0.02. The ionic strength of reaction mixtures containing aromatic salts was maintained at 0.05 M by downward adjustment of the acetate buffer concentration. In a typical experiment aliquots of a thermoequilibrated and appropriately buffered solution of myoglobin (0.1% protein) and an equilibrated 7.11 M urea solution containing the aromatic compound were mixed in a stoppered cuvet of 1-cm light path. Reaction proceeded without significant change in pH, and the protein did not precipitate. The concentration of aromatic compound in the reaction mixture was known from its concentration

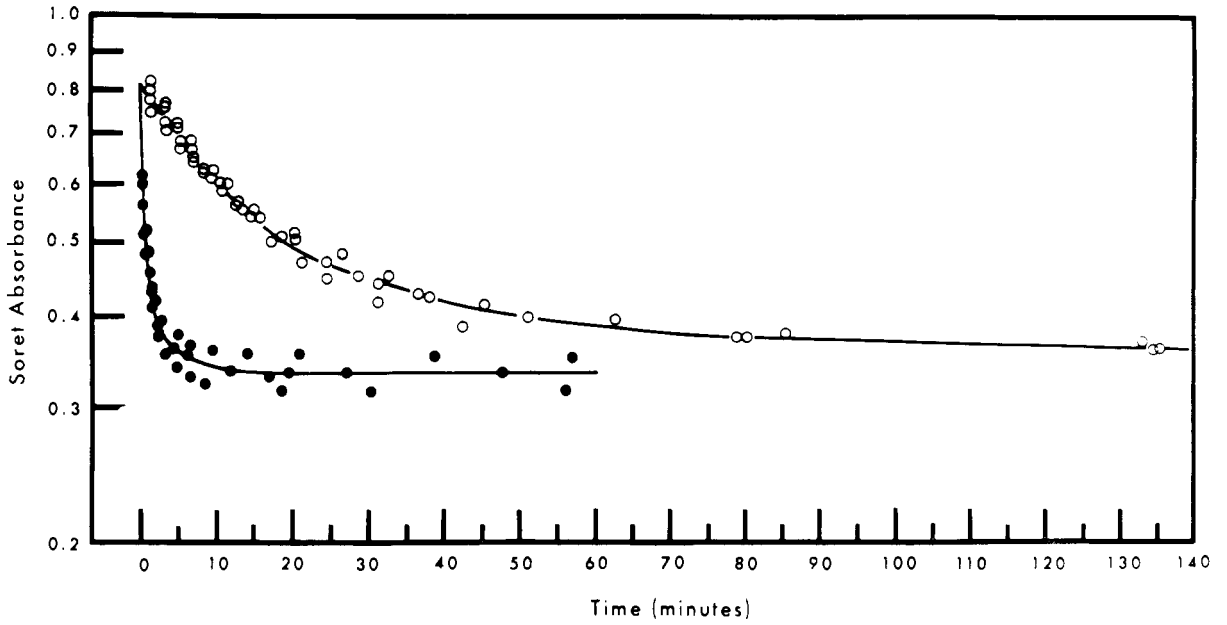

FIGURE 2: Effect of benzene on the time course of urea-induced suppression of the Soret band of myoglobin. Semilogarithmic plot of Soret absorbance *vs.* time in minutes. (○) No benzene in reaction mixture, half-time of 10.9 ± 0.8 min. (●) 0.035 M benzene, half-time of 0.4 min. In the absence of benzene the final absorbance obtained after 300 min was equal, within experimental error, to that in the presence of benzene.

in the stock urea solution or was determined, as in the case of the volatile hydrocarbons, from its ultraviolet spectrum in diluted aliquots of the reacted mixture. For many of the 18 different compounds examined, the concentration range was limited by their solubility in urea. In the case of benzene the highest concentration employed was about 3.4×10^{-2} M; toluene, 1.2×10^{-2} M; *m*-xylene, 8.2×10^{-3} M; and iodobenzene, 4.0×10^{-3} M. Values for the other compounds can be readily obtained from the several figures. Measurements on the effect of chlorpromazine were made at concentrations less than its critical micelle concentration except possibly for those at the highest concentration,[2] 1.4×10^{-2} M. In control experiments chlorpromazine was added to buffered myoglobin solutions in the absence of urea; 9×10^{-3} M chlorpromazine had very slight, if any, effect on Soret absorbance. A value of 1.4×10^{-2} M caused a time-dependent decrease in absorbance but at a rate sufficiently slow so as not to invalidate the urea-denaturation experiments in the presence of this concentration of chlorpromazine. Control experiments showing that near-saturating concentration of benzene in the absence of urea does not denature myoglobin have been discussed previously (Cann, 1965). The rate of urea denaturation is insensitive to sodium acetate concentration between 0.025 and 0.05 M.

In the absence of added aromatic compounds, semilogarithmic plots of Soret absorbance *vs.* time are linear during early stages of denaturation, after which the logarithmic rate decreases progressively with time. Denaturation is first order with respect to protein concentration over the fourfold concentration range examined (0.005–0.02%), as judged from constancy, within experimental error, of the initial logarithmic rate and of the half-time of reaction calculated using initial and final absorbance. In the presence of added aromatic compounds, the semilogarithmic plots are nonlinear over the entire time course of denaturation. This is true even for low concentrations of these compounds. Accordingly, all rates are expressed as the reciprocal of the half-time.

Denaturation is about 60% reversible, as judged from the extent of recovery of Soret absorbance upon (1) removal of the urea from completely reacted mixtures by dialysis against buffer or (2) tenfold dilution with buffer (10-cm light path). This same result was obtained when the reaction mixture contained near-saturating concentrations of benzene.

Ultracentrifugal analyses were made on 0.4% native myoglobin in 0.05 M acetate buffer (pH 6.0), the same concentration of denatured myoglobin in its buffered urea solution, and the protein after removal of urea by dialysis against buffer. The native myoglobin sedimented as a single boundary, $s_{20,w} = 1.85$ S. The sedi-

[2] Certain observations in this laboratory indicate a critical micelle concentration of 5×10^{-3} M chlorpromazine in 0.5 M sodium acetate buffer (pH 5.95), which is about twice the value reported for the same concentration of unbuffered NaCl (Scholtan, 1955). The critical micelle concentration in 6.4 M urea buffered with 0.05 M sodium acetate may very well be higher than the value of about 10^{-2} M in 0.05 M NaCl, particularly since urea increases the critical micelle concentration of the cationic detergent, *n*-dodecyltrimethylammonium bromide (Bruning and Holtzer, 1961).

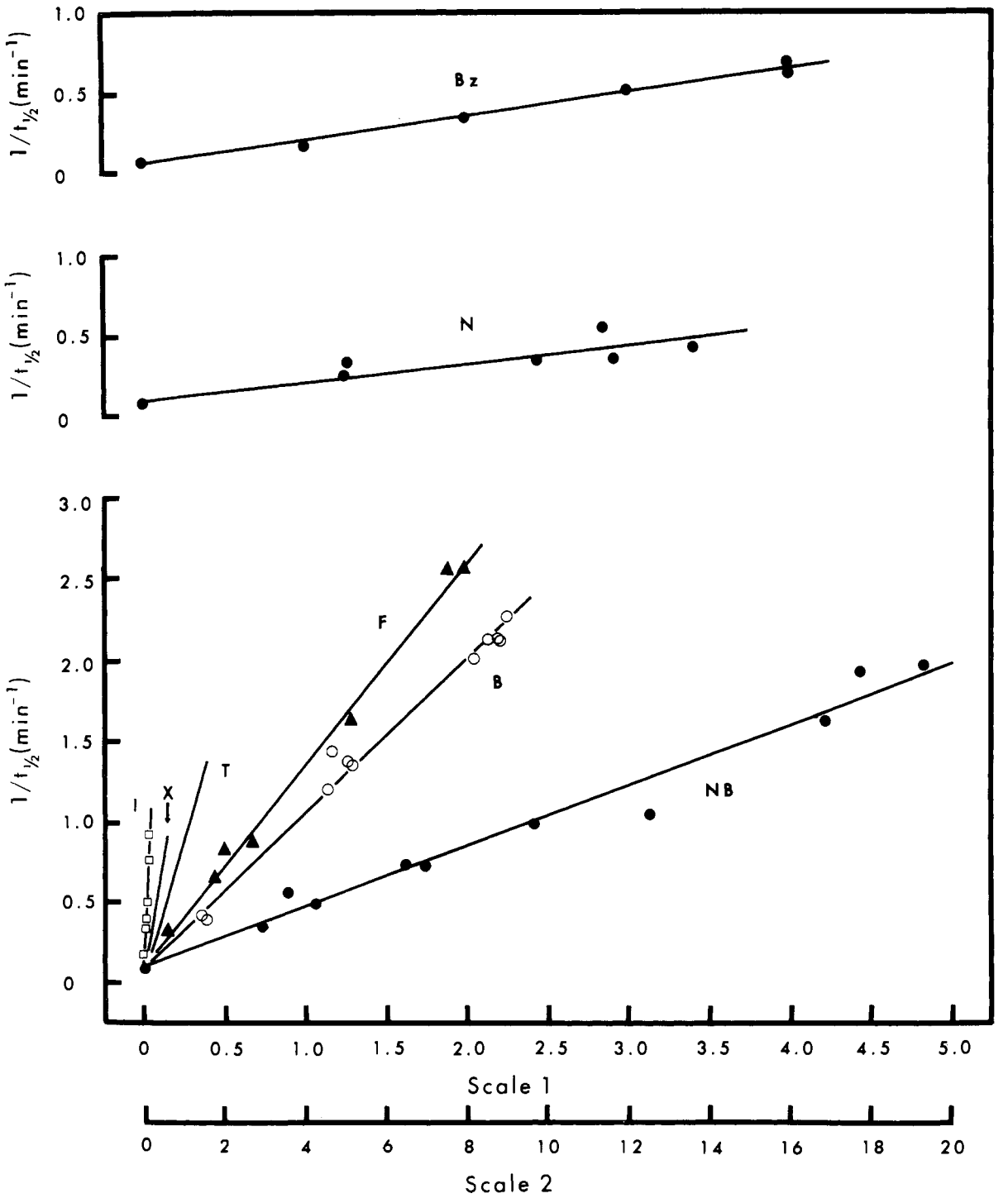

FIGURE 3: Order of the urea-induced suppression of Soret absorption with respect to rate-enhancing aromatic compounds: NB, nitrobenzene; B, benzene; F, fluorobenzene; T, toluene; X, *m*-xylene; I, iodobenzene; N, naphthalene; and Bz, benzoate. Abscissa: scale 1, $10^3 \times C^{1.8}$ for B, $10^4 \times C$ for N, and $10^2 \times C$ for Bz; scale 2, $10^4 \times C^2$ for NB, F, T, X, and I. For the sake of clarity the data points for T and X, which also show good second-order dependence, have been omitted from these plots. In this figure and in Figures 4, 5, and 8, $t_{1/2}$ symbolizes half-time of denaturation and C, molar concentration of aromatic compound. The straight lines are least-squared ones.

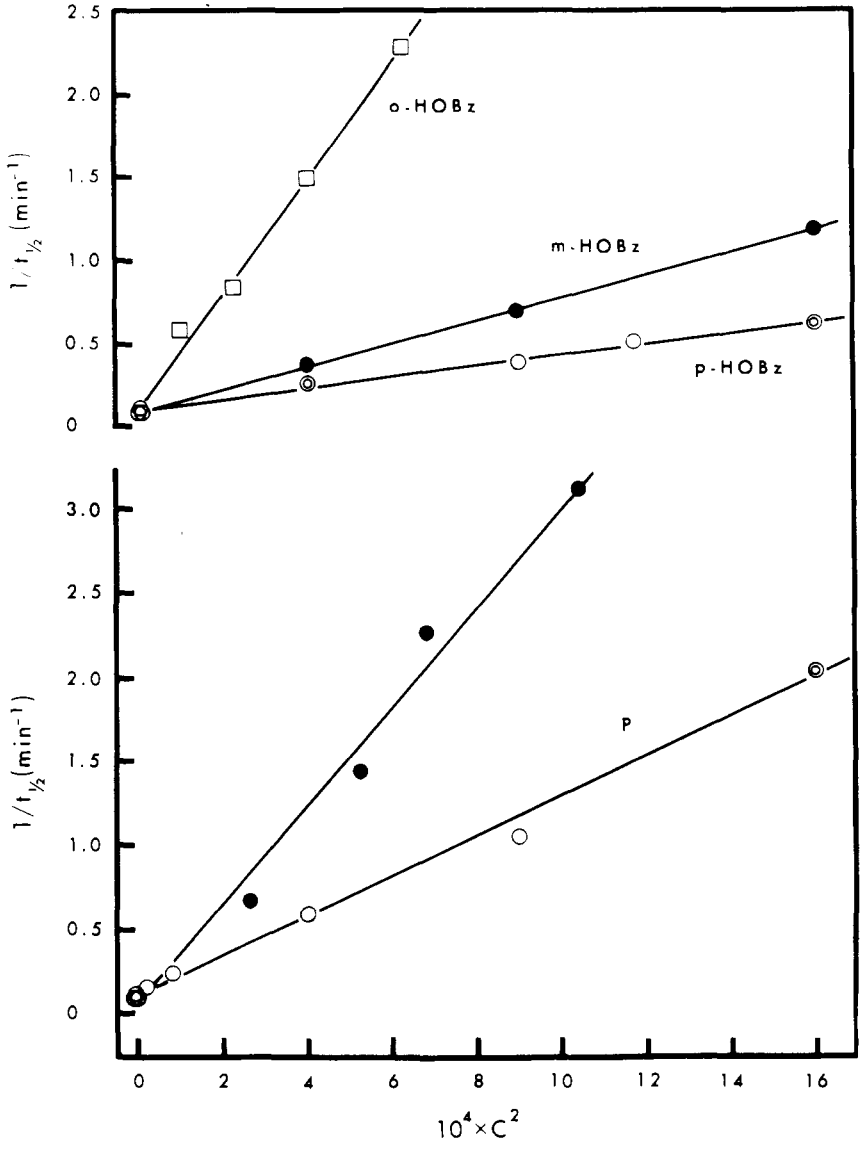

FIGURE 4: Order of the urea-induced suppression of Soret absorption with respect to rate-enhancing aromatic compounds: HOBz, hydroxybenzoate; A, anisole; and P, phenol. A circle within a circle symbolizes a duplicate determination with identical results.

mentation pattern of the denatured protein showed two boundaries, a major one with $s_{20,w} = 6.4$ S and a small one with 1.1 S, and the heme sedimented with the protein. After removal of the urea the pattern showed 60% of a boundary with $s_{20,w} = 1.75 \pm 0.05$ S and 40% 8.9 S, which increased to 12 S during aging for 4 days at room temperature. It is concluded that under the conditions of our experiments urea-denatured myoglobin is to a very large extent highly aggregated and that removal of the urea causes about 60% disaggregation into monomeric protein molecules having a sedimentation coefficient close in value to that of native myoglobin. The latter is consistent with the spectroscopic measurements which indicate that urea denaturation is about 60% reversible.

Finally, measurements on molecular complexing between chlorpromazine and methyl viologen were made in 0.5 M sodium acetate buffer (pH 5.95) at chlorpromazine concentrations less than its critical micelle concentration.[2] Methyl viologen was in large excess. Analysis of the data indicated a 1:1 complex, and the plot in Figure 7 is in accordance with the corresponding mass action relationship

$$\frac{[\text{chlorpromazine}]}{A} = \frac{1}{K\epsilon}\frac{1}{[\text{methyl viologen}]} + \frac{1}{\epsilon}$$

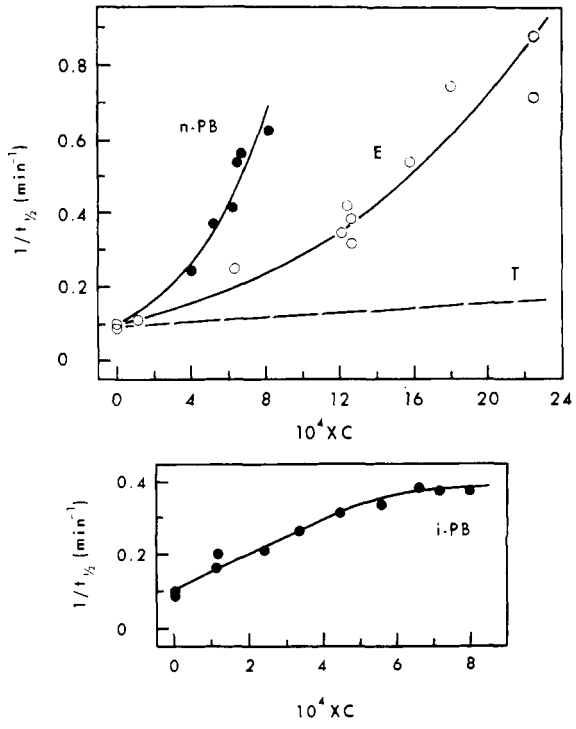

FIGURE 5: Comparison of the effectiveness of several alkylbenzenes in enhancing the rate of urea denaturation: toluene, T; ethylbenzene, E; *n*-propylbenzene, *n*-PB; isopropylbenzene, *i*-PB. The dashed curve for T is interpolated from Figure 3; the lowest concentration of toluene employed was 2.62×10^{-3} M; $1/t_{1/2} = 0.175$ min^{-1}.

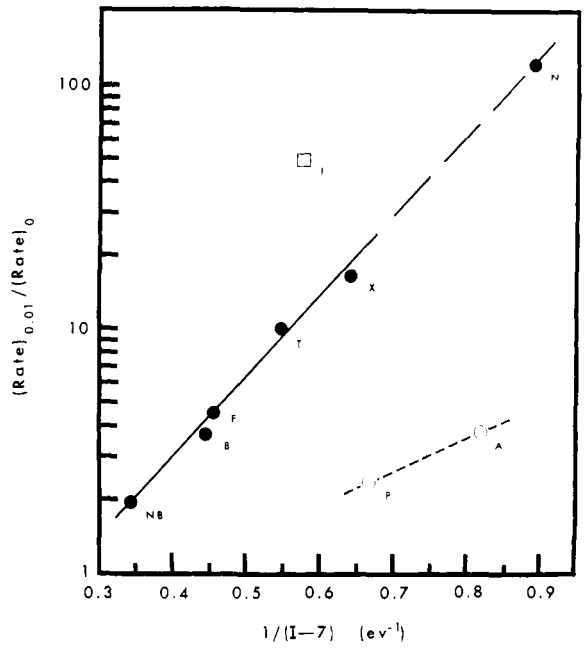

FIGURE 6: Relationship of enhancement of the rate of urea denaturation by a standard concentration of aromatic compound to molecular ionization potential, *I*. Semilogarithmic plot of the ratio, rate in presence of 0.01 M aromatic to that in absence of aromatic, *vs.* $1/(I - 7)$. The free energy of formation of acceptor–donor complexes is proportional to $1/(I$ − constant) (Briegleb, 1961). The data point for N was obtained by a long extrapolation, but its relative ranking is obviously qualitatively correct. Values of *I* were taken from Watanabe (1957) and Watanabe *et al.* (1962). Symbols as in Figure 3.

where the brackets denotes molar concentration; *K*, association constant; ϵ, molar extinction coefficient of the complex; and *A*, absorbance of the complex observed using 1-cm light path. The particular set of data displayed in Figure 7 was obtained at 2×10^{-3} M chlorpromazine. Another set of 21 measurements at 28° was made at several different chlorpromazine concentrations ranging from 1 to 3×10^{-3} M. These data give virtually the same line of regression as shown in the figure. In other experiments, the methyl viologen in the original complexing mixture was diluted from 0.1 to 0.02 M while maintaining a constant chlorpromazine concentration of 1.5×10^{-3} M. The absorbance of the diluted solution was, within experimental error, that predicted from the line of regression, which demonstrates reversibility of complex formation. The effect of temperature upon complex formation was also reversible within the temperature range employed.

Results

The effect of benzene on the time course of the urea-induced suppression of Soret absorption of myoglobin is illustrated in Figure 2. It is strikingly apparent that low concentration of benzene causes an enormous enhancement of the rate of denaturation. Since benzene also enhances the rate of the zinc-mediated suppression of Soret absorbance, the possibility existed that its effect on urea denaturation might be illusionary and simply due to metal ion contamination of the reaction mixture. This possibility was eliminated by addition to the initial reaction mixture of 10^{-4}–10^{-3} M ethylenediaminetetraacetate, which is known to reverse the benzene-enhanced zinc–myoglobin reaction (Cann, 1965) but which was found to have no effect on the benzene-enhanced rate of urea denaturation. Nor is benzene playing an irreversible denaturing role. This is clear from the reversal experiments described in the preceding section.

Many other aromatic compounds have also been examined and found effective, more so than benzene in most cases. Comparison of some of these with comparable concentration of their aliphatic analogs (benzoate with acetate, iodobenzene with methyl iodide, and phenol with ethanol) indicates the same specificity for the aromatic ring as found previously for enhancement of the zinc–myoglobin reaction (Cann, 1965). Furthermore, experiments on the dependence of rate upon concentration of aromatic compounds (Figures 3–5) show that they exert their action on a very

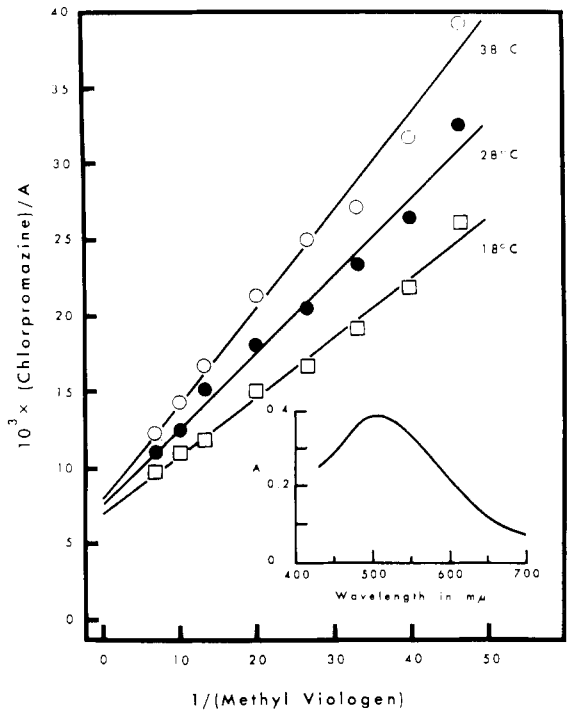

FIGURE 7: Molecular complexing between chlorpromazine and methyl viologen at indicated temperatures. Insert is spectrum of complex formed at 28° in solution containing 5×10^{-3} M chlorpromazine and 0.1 M methyl viologen, 1-cm light path. At 28°, $K = 15 \pm 2$ M^{-1} and $\epsilon = 130 \pm 10$ M^{-1} cm^{-1}; $\Delta H^\circ = -3 \pm 0.4$ kcal $mole^{-1}$.

limited portion of the protein molecule. Statistical analyses indicate that the reaction is second order with respect to most aromatic compounds, even the known protein denaturant, salicylate. The only clear exceptions are benzoate[3] and isopropylbenzene, for both of which the reaction shows first-order dependence. (The naphthalene data are displayed as a first-order plot, but the concentration range available is too limited to permit a decision as to actual order. Those for ethylbenzene and *n*-propylbenzene are fitted better by a second-order relationship than a least-squares straight line, the standard error of estimate being about 10% less for the former.) It is concluded, therefore, that aromatic compounds act by binding generally at two separate sites in the myoglobin molecule and that at least one of the sites has definite restraints. It is interesting that isopropylbenzene is the only compound examined which shows saturation, the data in Figure 5 in-

[3] In contrast to urea denaturation the benzoate-enhanced rate of the zinc–myoglobin reaction is second order in benzoate (Cann, 1965). This unexpected difference between the two systems is not understood but could conceivably be due to complexing of benzoate with Zn^{2+}. It may be pertinent that zinc benzoate complexes with aniline and heterocyclic bases (Taurugi and Nakabayashi, 1954; Tronov and Kharitonova, 1960).

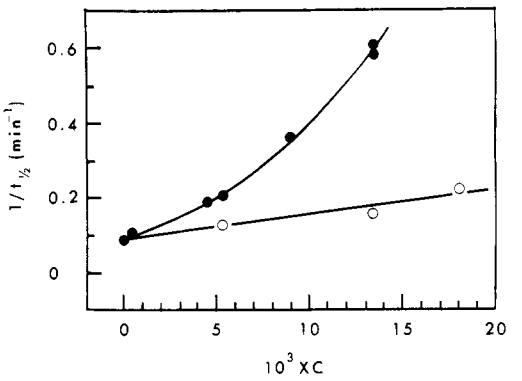

FIGURE 8: Comparison of the effectiveness of chlorpromazine (●) and methyl viologen (○) in enhancing the rate of urea denaturation. The chlorpromazine-enhanced rate shows a 1.6-order dependence on chlorpromazine concentration.

dicating a value of the order of 10^3 M^{-1} for its association constant. Other, less effective, compounds like benzene and toluene obviously bind much less strongly.

These results also provide information as to the forces involved in the binding. With the single exception of iodobenzene all the compounds fall naturally into one or another of four series each ordered with respect to increasing effectiveness in enhancing the rate of denaturation: (1) nitrobenzene < benzene < fluorobenzene < toluene < *m*-xylene < naphthalene, (2) phenol < anisole, (3) *p*-hydroxybenzoate < benzoate < *m*-hydroxybenzoate < *o*-hydroxybenzoate, and (4) toluene < ethylbenzene < *n*-propylbenzene. It is evident that the members of each series are ordered not only with respect to their effectiveness but also with respect to certain molecular parameters. Thus, in the first two series the compounds are also ordered with respect to decreasing molecular ionization potential, *i.e.*, the ordering is that to be expected if these molecules form electron donor–acceptor complexes with groupings in myoglobin. When one attempts to fit iodobenzene into the first series, it is found to be more effective than anticipated from its ionization potential possibly because iodobenzene is an n donor rather than π donor. These relationships of effectiveness to ionization potential are shown graphically in Figure 6. (The relatively low effectiveness of phenol and anisole is presumably related to their strong interaction with the solvent; *e.g.*, the solubility of anisole in about 7 M urea is more than an order of magnitude greater than in water.) In the case of the third series the several acid anions are also arranged according to decreasing carboxyl pK and increasing hydroxyl pK, and qualitative reasoning indicates that the ordering is with respect to increasing energy of the π electrons, hence, increasing electron-donating tendency. Finally, the members of the fourth series are ordered with respect to increasing hydrophobicity at approximately constant molecular ionization potential. These several considerations implicate both charge-transfer and hydrophobic forces

in the binding of aromatic compounds to the two sites in myoglobin with the aromatic compounds serving as donors.

A test of the conclusion that charge transfer plays an important role in binding is provided by the prediction that a donor molecule like chlorpromazine should enhance the rate of urea denaturation to a much greater extent than an acceptor such as methyl viologen. Important for the validity of this test is the preliminary observation that chlorpromazine and methyl viologen interact reversibly with each other to form a colored molecular complex with properties characteristic of electron donor–acceptor complexes, *i.e.*, a broad structureless absorption band, low molar extinction coefficient, and negative standard enthalpy of formation (Figure 7). The dependence of the rate of urea denaturation upon the concentration of each of these compounds is presented in Figure 8. It is evident that chlorpromazine enhances the rate much more so than methyl viologen, which confirms our prediction.

Discussion

The experiments described above show that, as in the case of the zinc–myoglobin reaction, aromatic compounds have a strong and specific enhancing effect upon the rate of urea denaturation of myoglobin. In most instances enhancement of the rate evidently involves complex formation between two aromatic molecules and two sites in the heme protein. One of these sites exhibits definite restrictions. Thus, while compounds like benzene, toluene, and the hydroxybenzoates bind at two sites, isopropylbenzene and benzoate bind at only one. This line of investigation is being continued in the hope that it may eventually permit mapping of the sites.

We propose that aromatic compounds disrupt π-bonding interactions between the two aromatic rings of the phenylalanine residues, CD1 and 15H, and the heme by complexing directly with the heme. The complexes are evidently of the electron donor–acceptor type with an important hydrophobic contribution to stability. These ideas are supported by the experiments on molecular complexing reported in the companion paper (Cann, 1967). Thus, chlorpromazine interacts with hemin, hematoporphyrin, and myoglobin to form complexes showing new visible absorption bands. Comparison of these spectra leaves little doubt that chlorpromazine complexes with the heme in myoglobin. Moreover, it complexes much more strongly with hemin than does methyl viologen, which correlates with their relative effectiveness in enhancing the rate of urea benaturation. Iodobenzene and nitrobenzene have also deen examined. Both form a 1:1 complex with hematoporphyrin, and the value of the association constant for iodobenzene is about four times that for nitrobenzene. This too correlates nicely with the greater effectiveness of iodobenzene in enhancing the rate of urea denaturation.

While it seems clear that the heme moiety provides at least one of the binding sites, the possibility cannot be ignored that the other site is located elsewhere in the protein molecule. Inspection of the myoglobin structure suggests two other possible sites of interaction: (1) the aromatic ring of the phenylalanine residue, G7, and the imidazole side chain of the histidine residue, C1, are parallel and quite close together; and (2) phenylalanines, CD1 and CD4, could conceivably rotate into parallel orientation. In any event, the experiments described in this paper give added credence to our previous conclusion that aromatic residue interactions, particularly those between heme and protein side chains, play a significant role in maintaining the structural integrity of myoglobin in solution.

Acknowledgment

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References

Briegleb, G. (1961), Elektronen-Donor-Acceptor-Komplexe, Berlin, Springer-Verlag.

Bruning, W., and Holtzer, A. (1961), *J. Am. Chem. Soc. 83*, 4865.

Cann, J. R. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 368.

Cann, J. R. (1964a), *Biochemistry 3*, 714.

Cann, J. R. (1964b), *Biochemistry 3*, 903.

Cann, J. R. (1965), *Biochemistry 4*, 2368.

Cann, J. R. (1967), *Biochemistry 6*, 3435 (this issue; following paper).

Kendrew, J. C. (1961), *Sci. Am. 205*, 96.

Kendrew, J. C. (1962), *Brookhaven Symp. Biol. 15*, 216.

Kendrew, J. C., Watson, H. C., Strandberg, B. E., Dickerson, R. E., Phillips, D. C., and Shore, V. C. (1961), *Nature 190*, 666.

Scholtan, W. (1955), *Kolloid Z. 142*, 84.

Stryer, L., Kendrew, J. C., and Watson, H. C. (1964), *J. Mol. Biol. 8*, 96.

Taurugi, J., and Nakabayashi, T. (1954), *Chem. Abstr. 48*, 12602*g*.

Tronov, B. V., and Kharitonova, R. I. (1960), *Chem. Abstr. 54*, 24087*a*.

Watanabe, K. (1957), *J. Chem. Phys. 26*, 542.

Watanabe, K., Nakayama, T., and Mottl, J. (1962), *J. Quant. Spectry. Radiative Transfer, 2*, 369.